Contribution of neuropathology to the understanding of human prion disease

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Neuropathology is an important tool for definitive diagnosis of sporadic, genetic, and acquired prion disease. Classical neuropathological hallmark is the highly disease-specific spongiform change accompanied by neuronal loss, astro- and microgliosis. Spongiform change of the neuropil consists of either microcystic or confluent vacuoles and varies greatly within the same brain. In addition, the most important aspect of confirmatory diagnosis is the demonstration of disease-associated prion protein (PrPd) by immunohistochemistry or Western blotting. Different PrPd immunostaining patterns include patchy/perivacuolar surrounding spongiform change, diffuse/synaptic, perineuronal, or plaque type. The latter includes unicentric kuru-type plaques or multincentric plaques as in the peculiar genetic prion disorder, Gerstmann-Sträussler-Scheinker disease. PrPd immunostaining patterns correlate well with phenotypes defined by the polymorphic codon 129 and the type of protease resistant PrPd seen on Western blots. PrPd immunoreactivity in the cerebellum may be highly informative about disease subtypes. Although the central nervous system is the major site of PrPd accumulation, it may also be observed in peripheral nerves as adaxonal deposits; in skeletal muscle as granular immunoreactivity in particular in abundance in a unique instance of concomitant Creutzfeldt-Jakob disease and inclusion body myositis; as well as associated with dendritic cells and macrophages in vessel walls. A subset of inhibitory GABAergic neurons is selectively affected in experimental and human prion disease. The central pathogenetic cascade includes oxidative stress and apoptosis. Deposition of terminal complement components on neurons accompanies tissue damage.

key words: prion protein, Creutzfeldt-Jakob disease, spongiform change, amyloid plaque

INTRODUCTION

It may appear surprising that in the genomic era of medicine prion diseases or transmissible spongiform encephalopathies (TSEs) can be diagnosed only after death. Neuropathology is not only needed for diagnostic confirmation, but also for all research on tissue, i.e. for brain banking programmes. In addition, neuropathological studies have greatly advanced the understanding of the pathogenesis of TSE. TSEs may be classified according to etiology, clinicopathological phenotype, constellation of the prion protein gene (PRNP), and Western blot characteristic of the protease resistant prion protein (PrP+) [7, 33]. Although the most frequent and the earliest described form of TSEs is in humans, a plausible theory for the etiology of sporadic Creutzfeldt-Jakob disease (CJD) is still lacking. A peculiar thalamic degeneration, fatal insomnia (FI) may also be sporadic [10]. Acquired forms include bovine spongiform encephalopathy (BSE) related variant CJD, iatrogenic CJD, and kuru of the cannibalistic Fore tribe in Papua-New Guinea. A family histo-
ry is not recognized in all cases with PRNP mutation, thus the term genetic TSE is more suitable to label disorders that are classified upon neuropathological basis as genetic CJD, fatal familial insomnia (FFI), and Gerstmann-Sträussler-Scheinker disease (GSS) [34].

Representatives of all groups have been transmitted, and by definition, all harbour an abnormal conformer of the cellular PrP (PrP^C) termed disease-associated PrP (PrP^D or PrP^Sc, where Sc indicates scrapie). In addition to PRNP mutations in genetic/familial forms, all disease phenotypes seem to be influenced by the codon 129 polymorphism [1, 6, 26]. Furthermore, the protease-resistant PrP shows variability as shown by Western blot isoform patterns. Therefore, protease-resistant PrP is classified on the basis of electrophoretic mobility as type 1 (non-glycosylated PrPres, 21 kDa) and type 2 (non-glycosylated PrPres, 19 kDa), and the based of differences in glycoform ratio [33]. Moreover, the literature distinguishes more PrP^res types on Western blots [19].

Several reviews have summarized the histopathological features of prion disease (e.g. in [5]). In addition to a summary of relevant changes, the present paper focuses mainly on neuropathological findings that have contributed to the understanding of these multifaceted, both neurodegenerative and transmissible, disorders.

**CLASSICAL HISTOPATHOLOGY**

**Spongiform change**

On a light microscopic level, spongiform change indicates vacuolization seen mainly in the neuropil of the gray matter. Ultrastructural investigations have initiated a debate whether it is mostly in neuronal/astrocytic processes or cytoplasm [31]. It is the most specific change for TSEs since other classical changes like neuronal loss and reactive astrocytosis are common features of other neurodegenerative disorders. Spongiform change may be diffuse or focally clustered (Fig. 1A, B), featuring small round or oval vacuoles, which must be differentiated from non-specific spongiosis observed in many other circumstances including ischaemic-hypoxic damage, oedema, or diffuse Lewy body, Alzheimer's disease, and dementia lacking distinctive histopathology [5]. Anatomically, it may appear in cerebral cortical and subcortical structures and less in the brainstem. In the cerebellum, the molecular layer may be typically affected (Fig. 1C), while the granular layer usually shows neuronal loss. When severe, end-stage burn-out lesions are observed with a collapse of the cytoarchitecture of the cortex (Fig. 1D), the change is usually referred to as status spongiosus. However, some TSEs have little or no spongiform change. These are mainly genetic cases like FFI or GSS.

**Loss of neurons**

**I. Selective vulnerability**

Neuronal vulnerability seems to be selective in TSEs similarly to other neurodegenerative disorders. The most striking observation is that a subgroup of GABAergic neurons immunolabeled by a calcium binding protein, parvalbumin, is one major target of neuronal loss in experimental TSE and CJD [14, 15]. Interestingly, calbindin immunoreactive neurons do not show loss, even though they are also calcium binding protein immunolabeled GABAergic neurons. Parvalbumin immunopositive neurons surrounded by isoleucine B4 perineuronal net are the most vulnerable, suggesting a role for the extracellular matrix in the pathogenic process [3].

Further morphological studies were focused on the serotonergic system. In FFI, it was shown that there is a significant increase in the pool of neurons that can synthesize serotonin in the brainstem raphe nuclei as compared to sporadic CJD and non-disease controls [40]. Recent observations suggest more widespread alterations of the serotonergic network, including astrocytic responses [9].

In contrast to more frequent degenerative dementia, the hippocampus and gyrus dentatus are relatively preserved in TSEs. However, this depends on the molecular and genetic subtype of TSE, e.g. the codon 129 Valine homozygote sporadic CJD cases are more susceptible to damage in hippocampal structures [23].

**II. Mechanisms of cellular damage**

a) Oxidative stress was shown to be present in both experimental models and human TSE. In scrapie-infected mouse brains, nitrotyrosine and heme oxygenase-1 (as markers) were immunolabeling neurons [16]. In human TSE, oxidative damage to nucleic acids has been shown in affected brains [17].

b) Apoptosis is the most likely form of neuronal death, even though the respective neuropathological studies have been based mainly on the detection of DNA fragmentation (Fig. 1E) [13], which is thought to reflect vulnerable neuronal populations instead of clear cut evidence of apoptotic cell death [8].

c) In addition to the importance of the complement system in the early steps of peripheral pathogenesis of experimental TSEs [21, 32], we have recently shown that deposition of terminal complement components on neurons accompanies tissue damage in human TSE forms irrespective of genotype or molecular phenotype (Fig. 1E, F) [29]. Apart from the link of the terminal complement components to oxidative damage and apoptosis, another aspect of the membrane attack complex is lytic damage of cells. Although complement activation is known in other neurodegenerative disorders, the ex-
tensive accumulation of the membrane attack complex on neurons suggests a novel pathway of neurodegeneration in TSEs.

d) To characterize the stress response, especially the role of the stress-related 70 kDa heat shock protein (Hsp) group in CJD, we have recently compared the distribu-

Figure 1. Neuropathology of Creutzfeldt-Jakob disease. Spongiform change in the cerebral cortex: focally confluent vacuoles and microcystic change dispersed throughout the cortex (H & E, A: × 40, B: × 400). Vacuoles are mainly localized in the molecular layer of cerebellum (H & E, C: × 200). "Status spongiosus": burnt-out lesion with a breakdown of the cytoarchitecture (H & E, D: × 400). TUNEL reaction showing DNA fragmentation in vulnerable neurons in the cerebral cortex (E: × 400). Membrane-attack complex (C5b-9) deposits in neurons in the cerebral cortex of a sporadic Creutzfeldt-Jakob disease case (F: × 400). Inset in upper right corner of F shows a confocal laser scanning image of membrane-attack complex in a neuron in sporadic Creutzfeldt-Jakob disease (× 750). Asterisk indicates the same capillary in E and F. Arrows indicate the same neurons in adjacent section shown in E and F.
tion of Hsp-72, Hsp-73, and PrP (both cellular and disease-associated) immunoreactivity with neuronal vulnerability detectable by the TUNEL method. Our observations support the hypothesis that elevated expression of the inducible Hsp-72 in affected brain areas is part of a cytoprotective, or “PrP sparing”, mechanism [24].

Reactive gliosis
This is represented partly by astroglia hyperplasia and hypertrophy, sometimes reaching the level of gemistocytic reaction. Additionally, microglia activation and cytokine production are also present, despite the lacking of typical inflammatory response and cellular infiltration [5, 39].

IMMUNOHISTOCHEMISTRY FOR PrP
Immunostaining for PrP in tissue is the gold standard for the definitive diagnosis of human TSEs. There is an abundance of commercially available available antibodies and pretreatment protocols for anti-PrP immunohistochemistry. To avoid confusion in this topic, we have compared the immunostaining patterns in different subtypes of human TSEs and control cases [25]. We have confirmed previous observations that the PrP deposition patterns are characteristic for the subtypes, especially those found in the cerebellum [36]. Some antibodies exhibit immunoreactivity restricted to pretreated sections of brains with TSEs but not to controls (e.g. 6H4, 12F10). Thus, these tools are more reliable for the specific immunolabeling of the abnormal form of PrP. Interestingly, antibodies directed against N-terminal epitopes react differently when compared to antibodies directed to other regions.

Pretreatment protocols used in different laboratories include a various mixture of steps like application of formic acid, proteinase K, guanidine-thiocyanate, hydrated and hydrolytic autoclaving, or citrate buffer. To exclude misinterpretations between laboratories, it is extremely important to use harmonized pretreatment protocols and it must be emphasized that any positive signal in immunohistochemistry for disease-associated PrP needs to be described with caution and experience. In our experience, a three-tiered protocol proves to be the most reliable. This includes formic acid pretreatment, hydrated autoclaving and guanidine-thiocyanate.

PrP immunostaining patterns

Characteristic Patterns of TSEs
a) Fine deposition (diffuse/synaptic pattern) (Fig. 2A);
 b) Pericellular deposits as dot-like and/or coarse granular immunoreactivity around unstained neuronal perikarya (Fig. 2B);

c) Coarser depositions (these include the granular, the patchy/perivascular deposits) (Fig. 2C);

Cerebellar cortex may show similar deposits (Fig. 2 D, E). Often intense PrP immunoreactivity is observed only focally (Fig. 2F).

d) Plaques: with amyloid characteristics, e.g. kuru-type (Fig. 2G), multicentric in GSS (Fig. 2H), and florid plaques in variant CJD, or without amyloid characteristics, as plaque-like deposits or so called focal deposits.

The most characteristic immunostaining patterns of sporadic CJD subtypes are summarized in Table 1. A mixture of patterns (Fig. 2C) might reflect coexistence of different isotypes of protease-resistant PrP within the same brain [25, 35]. Genetic TSEs represent further PrP immunostaining patterns. Multicentric amyloid plaques define GSS, however, there is a considerable variety of morphological appearance (Fig. 2H, I) according to the mutation. Base pair insertions may also show peculiar PrP deposits in the cerebellar molecular layer. In CJD cases with the PRNP mutation E200K, we showed that PrP immunoreactivity reminiscent of stripes perpendicular to the surface of the cerebellar molecular layer is highly characteristic [20].

A summary of different immunostaining patterns in genetic TSE is described in a recent paper [26]. Classification of PrP immunostaining patterns in different subtypes of TSEs is important for retrospective studies, for which materials for genetic or Western blot testing are often lacking.

Table 1. Characteristic immunostaining patterns of disease-associated PrP in sporadic Creutzfeldt-Jakob disease (sCJD) subtypes classified according to Parchi et al. [33]. 1–5 is for cerebral, 6–9 for cerebellar cortex. Black colour indicates highly characteristic, grey colour variably observed, and white colour unusual PrP immunoreactivity
Patterns common to TSE and control cases

In addition to extracellular disease-specific deposits, PrP immunoreactivity may be observed within neuronal somata [25]. In a systematic study, we distinguished the following neuron related types of PrP deposits: (1) diffuse type of immunoreactivity in the neuronal perikaryon; (2) large intracytoplasmic inclusions-like body in ballooned neurons in TSE cases; (3) multiple intracytoplasmic small dots in genetic TSE; and (4) somato-synaptic, seen as punctuate IR on the neuronal somata surrounded by diffuse/synaptic PrP deposits [27]. Only the diffuse type (1) is seen in controls and TSE, while the others are specific for TSE cases and might represent stages of intraneuronal processing of PrP. After a trial of pretreatment protocols, we found one which includes only citrate buffer (and excludes formic acid, autoclaving, proteinase K, and guanidine thiocyanate) that enhances specifically the diffuse (1) type of deposits, i.e. the PrP°.

In that study, we also observed an inverse correlation between the proportion of neurons with diffuse immunoreactivity of neurons, the intensity of disease-associated PrP immunoreactivity, and severity of lesions, supporting the notion that upregulation of PrP
might represent an early loss of function of the non-pathological form of PrP, in parallel with a neurotoxic effect of accumulating disease-associated isoform, as part of the pathogenesis of TSEs.

**PrP immunoreactivity in human neurodegenerative diseases**

Using immunohistochemistry with different pretreatment protocols, double immunolabeling and confocal laser microscopy, we evaluated the role of PrP in neurodegeneration, including Alzheimer’s, Parkinson’s, and diffuse Lewy body diseases, progressive supranuclear palsy, and multiple system atrophy [28]. We observed immunoreactivity only when we used citrate buffered pre-treatment. We did not see any immunostaining pattern reminiscent of TSE in any of the investigated disorders. In summary, the presumed cellular form of PrP decorates dystrophic neurites and focally co-localizes with tau and with alpha-synuclein in early, but not in fully developed disease-specific inclusions. However, diffusely PrP immunoreactive neurons may contain abnormal tau or alpha-synuclein aggregates. PrP accumulates mainly at the periphery but also throughout the β-A4 plaques of Alzheimer’s disease patients. We noted more neurons with a diffuse type of neuronal PrP immunoreactivity in neurodegenerative diseases than in controls. Our results suggest that expression of the normal, cellular form of PrP reflects a general response to cellular stress rather than specific co-operation in aggregation of other proteins.

**Extracerebral disease-associated PrP in sporadic CJD**

It is becoming increasingly evident that disease-associated PrP may be deposited outside the central nervous system (CNS). Within the CNS, disease-associated PrP is detected mainly in the gray matter, practically anywhere where synapses exist. It is thus not surprising that PrP is observed in the spinal cord gray matter, especially in the posterior horns [12].

As a major rule, tissues expressing PrP are believed to harbour PrP in case of a yet unidentified conformation change process affecting extracerebral sites. Sites of PrP deposits, based on recent studies aiming to map extracerebral disease-associated PrP, may be summarized as follows.

a) **Peripheral nerves**

Discrete PrP immunoreactive deposits were detected in posterior root nerve fibers in an adaxonal location in sporadic CJD and GSS [18].

b) **Muscle**

Glatzel and co-workers have shown that in sporadic CJD, similarly to experimental mouse and hamster models [4, 38], disease associated PrP may be present in skeletal muscle tissue in low concentration. Only highly sensitive Western blot detection methods can reveal this. Using immunohistochemistry, paraffin embedded tissue blot [37], and Western blot, we recently showed abundant PrP in the muscle of a patient with sporadic CJD and inclusion body myositis (IBM) [30]. The PrP level in muscle was about one third of that of a CJD brain. Extensive studies by Askanas and co-workers showed an increased expression of PrP in hereditary and sporadic IBM; however, it was only recently biochemically characterized as the cellular form of PrP in sporadic IBM [2, 41]. This supports the notion that extraneural PrP conversion into CJD becomes prominent when the cellular form of PrP is abundantly available as substrate, as in IBM muscle.

c) **Vessel wall**

We showed disease-associated PrP deposition in intracranial vessel walls, in both sporadic and variant CJD, in addition to extracranial vasculature in variant CJD. These deposits occurred together with HLA-DR and S-100 immunoreactive cells in the intima, which are components of the vascular-associated dendritic cell network, as well as with HLA-DR and CD-68 immunopositive macrophages of the intima and media. This suggests that mobile immunocompetent cells may contribute to the transport of disease-associated PrP, and potential infectivity, across vascular walls [22].

d) **Internal organs**

A systematic study on sporadic CJD is lacking, although Glatzel et al. [11] mentioned that spleen also harbours disease-associated PrP. However, the morphological substrate is not yet defined. Variant CJD harbours PrP in the lymphoid system, but this is not the topic of our present review.

**CONCLUDING REMARKS**

Human TSEs are rare disorders. Nevertheless, scientific ONNT research in this field have opened new dimensions. Neuropathology is one major part of research which contributed to the understanding of TSEs by showing the selectivity of cellular vulnerability, pathways of cellular damage, and variability of disease-specific deposits of PrP in CNS and extracerebral sites. On one hand (in CNS), these changes serve as a basis for phenotypic classification, and the other hand (in extraneural sites) they raise issues of public health. Thus, it is understandable that neuropathology is still very important in the genomic era of medicine.
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