Biochemical and Ultrastructural Study of Neurofibrillary Tangles in Amyotrophic Lateral Sclerosis/Parkinsonism-Dementia Complex in the Kii Peninsula of Japan

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Abstract. Amyotrophic lateral sclerosis/parkinsonism-dementia complex of the Kii peninsula (Kii ALS/PDC) is a neurodegenerative disorder endemic to natives in the southern coast area of the Kii peninsula of Japan. The disorder closely resembles Guamanian ALS/PDC clinically and neuropathologically. The characteristic neuropathological finding is abundant neurofibrillary tangles (NFTs) without amyloid deposition. To elucidate the biochemical properties of hyperphosphorylated tau protein, the major component of the NFTs, we examined Kii ALS/PDC brains by immunoblotting and immunohistochemical analysis using well-characterized anti-tau antibodies specific to phosphorylation-dependent or -independent epitopes. Hyperphosphorylated tau in Kii ALS/PDC had phosphorylated epitopes common to tau of paired helical filaments (PHFs) in Alzheimer disease (AD); immunoblot showed triplet bands composed of 6 tau isoforms. Ultrastructurally, NFTs revealed a twisted filamentous shape similar to PHF of AD. The biochemical properties of its phosphorylated tau protein and the ultrastructural characteristics of the NFTs of Kii ALS/PDC are very similar, if not identical, to PHF tau in AD, although they are different tauopathies.

Key Words: Amyotrophic lateral sclerosis/Parkinsonism-dementia complex; Kii peninsula; Neurofibrillary tangle; Paired helical filaments; Tau protein.

INTRODUCTION

There is a high prevalence of amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex (PDC) in the mountainous southern coast area of the Kii peninsula of Japan (1, 2). The clinical features and neuropathological findings of the ALS and PDC cases in this area are almost identical to ALS/PDC of Guam. Since manifestations of Kii ALS and PDC frequently overlap in a single patient or in members of a family, they are suspected to be part of a spectrum of a single disease entity—ALS/PDC of the Kii peninsula (Kii ALS/PDC)—as is ALS/PDC of Guam (3–5). Epidemiologically, Kii ALS/PDC differs from Guamanian ALS/PDC in its continuing high incidence and prevalence rates in the Kii area after the 1990s, in contrast to the marked decline in high incidence rates on Guam (1, 6, 7). In addition, the high rates of familial occurrence suggest the existence of some genetic abnormalities in Kii ALS/PDC.

Neuropathologically, ALS/PDC of Kii and Guam (especially PDC) is characterized by marked cortical atrophy of the anterior portion of the frontal and temporal lobes with neuronal loss. The neuropathological hallmark of ALS/PDC of Kii and Guam is widespread neurofibrillary tangles (NFTs) and neuropil threads, most predominantly in the hippocampal formation and frontal neocortex, and quite similar to that of Alzheimer disease (AD). ALS/PDC, however, differs from AD as follows: 1) absence of abundant senile plaques that are the most characteristic neuropathological hallmark of AD; 2) NFT laminar distribution patterns in the neocortical areas where NFTs are preferentially distributed within layers II and III in ALS/PDC, whereas they are more dense in layers V and VI in AD cases; 3) different NFT regional distribution in ALS/PDC in that NFTs are found not only in the hippocampal formation and neocortex, as in AD, but also in the basal ganglia and brainstem (1, 4, 5); and 4) the existence of pathologic features of classic ALS, including degeneration of the upper and lower motor neurons and Bunina bodies (1). Additionally, there are occasional NFTs in the spinal cord gray matter, including intermediolateral nucleus, posterior and anterior horns, and Clark’s column. These findings suggest that the etiopathogenesis of ALS/PDC may be different from that of AD.

Previously, we have reported findings of a single case of Kii PDC in which NFTs were similar to those of AD ultrastructurally and by immunoblotting with anti-tau protein antibodies (1). In this study, we characterize NFTs and tau protein from brains of 4 patients with Kii PDC, including 3 new cases with ultrastructural, immunohistochemical, and immunoblotting techniques using well-characterized anti-tau antibodies specific to phosphorylation-dependent or -independent epitopes.

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Fig. 1. Tau immunohistochemical staining of the frontal cortex of a Kii PDC case with AT8. NFTs distributed in a laminar patterns in the neocortical areas preferentially in the layers II and III.

MATERIALS AND METHODS

Brain Samples

The brains from 4 Kii PDC patients (PDC-1, 2, 3, and 4) were submitted for the present study. For comparison, we selected 1 brain sample from each of the following: AD, corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), Pick’s disease, and cognitively normal aged control. The clinical and neuropathological findings of the Kii PDC-1 have been described in a previous report (1) and those of the 3 new cases were almost the same. In PDC-1, samples from the basal ganglia and from the frontal, medial temporal, parietal and occipital cortices were analyzed. In PDC-2 and PDC-3, samples from the medial temporal cortex and basal ganglia were available and samples from the entorhinal cortex alone were available in PDC-4. The other tauopathy cases submitted showed typical clinical and neuropathological findings of the individual disorders, and samples for the present study were taken from the most severely affected sites of the brains, including the medial temporal cortex in AD, the basal ganglia in PSP, parietal cortex in CBD, and frontal cortex in Pick’s disease. The brain samples of the control were obtained from the frontal cortex.

Immunohistochemical studies were done with sections from paraaffin-embedded brain samples that had been fixed in 4% paraformaldehyde or in 10% formalin.

Immunoblot Analysis

Sarkosyl-insoluble tau was extracted from 1-gram samples of the frozen brains. Extraction and dephosphorylation of Sarkosyl-insoluble tau were done as described previously (8). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blot analysis of tau proteins were performed using 9% SDS-PAGE. Tau protein concentration of the brain samples was monitored by quantification of the intensities of tau protein bands of serially diluted samples with phosphorylation-independent antibodies using densitometry. We used 10 different epitope-specific anti-tau polyclonal antibodies that were prepared by one of the authors, Koichi Ishiguro. They included 8 polyclonal antibodies that were specific for phosphorylation-dependent epitopes (PS199, PS199/202, PT231/PS235, PS262, PS396, PS404, PS413, and PS422) in PHF tau, and 2 polyclonal antibodies specific for phosphorylation-independent epitopes (tau N and tau C) in normal tau and PHF tau. Nine antibodies other than PS199/202 were extensively characterized in a series of immunohistochemical and immunochemical studies, as reported previously (9–11). PS199/202 was newly developed and reacts with doubly-phosphorylated tau at residues Ser199 and Ser202 (data not shown), and the sequence of the antigen peptide is Lys-SSpPGSpPGTPGSR (Sp; phosphoserine) that corresponds to the residues 198 to 209 of the 441 amino acids of the human tau isoform. This antigen peptide was conjugated with keyhole limpet hemocyanin (Pierce, Rockford, IL) with glutaraldehyde and was used to immunize rabbits.

To compare the levels of phosphorylation at the tau protein epitopes, the same amount of tau protein samples from the brains of each tauopathy were analyzed by immunoblot studies.

For dephosphorylation, aliquots of Sarkosyl-insoluble tau were incubated with E. coli alkaline phosphatase type III (Sigma, St. Louis, MO) for 3 hours at 67°C, as described previously (12).

Immunohistochemistry

Paraaffin-embedded, 6-μm-thick sections from the hippocampus were cut for immunohistochemical analyses. Additionally,
to demonstrate laminar NFT distribution patterns in the neocortex, the frontal cortex sections were immunostained with phosphorylation-dependent anti-tau antibody AT8 (Innogenetics, Ghent, Belgium). Immunostaining was carried out with the Avidin-Biotin Kit (Vector Laboratories, Burlingame, CA) for rabbit antisera. The immunoreaction was visualized with 0.01% diaminobenzidine tetrahydrochloride (DAB). All antibodies were diluted to 1:1,000 for histochemical staining.

Electron Microscopy of Isolated PHFs from Kii PDC Brains

Sarkosyl-insoluble tau samples from the Kii ALS/PDC brains were adsorbed onto carbon-coated 400-mesh platinum grids and negatively stained in 4% aqueous uranyl acetate. Electron micrographs were taken with an electron microscope (Hitachi 800) at 80 kV as described previously (12).

**Fig. 2.** Immunohistochemical staining of the hippocampus from a case of Kii PDC (PDC-1) using various phosphorylation-dependent and -independent anti-tau antibodies. Panels (a–j) demonstrate NFTs and neuropil threads stained with 10 different antibodies: tau-N (a), PS199 (b), PS199/202 (c), PT231/PS235 (d), PS262 (e), PS396 (f), PS404 (g), PS413 (h), PS422 (i), and tau C (j). The other 3 cases (PDC-2, 3, 4) showed similar findings. Original magnification: ×120.
RESULTS

Immunohistochemistry

NFTs demonstrated with immunostaining with AT8 were preferentially distributed within layers II and III in frontal cortex in Kii PDC (Fig. 1). All 10 antibodies positively stained NFTs and neuropil threads in the Kii PDC brains (Fig. 2). In addition to intraneuronal and extracellular NFTs, occasional coiled-type glial cytoplasmic inclusions were seen in the cortical gray and subcortical white matter of the brain and the white matter of the pons and cerebellum (data not shown).

Western Blot of Tau Protein of Kii ALS/PDC and Other Tauopathies

Figure 3A shows the immunoblotting of Sarkosyl-insoluble tau extracted from cases of Kii PDC (PDC-1), other tauopathies, and normal control immunostained with phosphorylation-dependent and -independent antibodies. Sarkosyl-insoluble tau from the Kii PDC brain shows triplet band pattern (3 major bands of 60, 64, 68 kDa and a minor band of 72 kDa) quite similar to that from AD (PHF tau). Sarkosyl-insoluble tau from the CBD and PSP brains showed doublet band pattern (2 major bands of 64 and 68 kDa and a minor band of 72 kDa) and the Pick’s disease sample showed doublet band pattern (2 major bands of 60 and 64 kDa and a minor band at 68 kDa) as reported previously (13±16). The sample from the normal control brain did not show any of these tau bands. Sarkosyl-insoluble tau from Kii PDC was immunoreactive to 8 phosphorylation-dependent and 2 phosphorylation-independent anti-tau antibodies as intensely as that from AD. Sarkosyl-insoluble tau from Pick’s disease brain faintly reacted with PS262, which recognizes PHF tau phosphorylated at Serine 262 epitope, as described previously (13). Sarkosyl-insoluble tau from PSP and CBD brains reacted with PS262 less intensely than those of AD and Kii PDC.

Sarkosyl-insoluble tau from various sites of the individual Kii PDC brains disclosed triplet band pattern of various intensities, probably reflecting the densities and amount of NFTs in the individual samples (Fig. 3B). With the alkaline phosphatase treatment, the triplet bands of Sarkosyl-insoluble tau of the Kii PD brains were resolved into 6 immunoreactive bands that aligned with 6 normal tau isoforms, as those of PHF tau from the AD brain (Fig. 3C).

Electron Microscopy

Electron microscopic examination revealed dispersed filaments of twisted appearances with a diameter of 8 to 20 nm and crossover spacing of approximately 80 nm (Fig. 4).
Fig. 3. Continued. B: Tau samples from different areas of brains of the 4 Kii PDC cases (PDC-1, 2, 3, and 4) immunostained with tau C. Triplet bands of abnormal tau are detected in all PDC cases, but they are faint in the lanes of frontal and parietal cortex of PDC-1 and basal ganglia of PDC-2 and not shown in the occipital cortex in PDC-1 and basal ganglia in PDC-3, where no or few NFTs are present on histological preparations. Abbreviations: F, frontal cortex (entorhinal cortex); T, medial temporal cortex; BG, basal ganglia; P, parietal association cortex; O, occipital cortex (calcarine cortex). C: Tau samples from brains of AD, PDC-1, CBD, and PiD immunostained with tau C before and after alkaline phosphatase treatment. Triplet bands of both AD and Kii PDC are resolved into 6 bands aligned with the positions of the recombinant 6 tau isoforms. Samples from Kii PDC-2, 3, and 4 showed the same findings as that from PDC-1(data not shown). Doublet bands of CBD are resolved into 3 bands aligned with the positions of the 4-repeat tau isoforms and 3-repeat tau isoforms respectively. rec = recombinant tau; + = after dephosphorylation; − = before dephosphorylation.

DISCUSSION

The main component of neurofibrillary lesions in AD and other tauopathies is microtubule-associated protein tau, which is hyperphosphorylated and insoluble in a detergent such as Sarkosyl. The role of this abnormal protein in neurodegeneration remains to be clarified (17, 18). Recent genetic and biochemical studies on frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), a hereditary tauopathy caused by mutations in the gene encoding tau protein, have proved that tau dysfunction causes neurodegeneration (19–21). Kii ALS/PDC where there was proved no tau gene mutation (1) may be etiopathogenetically related to tau protein abnormalities.

Comparative biochemical studies of tau aggregates of some tauopathies showed that tau protein characteristics of individual diseases differ in tau protein isoforms and in the level of phosphorylation (12–16, 21–23). The biochemical characteristics of hyperphosphorylated tau protein of Guamanian ALS/PDC were reported to be similar to those of PHF tau of AD (24, 25), but those of Kii ALS/PDC have so far been investigated in only a single case (1).

To elucidate the properties of hyperphosphorylated tau protein of Kii ALS/PDC, we performed immunoblotting and immunohistochemical analysis with an extensively characterized panel of antibodies to defined phosphorylation-dependent or -independent epitopes that span nearly...
the entire length of AD PHF tau. These studies failed to demonstrate any substantial immunochemical differences between PHF tau protein of AD and the hyperphosphorylated tau protein of Kii PDC.

Hyperphosphorylated tau protein of the brain is composed of all 6 tau isoforms in AD (12), whereas it is composed of excessively 3-repeat tau isoforms in the Pick’s disease brain (13), and mainly 4-repeat tau isoforms in PSP, CBD, and FTDP-17 with intronic tau mutation (14, 21), as shown in Figure 3A. The most likely explanation for these phenomena is that the subsets of neurons or glial cells that degenerate in the individual diseases express tau isoforms peculiar to each one. Glial cells and small pyramidal cells of the layers II and III are affected in PSP and in CBD, whereas mainly smaller interneurons of the layers II-III and granule cells of the dentate gyrus are affected in Pick’s disease (14, 15).

Hyperphosphorylated tau protein of the 4 brains of Kii PDC, including the 3 new cases and the previously reported PDC-1 case, showed a triplet band pattern consisting of 6 tau isoforms on immunoblot, identical to that of AD PHF tau. In Kii PDC, dense bands of triplet tau protein were shown not only in the hippocampal formation and neocortical association areas, but also in the basal ganglia. In PSP, NFTs distribute in the cerebral cortex and subcortical nuclei as in Kii PDC, but NFTs biochemically consist of doublet tau (26). An atypical parkinsonism with PSP-like clinical features that clusters in the French West Indies is suspected to be caused by habitual intake of neurotoxic tropical plants native to these areas and partly by genetic background (27). Although this endemic disorder resembles Kii and Guamanian ALS/PDC in clinical features, the neuropathological and biochemical features more closely resemble those of PSP (28).

Immunoblot and immunohistochemical studies have revealed that the levels of tau phosphorylation at the epitopes examined here are similar to that of PHF tau of AD. Tau protein kinase I/glycogen synthase kinase-3 β (TPK I/GSK-3 β) and TPK II/cyclin-dependent kinase 5 (cdk5) are candidate enzymes responsible for hyperphosphorylation of tau that induce the formation of PHF. TPK I/GSK-3 β phosphorylates proline-directed tau epitopes of SP199, TP231, SP396, and non-proline-directed epitope S413. TPK II/cdk5 phosphorylates proline-directed tau epitopes of SP202, TP205, SP235, and SP404 in vitro. The Serine 262 epitope is phosphorylated by p110/microtubule affinity regulating kinase (MARK) or PKA in vitro (29, 30). The present study has shown that these enzymes might play similar roles in hyperphosphorylation of tau protein in both AD and Kii ALS/PDC.

Ultrastructurally, NFTs of AD consist mainly of PHF and those of PSP and CBD consist of straight filaments. Extracted NFTs from Kii ALS/PDC brains were twisted filaments similar to those of AD PHF.

The present series of immunoblotting, immunohistochemical, and ultrastructural studies have indicated that the abnormal tau protein in NFTs of Kii ALS/PDC are very similar, if not identical, to AD PHF. Similar findings
were reported in tau protein of Guamanian ALS/PDC (32).

Some FTDP-17 cases show clinical and neuropathological features of motor neuron disease, and transgenic mice of the particular tau gene mutation demonstrate some features akin to human tauopathies, including behavioral problems, amyotrophy, and developing intraneuronal tau-immunoreactive inclusions with neuronal loss and spinal anterior horn cell loss (31–37). Tau dysfunction, therefore, can induce clinical and neuropathological features resembling those of ALS/PDC of Kii peninsula and Guam. Further genetic and biological studies are necessary to elucidate the roles of these abnormal tau proteins in neurodegeneration in Kii ALS/PDC, and their findings may provide important clues to aid in understanding the etiopathogenesis of AD and other tauopathies.

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REFERENCES


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