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ELISA-Quantitation of Phosphorylated Tau Protein in the Alzheimer's Disease Brain

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Key Words
Dementia · Biochemical markers · Early diagnosis · Neurofibrillary tangles

Abstract
A reliable, sensitive and specific sandwich ELISA for the quantitation of paired helical filament (PHF) tau in human brain was developed using well-defined monoclonal antibodies. We examined rapid-autopsy-derived brain tissue from 21 neuropathologically confirmed Alzheimer's disease (AD) patients and 14 nondemented controls, matched for age, sex and postmortem delay times. We demonstrated significant elevations of phosphorylated tau levels in the frontal and parietal cortex as well as in the hippocampus of AD patients as compared to the nondemented controls. No difference was observed in the cerebellum. Phosphorylated tau levels measured by ELISA were significantly correlated with the presence or absence of neurofibrillary tangles.

Introduction

The formation of neurofibrillary tangles (NFTs) with highly insoluble paired helical filaments (PHF) is one of the pathological hallmarks of Alzheimer's disease (AD). The major component of PHF is a posttranslationally modified form of the protein tau which has been shown to be abnormally phosphorylated in the AD brain [1]. The mechanisms of phosphorylation and dephosphorylation of tau in autopsy- and biopsy-derived brain from AD are still controversially discussed, but appear to be time dependent [2, 3]. Several previous studies using Western blot or enzyme-linked immunosorbent assay (ELISA) techniques, for example, demonstrated the presence of abnormally phosphorylated tau in the AD brain. However, these studies were hampered by several limitations, such as the presentation of relative values of phosphorylated tau due to lack of standard protein solutions [4, 5], the use of semiquantitative Western blot, radioimmunoslot-blot or relative immunoreactivity measurement techniques [6–8], the limited sample sizes or lack of control brain tissue [4, 7, 8], or rather long postmortem delay times [5, 7–9].

In order to more accurately quantitate the levels of phosphorylated tau in AD brains as compared to controls,

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Table 1. Characterization of autopsy brain samples

	Cerebellum		Frontal cortex		Parietal cortex		Hippocampus	
	AD	Ctr	AD	Ctr	AD	Ctr	AD	Ctr
Sex	7 m, 7 f	4 m, 4 f	6 m, 9 f	5 m, 6 f	9 m, 7 f	5 m, 7 f	3 m, 4 f	1 m, 5 f
Age, years	75 ± 2	78 ± 3	76 ± 3	75 ± 4	73 ± 2	75 ± 3	77 ± 4	70 ± 7
p.m., min	84 ± 12	103 ± 18	94 ± 11	123 ± 16	80 ± 10	96 ± 15	88 ± 15	127 ± 24

Within each brain region there was no significant difference in sex, age or postmortem delay time using the Mann-Whitney U test. The values represent means ± SEM. m = Male; f = female; p.m. = postmortem delay time.

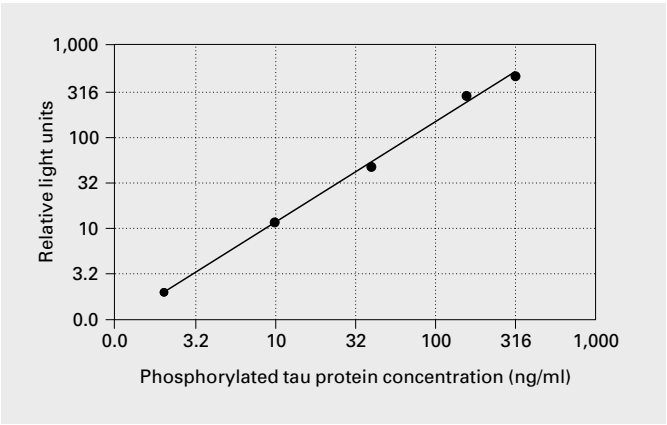

Fig. 1. The standard curve shows the dilution series of phosphorylated human recombinant tau ranging from 2.5 to 320 ng/ml versus the relative light units (blank subtracted; r = 0.99).

we established a sensitive and reliable ELISA specific for phosphorylated tau, and examined brain tissue with extremely short postmortem delay times (95 ± 8 min, range 30–180) to minimize effects of postmortem dephosphorylation.

In addition, we investigated the potential clinical use in terms of diagnostic properties of the present ELISA method and screened for phosphorylated tau protein levels in cerebrospinal fluid (CSF) samples from AD patients and age-matched nondemented control patients.

Methods

Brain Tissue Samples

Autopsy brain samples from a total number of 21 clinically demented AD patients and 14 nondemented individuals were examined. From these subjects, different numbers of samples were available from the frontal and parietal cortex, hippocampus and cerebellum of the right half of the brain and stored at –80°C (table 1). The postmortem delay times ranged from 30 min to 3 h. Postmortem intervals were defined as the delay from death of the patients until freezing of the brain tissue. The rapid autopsy program of the Duke University Brain Bank was recently described in detail by Hulette et al. [10]. The formalin-fixed left half of the brain had been assessed according to the CERAD criteria [11], and AD had been confirmed neuropathologically in all cases. Table 1 summarizes the characteristics of the groups which were matched for sex, age and postmortem delay times within each brain region (Mann-Whitney U test, p = n.s.). NFTs were counted per 20× field in an area of the section that appeared the most severely affected. Histopathological examination was performed by only 2 well-trained neuropathologists, thus guaranteeing consistency. In order to compare phosphorylated tau values obtained from the ELISA with the number of NFTs detected by immunohistochemistry in the corresponding left half of the brain, we categorized each single brain sample according to presence (category 1) or absence (category 0) of NFTs, irrespective of the CERAD diagnosis. Brain tissues were homogenized in ice-cold homogenization buffer (50 m*M* Trizma base/HCl buffer, pH 7.4 containing protease inhibitors (Complete™, Mini; Boehringer Mannheim, Germany) and 2 μ*M* of the phosphatase inhibitor okadaic acid), according to the method described by Song et al. [3]. After centrifugation, the supernatants were adjusted to a total protein concentration of 1.2 mg/ml with homogenization buffer and stored in aliquots at –20°C. These brain homogenates were assayed in triplicates.

CSF Samples

We assayed CSF samples from patients with probable AD (according to the NINCDS-ADRDA criteria, n = 7, 5 male, 2 female, age 67.7 ± 3.3 years, SEM, MMS 20 ± 1) and control subjects (elderly patients with major depression according to ICD-10 (F32.0x/1x, F33.0x), n = 8, all female, age 72.9 ± 3.3 years, MMS 27 ± 1). CSF was obtained by lumbar puncture, and 0.5 ml aliquots were immediately frozen and stored at –80°C. Informed consent was given by all patients and their caregivers.

Phosphorylated Tau ELISA

A solid-phase, noncompetitive sandwich ELISA was developed: 96-well black polystyrene microtiter plates (Black Cliniplate EB; Labsystems, Helsinki, Finland) were coated overnight at 4°C with monoclonal antibodies (mAb) AT 180 and AT 270 (Innogenetics, Zwijndrecht, Belgium; both antibodies used at a concentration of

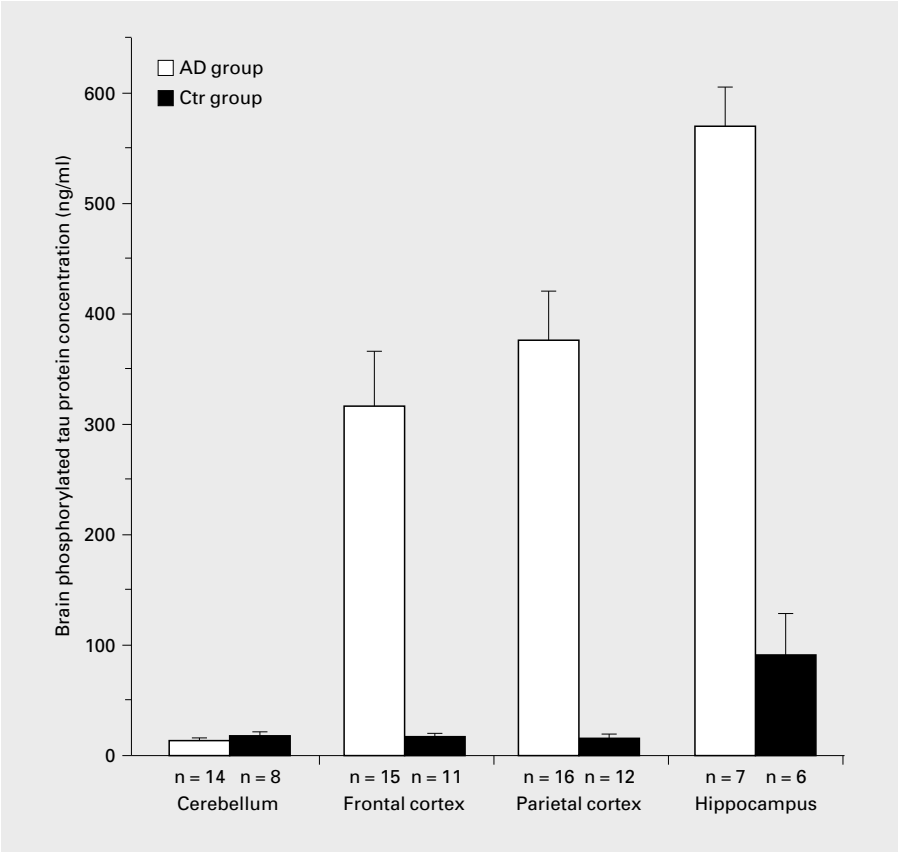

Fig. 2. Phosphorylated tau levels measured by ELISA in homogenates of rapid-autopsy-derived brain tissue. Region-specific comparisons of samples from AD patients (AD group) with age-matched controls (Ctr group). Levels (ng/ml) are given in means ± SEM. Significantly increased levels in the AD group were obtained in the frontal cortex ($z = 4.3$, $p < 0.0001$), the parietal cortex ($z = 4.1$, $p < 0.0001$) and the hippocampus ($z = 3.0$, $p < 0.003$). No difference was observed in the cerebellum (p = n.s.; Mann-Whitney U test). Within the AD as well as the control group, the brain region with the highest levels of phosphorylated tau was the hippocampus.

5 μg/ml) in 50 m*M* $NaHCO_3$, pH 9.6. The mAb AT 180 and AT 270 directed against epitopes containing phosphorylated Thr-231 and Thr-181, respectively, are weakly cross-reactive with normal tau [12]. After washing 3 times with PBS containing 0.05% Tween (PBS-T), 100 μl of brain homogenate per well (total protein amount 120 μg/well) were incubated for 3 h at room temperature. After washing 3 times with PBS-T, the horseradish-peroxidase-labeled mAb AD2 (1:1,000; Prof. Pau, Montpellier) in PBS-T was added for 2 h at room temperature, followed by another 3 washing steps with PBS-T. The reporter mAb AD2 shows no cross-reactivity with normal human adult brain tau and detects phosphorylated Ser-396 and Ser-404 [13]. One hundred microliters of PowerSignal™ chemiluminescent substrate (PIERCE, Rockford, USA) was added to the wells and the relative light units were read after 5 min on an ELISA Luminometer (Fluoroskan Ascent FL; Labsystems). Each plate included a standard dilution series of phosphorylated human recombinant tau (htau40, 4 repeats) in homogenization buffer. The recombinant tau protein had been phosphorylated with brain homogenate according to the method of Goedert et al. [14]. A typical standard curve is shown in figure 1. The correlation coefficients of the standard curves were within the range of r = 0.96–0.99. Concentrations were calculated after the mean blank value had been subtracted (values higher than 320 ng/ml were extrapolated). The blank value was obtained from 3 additional wells containing all reagents except for the sample. The lower detection limit was 2.5 ng/ml phosphorylated tau, the mean recovery 105%, the intra- and interassay coefficients of variation were 5.9% (± 4.4 SD, range 0.3–15.5%; n = 89) and 9.9% (n = 6), respectively. Addition of normal human recombinant tau (htau40, 4 repeats) did not interfere with the phosphorylated tau measurements. CSF levels of total tau were determined using a commercially available sandwich ELISA system (Innogenetics).

Statistical Analyses

Statistical analysis of data was performed using the Mann-Whitney U test for group comparisons. Spearman type of correlation was performed using levels of phosphorylated tau protein measured by ELISA and NFT counts. Regression analysis was complemented with analysis of variance (ANOVA) by using SPSS for Windows (version 8.0). Statistical significance was assumed at $p < 0.05$.

Results

Figure 2 shows that levels of phosphorylated tau were significantly increased in several brain regions in the AD group as compared to the control group (Ctr), e.g. in the frontal cortex (AD, ng/ml ± SEM, 316 ± 50 ng/ml, Ctr 16 ± 2 ng/ml; $z = 4.3$, $p < 0.0001$; Mann-Whitney U test), the parietal cortex (AD 374 ± 46 ng/ml, Ctr 14 ± 1 ng/

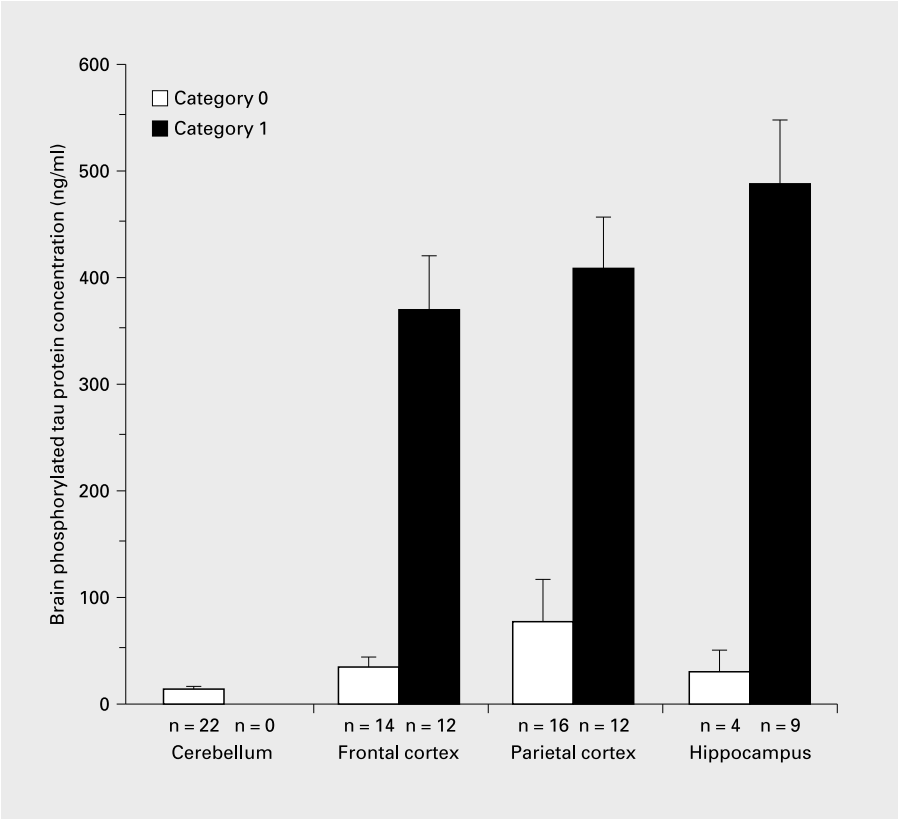

Fig. 3. Rapid-autopsy-derived brain samples have been categorized according to the presence (category 1) or absence (category 0) of NFTs detected by immunohistochemistry. Comparison of phosphorylated tau levels (ng/ml ± SEM) measured by ELISA in various brain areas. Levels (ng/ml) represent the means ± SEM. Except for the cerebellum, where category 1 was absent, phosporylated tau levels were significantly increased in category 1 in the frontal cortex ($z = 4.2$, $p < 0.0001$), the parietal cortex ($z = 3.9$, $p = 0.0001$) and the hippocampus ($z = 2.8$, $p < 0.006$; Mann-Whitney U test).

ml; $z = 4.1$, $p < 0.0001$) and the hippocampus (AD 570 ± 35 ng/ml, Ctr 89 ± 39 ng/ml; $z = 3.0$, $p < 0.003$). However, no difference was observed in the cerebellum (AD 10 ± 1 ng/ml, Ctr 16 ± 4 ng/ml; $z = 1.8$, p = n.s.). The highest mean values of phosphorylated tau were observed in the hippocampus of both AD and controls.

Figure 3 shows significantly increased levels of phosphorylated tau in the category 1 group (presence of NFTs) as compared to the category 0 group (absence of NFTs) in all brain areas investigated, except for the cerebellum, where no NFTs had been observed in either AD brains or controls (all category 0). Phosphorylated tau mean values are given in ng/ml ± SEM (Mann-Whitney U test) in the frontal cortex (cat. 1: n = 12, 373 ± 49 ng/ml; cat. 0: n = 14, 32 ± 11 ng/ml; $z = 4.2$, $p < 0.0001$), the parietal cortex (cat. 1: n = 12, 410 ± 49 ng/ml; cat. 0: n = 16, 77 ± 36 ng/ml; $z = 3.9$, $p = 0.0001$) and the hippocampus (cat. 1: n = 9, 489 ± 59 ng/ml; cat. 0: n = 4, 30 ± 17 ng/ml; $z = 2.8$, $p < 0.006$). The mean level of phosphorylated tau in the cerebellum was 12 ± 2 ng/ml (n = 22, all cat. 0). Elevated mean levels of phosphorylated tau in category 0 were found in 2 frontal cortex samples (82 and 163 ng/ml) and in 3 parietal cortex samples (207, 364 and 486 ng/ml) from AD patients as compared to controls. One hippocampal sample from a control subject with intense tangle staining had a very low level of phosphorylated tau (4 ng/ml) in the ELISA, whereas another without any detectable tangles had a level of phosphorylated tau of 79 ng/ml. Apart from these discrepancies, all category 0 samples had a phosphorylated tau level of 41 ng/ml or lower. Category 1 samples had 140 ng/ml or more.

Regression analysis demonstrated that levels of phosphorylated tau protein measured by ELISA correlated positively with NFT counts ($n = 79$, $r = 0.75$, $p < 0.01$, Spearman's correlation).

Phosphorylated tau was not detectable in the CSF of AD patients as well as in the CSF of the age-matched control patients with major depression. The mean CSF levels of total tau determined using a commercially available ELISA system (Innogenetics) were 624.9 ± 244.8 pg/ml (SEM) in the AD group and 182.9 ± 37.6 pg/ml in the control group, respectively.

Discussion

We established a sensitive ELISA for the quantitation of phosphorylated tau in human brain using specific mAb. This study demonstrated markedly elevated levels of phosphorylated tau in several regions of the AD brain such as the frontal and parietal cortex, as well as the hippocampus. In contrast, phosphorylated tau levels were normal in the cerebellum. Moreover, the phosphorylated tau levels were significantly increased in samples with positive neuropathological staining for NFTs in the cortical areas and the hippocampus. These findings are in good agreement with previous reports on distribution patterns of NFTs in the AD brain. Highest densities of NFTs were consistently found in the medial temporal lobe, including the hippocampus, and less severe changes were observed in the parietal and frontal lobes [15, 16]. In most cases, the cerebellum is spared from neurofibrillary changes [17].

Interestingly, in the nondemented control group of the present study, we found higher levels of phosphorylated tau in the hippocampus, as compared to the cortical areas and the cerebellum. This finding supports the hypothesis that the hippocampus is one of the first affected areas during preclinical development of NFT deposition, while the neocortical regions may be affected later [16].

The regression analysis showed that the levels of phosphorylated tau protein measured by ELISA correlated positively with NFT counts, but also showed that the ELISA levels did not completely match with the NFT counts. These discrepancies might be due to an altered epitope presentation of tau within the structure of NFTs. As a consequence, several phosporylated epitopes of tau may no longer be available for the antibodies used in the ELISA.

Phosphorylated tau was not detectable in the CSF of live AD patients and age-matched controls. This is in contrast to the findings of Blennow et al. [18], who reported the presence of phosphorylated tau in the CSF of normal controls and elevated phosphorylated tau in the CSF of AD patients. The reason for this discrepancy may be that either the phosphorylated tau concentrations are below the detection limit of our assay, or phosphorylated tau might be proteolytically degraded in the CSF resulting primarily in N-terminal tau fragments [19]. Such fragments might not be detectable with the present assay which uses a C-terminal-directed detection antibody. The ELISA system of Blennow et al. [18] included the same capturing antibodies, but the two reporter antibodies are directed against epitopes in the middle portion and N-terminal part of the molecule. Moreover, the present assay requires the phosphorylation of at least two different sites of tau since the capturing antibodies as well as the detection antibody are phosphorylation dependent. Thus, tau molecules that are phosphorylated only at one of these sites will not be detected. The use of phosphorylation-independent detection antibodies in the ELISA of Blennow et al. [18] may also allow for the detection of only partially phosphorylated tau proteins. These findings suggest that the length and phosphorylation state of the tau molecules could be markedly different in the brain as compared to CSF. Thus, the present ELISA method does not add to the repertoire of diagnostic tools for AD in live patients. However, the promising ELISA results reported recently [18] showed unexpected high levels of phosphorylated tau protein in CSF of AD patients that even exceeded the levels reported for total tau protein. In spite of continuous efforts of several investigators, these findings have not been replicated independently to date.

In conclusion, the present specific ELISA for phosphorylated tau may constitute a valuable tool to accurately quantify phosphorylated tau and to study abnormal phosphorylation of tau in ageing and neurodegenerative diseases in the human brain.

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