Charles University

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SUMMARY OF DOCTORAL THESIS

TAU PROTEIN AND ITS VARIANTS IN THE DIAGNOSIS OF ALZHEIMER'S DISEASE

TAU PROTEIN A JEHO VARIANTY V DIAGNOSTICE ALZHEIMEROVY NEMOCI

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The full text of the doctoral thesis is available at Charles University, Third Faculty of Medicine, Ruska 87, 100 00, Prague 10.

Abstrakt

Vláknité struktury tvořené proteinem tau byly doposud prokázány jako nejvhodnější histopatologický ukazatel nástupu a rozvoje Alzheimerovy nemoci (AN). Tau protein byl zpočátku považován za intracelulární protein regulující výstavbu mikrotubul. Nejnovější nálezy však prokázaly sekreci tau do extracelulárního prostoru. Ukazuje se, že pravděpodobně, jak intracelulární, tak i extracelulární formy tau přispívají k neurodegeneraci. V poslední době se uvažuje o rozpustných agregátech tau (oligomerech) jako o příčině šíření patologie AN a dalších tauopatií. Kromě toho byly u pacientů trpících AN prokázány i změny v regulaci imunitního systému a projevy autoimunitních poruch. Proto jsme se zaměřili na roli různých extracelulárních variant proteinu tau a anti-tau protilátek ve vztahu k AN.

Nejprve jsme studovali výskyt a charakter přirozeně se vyskytujících plasmatických anti-tau protilátek. Zjistili jsme, že anti-tau protilátky izolované z produktu intravenózních IgG (IVIG, Flebogamma) a z plazmy kognitivně zdravých starých lidí vykazují reaktivitu s patologickými agregáty (oligomery) tau proteinu. Naproti tomu protilátky z plazmy pacientů s AN reagovaly především s nízkomolekulárními (monomerními) formami proteinu tau. Anti-tau protilátky od kognitivně zdravých seniorů a z IVIG navíc silně reagovaly se zkrácenou formou tau (155-421). Z literatury i našich experimentů vyplývá, že zkrácené formy tau podléhají snadno agregaci a tvoří reaktivní oligomery. Výše uvedené koreluje s hypotézou „peripheral „sink", která uvádí, že protilátky v krvi mohou podpořit vyplavování a odstranění agregovaných a zkrácených forem tau proteinu z mozku, aniž by prošly přes hematoencefalickou bariéru.

Následně jsme detekovali samotné oligomery tau proteinu v krevním séru. Pomocí námi vyvinuté metody ELISA jsme zjistili, že se vyskytují tau oligomery v krevním séru zdravých starších lidí, kde se jejich hladiny navíc zvyšují s věkem. Oproti tomu jsme změřili snížené hladiny tau oligomerů u pacientů s mírnou kognitivní poruchou. Snížené hladiny tau oligomerů mohou souviset se zvýšenou hladinou sérových anti-tau protilátek detekovaných u stejné skupiny lidí a také s narušeným vyplavováním tau proteinu z intersticia do krve a následné hromadění tau agregátů v mozku. Kromě kvantitativního měření jsme pomocí Western blot techniky ohodnotili tau oligomery v krevním séru i kvalitativně. V séru pacientů s AN jsme našli stabilní vysokomolekulární tau oligomery,

kdežto u zdravých starších lidí jsme anti-tau oligomerní protilátkou pozorovali výskyt i nízkomolekulárních oligomerů tau proteinu, které pravděpodobně vznikly disociací nestabilních vysokomolekulárních tau oligomerů.

Tyto nálezy nás vedou k hypotéze, že extracelulární tau může být cíleně odváděn do krevního řečiště, kde je dále degradován. U AN mohou tyto mechanismy selhávat a umožnit tak agregování tau a následné šíření patologie.

Abstract

It is accepted that fibrillar aggregated tau protein is the best histopathological correlate of the onset and progression of dementia. Tau protein was long regarded as an intracellular protein with several functions inside of cells. New evidence suggests tau secretion into the extracellular space. It is plausible that both intracellular and extracellular forms of tau protein contribute to AD neurodegeneration. The truncated/fragmented forms of tau protein are prone to self-aggregate and form soluble oligomers which are now considered the toxic agents that spread the pathology in AD and other tauopathies. In addition, immunologic abnormalities including defective immune regulation and autoimmunity have been demonstrated in AD patients. Therefore, we have studied the role of various extracellular forms of tau protein and antibodies against them in AD.

Firstly, we showed that antibodies isolated from intravenous IgG (IVIG, product Flebogamma) and plasma of older cognitively healthy persons (controls) were reactive with pathological soluble aggregates of tau protein present in the brain of AD patients. On the contrary, isolated antibodies from the plasma of AD patients revealed reactivity with lower molecular weight (LMW, monomeric) tau forms found in brain tissue. Moreover, the antibodies from control subjects showed strong binding to the fragment of tau (155-421 aa). Thus, our findings with the hypothesis of peripheral sink in mind may indicate the participation of blood antibodies in clearance of the aggregated and truncated tau structures from the brain without the need to cross the blood-brain barrier. However, the levels of anti-tau antibodies itself have not proved as suitable biomarkers of AD.

Secondly, we have found tau oligomers in the sera of controls and their levels correlated with aging. On the contrary, the levels of serum tau oligomers were lowered in patients with mild cognitive impairment due to AD in comparison to controls. This result may be related to elevated serum levels of tau-reactive antibodies found in this study and/or to impaired clearance of tau protein from interstitium to blood and consequent accumulation of tau aggregates in the brain. By western blot, we found that serum of AD patients contained stable HMW oligomers while in the serum of controls the HMW oligomers were unstable and dissociated into LMW oligomers. We suppose extracellular tau proteins are cleared from the brain to the periphery where are subjected to degradation. In some cases as for the AD pathology, this clearance pathway could fail, thus contribute to form oligomers and spread the pathology.

Content

Introduction

In connection with the global trend of prolonging human life and the increasing number of older persons in the population, age-related neurodegenerative diseases become one of the most serious health and socioeconomic problems. Neurodegenerative diseases are characterized by specific protein inclusions [1]. This hallmark of age-related neurodegenerative disorders is evident in tauopathies characterized by the presence of abnormally phosphorylated tau aggregates. Alzheimer's disease (AD), the most common tauopathy, is characterized by two main pathological features in the tissue of the brain: firstly, by extracellular amyloid plaques, which are formed by insoluble amyloid beta and secondly, by intraneuronal neurofibrillary tangles (NFT) containing fibrillar tau protein [2,3]. We have chosen to study tau protein because neocortical NFT made out of this protein correlates with cognitive decline in AD patients [4,5].

Tau protein, under physiological conditions, promotes tubulin assembly into microtubules (MTs), one of the major components of the neuronal cytoskeleton that defines the typical morphology and provides the structural support to the neurons [6]. Physiological binding of tau to tubulin is regulated by its phosphorylation state, which is regulated by the coordinated action of kinases and phosphatases on tau molecule [7,8]. In AD, tau protein is subjected to the cascade of post-translational modifications. These modifications cause conformational changes in the structure of the protein, its aggregation into the paired helical filaments (PHF) and formation of NFT in neuronal cells. This cascade of events leading to NFT was suggested to begin with a toxic PHF-core [9] containing aggregates/oligomers of modified tau proteins (i.e. phosphorylated and cleaved tau protein forms) [10]. These toxic oligomers have a high affinity to intact tau molecules, and cells in an attempt to hide these oligomers may trigger processes like phosphorylation. In AD, these protective mechanisms lead to increased

amounts of tau molecules freed for aggregation and further truncations by activated caspases, which in turn re-expose the toxic PHF-core [11].

Tau protein was long regarded as an intracellular protein with several functions inside of cells. New evidence suggests tau secretion into the extracellular space [12]. At first, the occurrence of tau in the interstitial and the cerebrospinal fluid was believed as a consequence of dying neurons and their released cell content. Lately, several research groups, employing cell cultures and transgenic mouse models, have shown tau to be actively secreted from cells in membrane-free "naked" form [13] or included in microvesicles/exosomes [14–16]. Once tau secreted extracellularly, it can be taken up by other connected neurons. Several studies proved propagation of tau pathology trans-synaptically [17–20]. The group of Dr. Hyman from the MassGeneral Institute of Neurodegenerative Diseases showed that modified tau forms are accumulating at the pre and post-synaptic terminals in the AD brain [21]. It still remains unknown which forms of tau can be endocytosed by recipient cells. While some groups have shown uptake of toxic aggregates and others even full-length forms of tau protein, a growing number of studies indicate that only pathological tau can induce the seeded transmission and spread of pathology [22–24].

Taken together it is plausible that both intracellular and extracellular forms of tau protein contribute to AD neurodegeneration. The current state of research demonstrates that tau protein is a ubiquitous, highly dynamic, potentially broad range functional protein whose functions and localization are altered in neurodegenerative disease (for thorough review see [25]).

Autoantibodies in Alzheimer's disease

Over the past decade, there is an ongoing debate if AD can be a consequence of autoimmune processes. Immunologic abnormalities including defective immune regulation and autoimmunity have been demonstrated in AD patients [26–30]. Numerous reports of the presence of auto-antibodies against neuronal and non-neuronal antigens in sera of

AD patients were published [31–33]. However, it remains unknown whether the function of natural auto-antibodies in AD is a protective mechanism or contributes to pathology. While these natural antibodies may mainly play a protective role against microbial infections in individuals with an intact blood-brain barrier (BBB) [34], the same antibodies can cross-react with neuronal proteins and bind to the neuronal cells [35] if the BBB is impaired. Invasion of these "auto-antibodies" to the brain parenchyma can activate inflammatory cells, astrocytes and glial cells, which results in nerve damage [34,36] and consequently release of neuronal proteins. There are several reports of immunoglobulins bound to neurons in AD [37–39]. However, not all of these auto-antibodies must trigger pathological reactions in the brain [34]. Naturally occurring antibodies specific against toxic protein aggregates are desired to participate in the removal of these inclusions. This effect is now closely studied as a therapeutical approach to neurodegenerative diseases [40–44].

Nevertheless, naturally occurring antibodies against brain antigens circulating in serum and CSF could be useful biomarkers of developing neurodegeneration.

Aims of the thesis

Tau protein is strongly associated with AD because it forms the main part of pathological inclusions called neurofibrillary tangles (NFT) in the brains of AD patients. Moreover, the progress of neurodegeneration and a decline of cognitive functions of AD patients correlate with loads of NFT. Tau protein, conventionally regarded as intracellular, can be secreted in the healthy brain from active neurons into the brain interstitium. Above that tau is released after traumatic brain injury or during AD pathology by dying neurons into the extracellular space in the brain and consequently may appear in the cerebrospinal fluid (CSF) and blood. Once tau secreted extracellularly, it is most probably further modified by truncation and other post-translational modifications, and

that can be recognized by the immune system as a toxic antigen. Here we focus on the immune response to the occurrence of extracellular tau protein.

The aims of this thesis were **1)** to characterize reactivity of naturally occurring auto-antibodies against various forms of tau protein in relation to developing AD pathology. **We hypothesized that patients with mild cognitive impairment and dementia due to AD could have elevated levels of antibodies specific for pathological tau forms. 2)** We aimed to look into levels of tau modified forms in different biofluids as possible biomarkers of AD. **We expected elevated levels of tau oligomers in biofluids of AD patients.**

The specific aims of this thesis:

- Preparation of various recombinant forms of tau protein
- Preparation and characterization of antibodies against tau protein
- Developing of ELISA method for establishing tau-reactive antibodies levels in different body fluids
- Optimization of ELISA method for measurement of tau oligomers in serum

Materials and Methods

For the here presented research we employed all standard methods and techniques commonly used in our laboratories, including HPLC, SDS-PAGE electrophoresis, Western and dot blot techniques, ELISA assays, immunoaffinity chromatography, preparation of extracts from cells and tissues, (for detailed description see publications related to this thesis / section List of publications).

Participants

Serum/plasma and cerebrospinal fluid samples were obtained at AD Center, Charles University in Prague, Department of Neurology or Memory Clinic, Czech Republic. Serum and matched CSF samples for

antibody measurements were collected from 134 participants (Table 1). For tau oligomers and conformers, serum samples from 186 participants (Table 2). The participants were divided into five and four groups for each study, respectively. The first group of non-demented controls consisted of neurological patients with normal the Mini-Mental State Examination [45] and normal basic CSF findings [46]. The second group consisted of patients with MCI not fulfilling the criteria for MCI–AD (MCI) [47], and the third consisted of patients with MCI due to AD (MCI-AD) [48]. The fourth group consisted of patients with dementia due to AD (AD-dementia) according to the NIA-AA criteria [49]. The fifth group comprised demented patients with other types of dementia (frontotemporal dementia, progressive supranuclear palsy, Creutzfeld-Jakob disease, vascular dementia and mixed types of dementia were found in this group). The patients had an established diagnosis of AD from an experienced neurologist (MUDr. Aleš Bartoš). We measured concentrations of total tau protein, phosphorylated tau at Thr 181 and $A\beta_{42}$ peptide in CSF of participants using ELISA kits from Fujirebio (Malvern, Pensylvania, USA) according to manufacturer's instructions and in line with our previous research [50].

In total, four human autoptic brains were evaluated for the clinical diagnosis of AD using a silver staining technique in accordance with a study [51]. Human brain tissues of two control individuals (two men at the age of 71 and 79 whose cause of death was cancer and myocardial infarct, respectively) and two AD patients (two men at the age of 82 and 83 whose cause of death was cardiac insufficiency) were obtained by autopsy.

Table 1: *Demographic, cognitive and cerebrospinal fluid characteristics of subjects enrolled for measurement of tau-reactive antibodies*

	Control subjects	Mild Cognitive Impairment	Mild Cognitive Impairment due to Alzheimer's disease	Dementia due to Alzheimer's disease	Other dementias than Alzheimer's
N per group	46	13	19	30	26
Age (years)	65 (71-61)	60 (67-59)	**72** (77-67)**	**74*** (78-70)**	63 (74-58)
Female sex	43%	54%	37%	50%	43%
MMSE score	29 (29-28)	**26*** (27-24)**	**25.5*** (28-23)**	**21.5*** (24-17)**	**20*** (25-17)**
Total tau (pg/mL)	195.9 (255.2-155.0)	207.6 (263.0-187.0)	**301.8** (573.0-217.3)**	**557.5*** (687.3-363.5)**	**352.0** (485.4-197.9)**
Phospho-tau$_{181}$ (pg/mL)	32.9 (47.3-25.8)	43.0 (51.1-37.5)	**67.2** (106.0-38.9)**	**60.0*** (89.0-47.4)**	32.0 (42.0-23.0)
Aβ$_{42}$ (pg/mL)	928.2 (1121.0-802.1)	985.0 (1192.1-852.8)	**680.5* (1074.1-477.2)**	**578.5*** (815.4-467.0)**	**727.0** (950.5-622.0)**
Total CSF IgG (mg/L)	37.9 (53.1-23.5)	28.3 (40.7-22.9)	26.5 (71.0-21.6)	29.6 (58.1-22.9)	41.1 (56.2-21.5)
Total Serum IgG (g/L)	8.9 (10.8-7.9)	11.5 (11.8-7.8)	11.0 (12.4-7.9)	10.2 (11.5-6.7)	9.7 (11.7-7.7)

Data are presented as the median with interquartile range (Q_{75}-Q_{25}). Statistical significance (Mann-Whitney test) was calculated with respect to controls (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 2: *Demographic, cognitive and cerebrospinal fluid characteristics of subjects enrolled for measurement of tau oligomers.*

	Control subjects	Mild Cognitive Impairment	Mild Cognitive Impairment due to Alzheimer's disease	Dementia due to Alzheimer's disease
N per group (CSF/serum)	86	14	18	68
Age (years)	63 (68-58)	63 (77-59)	**72*** (77-67)**	**76*** (81-72)**
Female sex	51% / 47%	60%	41%	67% / 68%
MMSE score	29 (30-28)	**26.0*** (28.0-24.0)**	**25.5*** (28.0-24.0)**	**20.0*** (23-18)**
Total tau (pg/mL)	194.7 (263.0-161.0)	207.6 (240.1-185.6)	**301.8** (458.9-213.3)**	**599.0*** (967.0-325.0)**
Phospho-tau$_{181}$ (pg/mL)	35.5 (43.0-27.8)	39.7 (55.1-29.9)	**57.9** (80.2-39.2)**	**63.2*** (93.4-43.3)**
Aβ$_{42}$ (pg/mL)	911.1 (1125.0-729.9)	839.6 (988.7-643.3)	745.5 (1091.7-459.2)	**533.0*** (814-9-377.9)**
Total protein (mg/mL)	0.36 (0.42-0.27)	0.36 (0.41-0.32)	**0.45* (0.68-0.34)**	0.38 (0.61-0.28)

*Data are presented as the median with interquartile range (Q_{75}-Q_{25}). Statistically significant differences highlighted in bold were calculated with respect to controls (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).*

Results

We prepared five anti-tau antibodies in this study. One rabbit polyclonal anti- tau antibody and four human naturally occurring polyclonal tau-reactive antibodies. The rabbit anti-tau antibody was subjected to epitope mapping in collaboration with Department of Biological and Biochemical Sciences at the University of Pardubice to identify immunodominant epitopes of tau protein. By mass spectrometry were identified two epitopes regarding the longest isoform of tau protein: 299-317 aa and 171-194 aa [52].

Interactions between Amyloid-β and tau in cerebrospinal fluid

Partially characterized rabbit polyclonal antibody was used as a capture antibody in semi-quantitative sandwich ELISA to estimate levels of Aβ-tau complexes in cerebrospinal fluid as a prospective biomarker of AD [53]. We observed significantly lower levels of Aβ-tau complexes in the CSF of people with Mild cognitive impairment due to AD (MCI-AD) (84.5% of control levels) and AD (80.5% of control levels) as compared to cognitively normal controls. No significant changes were found in MCI, Frontotemporal dementia and other types of dementia.

Isolation and characterization of human naturally occurring polyclonal tau-reactive antibodies

The first two human polyclonal antibodies were purified from intravenous immunoglobulins product Flebogamma (nTau-IVIG-1 and -2) [54]. The other two were purified from plasma pool of seven cognitively normal older individuals (nTau-Ctrl) and four patients with AD (nTau-AD). We carried out ELISA, dot blot, and Western blot immunoassays to characterize the natural tau-reactive antibodies. The characterization of isolated nTau-IVIG-1 antibodies was firstly carried out against recombinant forms of tau protein and their phosphorylated equivalents (Hromadkova et al., 2015).
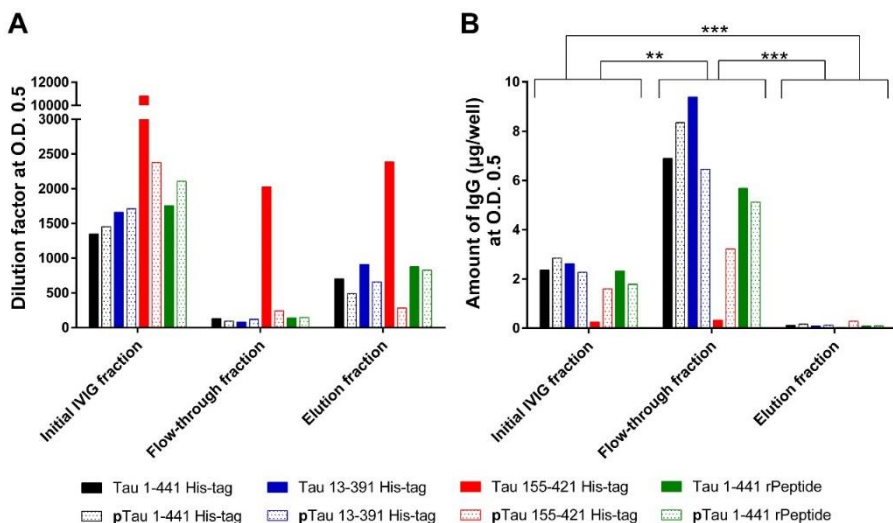
Figure 1: Reactivity of natural antibodies presented in individual IVIG purification fractions: initial Flebogamma IVIG fraction (50 mg/ml), flow-through fraction (7.9 mg/ml) and elution fraction (0.8 mg/ ml), against different forms of tau protein. The comparison is expressed as a dilution of each fraction (A) and the amount of IgG (B) required to obtain OD 0.5 at wavelength 450 nm. Bars represent a mean value from duplicate wells. Statistical analysis was performed by Student t-test at significant p levels 0.01 (**), 0.001 (***).

Table 3: The avidity of isolated antibodies towards different tau antigens.

		Isolated natural anti-tau antibodies from human plasma against	
		Tau 1-441	Tau 155-421
Avidity Index* Dot-blot	**nTau-IVIG 1**	0.74	1.67
	nTau-IVIG2	1.54	1.78
	nTau-AD	0.29	1.50
	nTau-Ctrl	1.27	1.56

* Avidity index is expressed as molarity of ammonium thiocyanate (M) that causes the decrease of initial sample signal (in the absence of thiocyanate) on the value of 50%. All forms of tau protein contained His-tag.

The nTau-IVIG-1 antibodies appeared to be most reactive with recombinant fragments of tau with sequence 155-421 aa and 13-391 aa in comparison to ligand used for purification. We have found that the phosphorylation of 155-421 aa tau fragment had markedly attenuated its antigenicity (Fig. 1). The avidity of nTau-IVIG (first and second batch), nTau-AD and nTau-Ctrl antibodies is summarized in Table 3.

By Western blot, we assessed isolated nTau Abs against native brain-derived tau protein forms present in homogenates of left hemisphere hippocampi of two AD patients and cognitively normal individuals. The samples were prepared as PBS-soluble (AD1, AD2 and C) and SDS-soluble (AD1´, AD2´and C´) protein fractions from brain homogenate and were probed with widely used monoclonal and polyclonal antibodies specific for different epitopes/forms of tau protein. We found monomeric isoforms of tau in control brain and pathological forms in AD brains (Fig. 2-D, E). According to the reactivity of specific antibodies against pThr231 and pSer396 of tau molecule in homogenates of brain tissue from two AD patients (Fig. 2-F, G), these two patients were at different stages of the disease.

The nTau Abs were biotinylated to avoid added signal from secondary anti-human IgG antibody alone, which we have observed (data not shown). The biotinylated nTau-IVIG2 antibodies exhibited weak staining of proteins on the membrane (Fig. 2-A). The biotinylated nTau-AD antibodies showed reactivity towards lower molecular forms of tau protein (Fig. 2-B). However, the biotinylated nTau-Ctrl antibodies reacted strongly with the higher molecular weight (HMW) forms but stained lower forms of tau protein as well (Fig. 2-C).
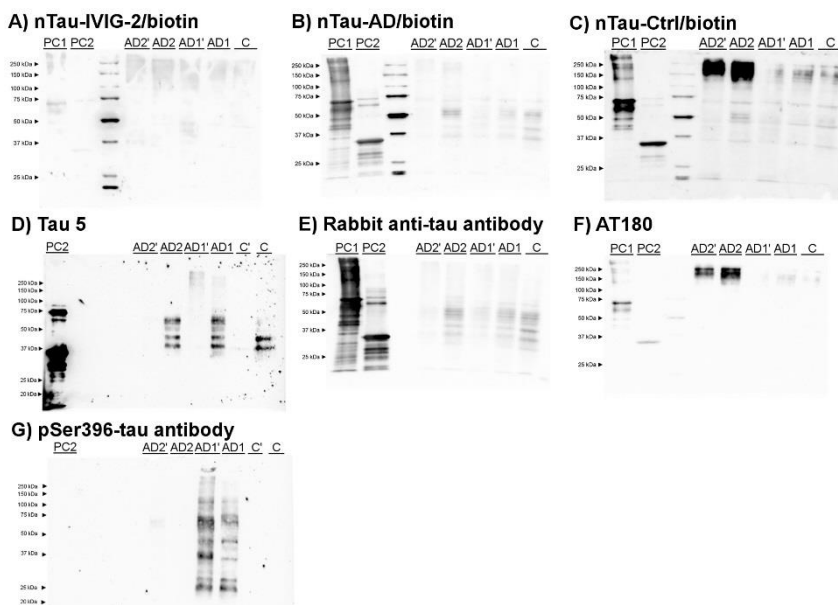
Figure 2: Western blot analysis of human brain homogenates (25 µg of total protein/lane) was carried out using biotinylated isolated naturally occurring tau-reactive antibodies from IVIG (nTau-IVIG2) product Flebogamma (A), isolated naturally occurring tau-reactive antibodies from plasma of AD patients (nTau-AD) (B) and naturally occurring tau-reactive antibodies from plasma of older cognitively normal subjects (nTau-Ctrl) (C), monoclonal Tau 5 antibody (D), rabbit polyclonal anti-tau antibody (E), monoclonal phospho-tau (pThr231) antibody (AT180, early stage of AD; F) and polyclonal pSer396-tau antibody (late stage of AD; G). Left hemisphere hippocampi of one control brain and two histopathologically proven AD patients were homogenized in PBS buffer (C-control brain and AD- AD brains) or PBS buffer containing 2% SDS (C'- control brain and AD'- AD brains), respectively. A recombinant fragment of tau 155-421 aa with the theoretical molecular weight of 30 kDa (2 µg/ well) (PC2) and full-length recombinant form of tau protein (1-441 aa) appearing on the gel around 75 kDa (PC1) were included as positive controls.

Comparison of levels of naturally occurring antibodies against tau proteins in serum and CSF samples

We extended the evaluation of humoral immunity associated with tau protein to two different compartments (serum and CSF) and between them (intrathecal synthesis). Table 1 shows basic characteristics of the

134 study participants who were divided into one group of cognitively healthy controls and four groups of cognitively impaired patients. The antibody levels were measured against three forms of tau protein: two His-tagged recombinant forms- full-length form of tau protein (1-441 aa) and one fragment (155-421 aa) and presumably "native" form of tau protein from bovine brain because of the presence of post-translational modifications in contrast to recombinant proteins. The levels of tau-reactive antibodies were ranging from 0.3 to 15 μg/ml in CSF and 2 to 300 μg/ml in serum samples of all investigated groups. The Kruskal-Wallis test revealed no significant difference in serum anti-tau antibodies among particular groups, whereas the Mann-Whitney test indicated an elevation of serum antibodies against the tau (1-441) in a group of MCI-AD when compared to controls (Mann-Whitney test, p= 0.020). There was no significant change in the intrathecal synthesis of specific CSF antibodies against all antigens.

We have observed a negative correlation between serum nTau (1-441) antibodies and CSF total tau levels in the MCI-AD group (r= -0.50, p= 0.049). We found negative link to intrathecal synthesis of nTau (1-441) antibodies (AD group: Spearman r= -0.61, p <0.001; OD group: r= -0.53, p=0.007) and positive correlation to total serum IgG levels (AD group: r= 0.60, p <0.001; OD group: r= 0.59, p=0.001) only in groups of patients with dementia. Thus, the elevated levels of serum nTau (1-441) antibodies in the MCI-AD group are not due to the overall increase in IgG levels, but rather due to specific immune response in blood.

Levels of tau oligomers and conformation-specific tau forms in serum

The levels of tau oligomers and conformers were measured by ELISA assay using unique antibody T22 specific only to oligomeric forms of tau protein [55] and antibody TTC-99 recognizing oligomers and misfolded monomers (conformers) (unpublished data). Table 2 shows demographic and cognitive characteristics of 186 individuals divided into groups including measured levels of current CSF biomarkers (total tau,

phospho-tau$_{181}$ and Aβ_{42}) by commercial ELISA kits if they were available. By comparing the average age of subjects in groups we found that individuals in MCI-AD and dementia due to AD groups were older than cognitively normal subjects. We found that the age of participants is an important variable in the analysis, thus it was included as a covariate into statistical comparisons of groups. The levels of serum tau oligomers were decreased in MCI group (GLM, p= 0.033) and MCI-AD group (GLM, p=0.006) with respect to control subjects. In sera of patients with dementia due to AD, we observed an elevation of tau oligomers levels in comparison to MCI and MCI-AD groups, but it did not reach statistical significance. Tau conformers did not show any difference between groups (Kruskal-Wallis test, p= 0.486). The results of the correlation analysis of measured levels of T22-tau oligomers with basic groups' characteristics indicated a positive correlation between aging and levels of tau oligomers in the group of controls (Spearman r= 0.26, p= 0.016) suggesting increasing levels of tau oligomers with aging.

We looked into the distribution of T22- reactive tau oligomers in the serum of randomly selected samples by Western blot. Representative western blot detection of tau oligomers in serum (Fig. 3) of three controls and three patients with dementia due to AD is presented. T22-immunoreactive tau oligomers higher than 250 kDa were observed in the serum of AD samples, but also in some of the control samples. Densitometric quantifications of higher molecular weight (HMW) bands from the Western blot of serum probed with T22 revealed significantly increased tau oligomers in AD (* $p < 0.05$, Student's t-test; Fig. 3-B). When the density of all protein bands was compared between groups, the AD patients had significantly more of tau oligomers detected with T22 when compared to controls (* p<0.05, Student's t-test; Fig. 3-C). We attribute the bands around 120-150 kDa to the tau dimers/trimers and probably non-specific immunoglobulins. The lower molecular weight oligomers (under 50 kDa) could be a result of dissociation of less stable HMW oligomers in the presence of SDS in the samples.
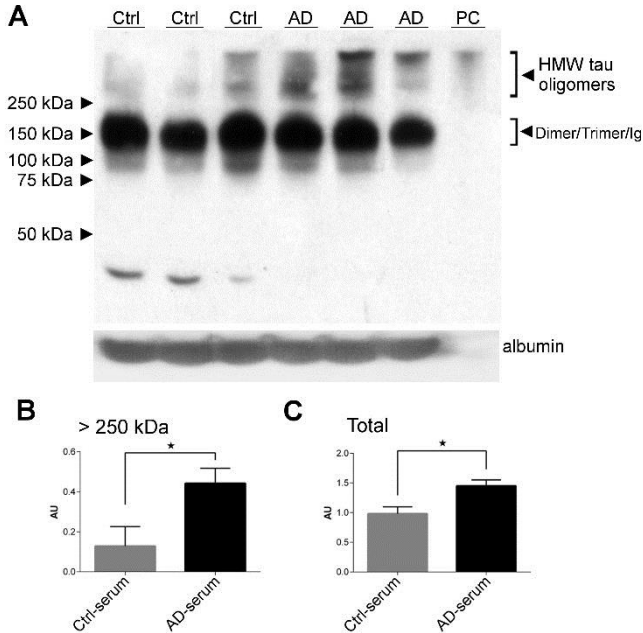
Figure 3: Western blot analysis of serum samples (50 μg of total protein/lane) from three patients with dementia due to Alzheimer's disease (AD) and three cognitively normal controls (Ctrl)(A) was carried out. In vitro prepared recombinant tau oligomers were used as a positive control (PC). Tau oligomers detected by rabbit polyclonal anti-oligomer antibody T22 were quantified by densitometry in higher molecular weight bands (>250 kDa) (B) and the whole lane (C). Densitometric quantifications (AU= arbitrary unit) were normalized to albumin, used as loading control. Bars represent mean ± SD.

Discussion

A number of reports analyzing the brain of AD patients are in agreement that fibrillar aggregation of tau is the best correlate of the onset and progression of dementia. Tau protein occurs in numerous post-translationally modified forms in the brain tissue. Site-specific phosphorylation and truncation are connected with the balance between physiological and pathological tau function. These post-translational modifications can significantly change the soluble form of tau protein in

paperclip-like arrangement to promote the aggregation [56–58]. It is proven that truncation is an early event in tangle formation [59]. Truncation in the C-terminal part of tau protein at cleavage points Asp421 [60,61] and Glu391 [62] was well defined and is even considered as a triggering factor in the development of tau pathology [59,63,64].

We prepared and studied several tau forms varying in length and phosphorylation state. We identified antigenic epitopes of tau protein by epitope mapping of our rabbit polyclonal antibody raised against the recombinant His-tagged full-length form of tau protein (1-441 aa). The binding epitopes of thoroughly purified rabbit polyclonal anti-tau antibody have been found in the proline-rich part of the protein (171-194 aa) and the microtubule-binding domain R3 (299-317 aa) that contains sequence [306]VQIVYK[311] with β-structure [25]. This repeat domain was described to promote self-aggregation of tau protein molecules in pathological conditions and *in vitro* experiments [65–67]. However, the epitope 299-317 aa was identified as a non-specific due to the undesirable adhesiveness of this fragment [68]. All forms of tau protein prepared for this study contained both, 171-194 aa and 299-317 aa, sequences and moreover the fragments were C-terminally truncated at sites described in the staging of NFT [69]. Above that the fragment 155-421 aa is N-terminally truncated at the Arg155, which is a cleavage site of thrombin protease [70,71] that is abundant in the blood. As a result of this, the fragment 155-421 aa can represent new epitopes for antibodies circulating in the blood.

Complexes of tau protein with Aβ peptide in the cerebrospinal fluid

The prepared rabbit polyclonal anti-tau antibody was used to study the occurrence of tau-Aβ complexes in CSF of AD patients, cognitively healthy controls, people with MCI-AD and other neurodegenerations [53]. The drops in levels of complexes to 80 % in the CSF of AD patients in contrast to controls could be interpreted as enhanced interactions between Aβ and tau and subsequent accumulation of the complexes in the brain,

in accordance with data in the literature [72,73]. The complexes distinguished between controls and AD patients with a sensitivity of 68.6 % and a specificity of 73.3 %. Nevertheless, the complexes haven't proved as a significantly better biomarker of AD than current biomarkers [53].

Reactivity of naturally occurring plasma antibodies with various forms of tau protein

Although AD is a neurodegenerative disorder, the evidence is accumulating that it involves alterations in humoral immunity and autoimmunity. Anti-neuronal antibodies have been detected in sera, CSF and brains of patients with AD [32,33,35,38,74–76]. Serum auto-antibodies have also been evaluated as potential diagnostic biomarkers for AD [31,77–79]. The IVIG products containing IgG molecules pooled from several thousand healthy donors are now in the spotlight because of ongoing clinical trials for AD therapeutic interventions. It has been proven that these products contain antibodies against neuronal tau protein [80], but their reactive character has been partly established [80,81]. Therefore, we characterized these naturally occurring antibodies against recombinant proteins [54] and moreover against native brain-derived forms of tau protein.

Interestingly, we have found marked reactivity of all three nTau Abs to a fragment of tau protein 155-421 aa despite the isolation against the full-length form of tau protein. The strong binding was confirmed by avidity measurements where we observed the highest avidity index of these antibodies (~1.6 M) for fragment 155-421 aa in contrast to other forms. This antigenicity was partly abolished by phosphorylation of this fragment pointing to epitopes related to truncation of tau protein rather than phospho-specificity [82]. This finding is interesting because in literature truncated tau is described as highly prone to aggregate and form reactive intermediates [64,83,84]. Although, the amount of isolated antibodies against truncated and phosphorylated tau forms could be

affected by using non-phosphorylated full-length tau as a ligand and these antibodies may not be sorted out during our isolation procedure.

Nonetheless, to obtain insight into the reactivity of these isolated antibodies with native proteins, we probed brain homogenates of histopathologically proven AD patients and control using nTau antibodies and compared their reactivity with other selected polyclonal and monoclonal anti-tau antibodies by Western blot. The homogenates contained different forms of tau protein ranging from presumable monomers of different isoforms (characteristic triplet, [85]) and fragments to HMW protein forms. The HMW proteins were found mostly in AD brains in contrast to control brain tissue. Interestingly, the isolated biotinylated nTau-IVIG and nTau-Ctrl plasma antibodies stained the HMW protein bands mostly, although the staining with nTau-IVIG was weak. On the contrary, the nTau-AD/biotin antibodies showed staining of LMW bands in the brain homogenates. The latest studies revealed that aggregated oligomeric tau forms are the toxic species of this protein. They can spread tau pathology and are responsible for cognitive impairment in AD mouse models which can be alleviated by passive immunotherapy specifically targeting tau oligomers [41,86,87]. Our findings may refer to immune control of tau truncation and related aggregation in healthy individuals thus providing clearance of these structures from the brain as was suggested by Castillo-Caranza DL et al. 2014 [86] and others [87–89]. This explanation would warrant the immunotherapy as a promising approach to AD treatment through the enhancement of the probable insufficient clearance of toxic tau aggregates. However, more experiments are needed to evaluate the character of these HMW proteins.

Levels of naturally occurring anti-tau antibodies in serum and CSF samples

In agreement with the latest studies and our previous results, we have found naturally occurring antibodies in all investigated groups. We have observed only a slight increase of serum tau (1-441 aa)-reactive antibodies in the group of patients with MCI-AD and their link to total

levels of tau protein present in CSF. This finding may point to an involvement of immune system in controlling higher amounts of tau proteins occurring in the biofluids as a consequence of developing pathology. Our results confirmed that auto-antibodies are ubiquitous in human serum, can be influenced by disease, and are remarkably stable over time [79,90]. However, these naturally occurring tau-reactive antibodies have not proved to be suitable biomarkers of AD.

Tau oligomers in serum of AD patients and aged cognitively normal individuals

There are studies concerning tau occurrence and its modified forms in CSF because this fluid is in direct contact with the brain parenchyma. Elevated CSF total tau, phospho-tau$_{181}$, and lower Aβ_{42} levels are now considered the only biochemical markers of AD pathology. However, detection of tau protein forms circulating in the blood could be a valuable diagnostic marker of AD. Several studies have reported plasma and serum levels of tau protein, but the results are contradictory. While one study showed lowered levels of plasmatic tau proteins in AD patients [91], Zetterberg et al. (2013) on the contrary, using their digital array technology, found higher plasma levels of tau protein in AD patients [92]. The serum levels of tau protein were reported to be elevated after mild traumatic brain injury [93] and in acute ischemic stroke [94]. Moreover, recent studies reported that release of endogenous tau protein from neurons into the interstitial fluid (ISF) is a physiological process which is mediated by neuronal activity (Pooler et al., 2013; Yamada et al., 2014). However, the basal levels of tau have to be restored upon release. The secreted tau could be washed out of ISF through glymphatic pathway into the blood where it can be subjected to fast degradation by proteases or targeted by antibodies.

In our study, we detected T22-reactive tau oligomers in the human serum, interestingly their levels positively correlate with aging [95]. However, the exact cause of this aggregation remains unidentified. It is reported that metabolism slows down and coagulation activity in blood

increases with aging (Sagripanti and Carpi, 1998). Therefore, the increased tau oligomer burden detected in the serum of patients with dementia due to AD, but also of cognitively normal persons could be a consequence of the higher accumulation of tau, thrombin and clotting agents in the blood. The observation of tau oligomers detected in the serum was also supported by Western blot assay. The detection of HMW tau oligomers in serum of AD patients and to some extent in control individuals in our study is in agreement with the study of Neumann et al. and Farías et al. where the researchers observed similar HMW forms of tau protein in the platelets of both AD patients and control subjects (Farías et al., 2012; Neumann et al., 2011). Moreover, we observed LMW bands in the serum of aged controls. We suppose that LMW bands could be the products of desired enzymatic degradation of tau protein aggregates in the blood under physiological conditions or unstable HMW aggregates/oligomers that can be dissociated. These observations are supporting the hypothesis that extracellular tau can be partly cleared on the periphery. When the clearance pathway is disturbed, the oligomers may accumulate in CSF or brain. In accordance with that we have found decreased levels of tau oligomers in the MCI-AD group. In fact, the impaired clearance of tau protein from the ISF through glymphatic pathway appearing after TBI has recently been described in a TBI mouse model (Iliff et al., 2014), which is an established risk factor for the development of neurodegeneration, including AD. In addition, the physiological efflux of proteins from CSF to blood through the choroid plexus was described as a clearance pathway for Aβ peptide (Crossgrove et al., 2005; Marques et al., 2013; Tarasoff-Conway et al., 2015). No studies describing clearance of tau protein through this barrier have been published thus far (Tarasoff-Conway et al., 2015). However, pathological changes in the choroid plexus related to neurodegeneration were described as enhancing tau oligomerization (Preston, 2001; Redzic et al., 2005; Serot, 2003) and that may be reflected later on in the course of the disease as an increase of stable tau oligomers in blood. On the other hand,

the accumulation of HMW oligomers could correlate to the pathophysiology of the disease in both the CNS and the peripheral blood cells. Nevertheless, the results of decreased levels of tau oligomers in the serum of MCI-AD patients may be related to elevated levels of tau-reactive antibodies found in this study. Thus, the immune system may play a significant role in protection against toxic protein aggregates. This assumption is supported by the reactivity and high avidity of antibodies isolated from plasma of older cognitively normal subjects to various forms of tau protein presented above. Although, the mechanisms involved in the clearance of tau are not completely understood when compared to Aβ clearance, the changes in the levels of tau oligomers with the manifestations of cognitive decline could be a valuable tool for future diagnostics.

Conclusion

The work presented here outlines the role of the peripheral immune system in the protection against toxic tau aggregates early in the development of neurodegeneration. However, our hypothesis of elevated levels of tau oligomers in biofluids of AD patients was confirmed only partly.

We characterized reactivity profile of anti-tau antibodies naturally occurring in plasma of healthy subjects contained in the product of intravenous immunoglobulins Flebogamma (nTau-IVIG), older cognitively normal subjects (nTau-Ctrl) and AD patients (nTau-AD). Interestingly, nTau-Ctrl antibodies showed strong reactivity against higher molecular weight (HMW) forms of tau protein (aggregates/oligomers) present in the brain homogenates of AD patients in comparison to nTau-IVIG were the reactivity was weak. On the contrary, nTau-AD antibodies reacted preferentially with lower molecular weight (LMW, presumable monomeric) forms of tau protein. Moreover, we found elevated levels of serum antibodies against human full-length tau protein (1-441 aa) in the group of patients with mild

cognitive impairment due to AD (MCI-AD) when compared to controls (Mann-Whitney test, p= 0.02). This finding partly supports our hypothesis stated in the aims. The serum levels of anti-tau (1-441 aa) antibodies negatively correlated with levels of total tau in CSF for this group. These results altogether likely point to the involvement of immune system in protection against the forming toxic tau forms probably via their clearance during normal aging or in the early stage of neurodegeneration, but not in the late stage of pathology.

These assumptions are in agreement with our findings of tau oligomers in the blood. We found tau oligomers in the sera of cognitively normal subjects, and these levels were increasing with age. The amounts of tau oligomers were decreased in the group of MCI-AD in comparison to controls (GLM, p= 0.015) and then increased in the group of AD patients. By western blot, we found that serum of AD patients contained stable HMW oligomers while in serum of controls the HMW oligomers could be partly dissociated. Our findings supports the assumption that extracellular tau protein can be partly cleared from the brain to the periphery. In the case of patients with MCI and MCI due to AD, the lower levels of tau oligomers could be the result of impaired clearance of tau protein from interstitium to blood and consequent accumulation of tau aggregates in the brain. However, the mechanisms involved in the clearance of tau from the brain to the periphery or the role of peripheral tau are not completely elucidated. Thus, further research on heterogeneous tau oligomer population and tau trafficking could help us better understand the role of tau protein in AD development.

Nevertheless, our findings stated above support the latest approaches to the treatment of AD via immunotherapy as an enhancement of the probable insufficient clearance of toxic tau aggregates.

List of publications

Publications related to the thesis (with IF)

Kolarova M., Sengupta U., Bartos A., Ricny J., Kayed R., 2017. Tau oligomers in sera of patients with Alzheimer's disease and aged controls. J Alzheimers Dis JAD-170048.

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Publications related to the thesis (without IF)

Krestova, M., Hromadkova, L., Ricny, J., 2017. Purification of natural antibodies against tau protein by affinity chromatography. In: Natural Antibodies, Methods in Molecular Biology. Humana Press, p. VIII, 324.

Kolarova, M., García-Sierra, F., Bartos, A., Ricny, J., Ripova, D., 2012. Structure and pathology of tau protein in Alzheimer disease. Int. J. Alzheimers Dis. 2012, 731526

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