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Seed-competent HMW tau species accumulates in the cerebrospinal fluid of Alzheimer's disease mouse model and human patients

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Abstract

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Objective—Cerebrospinal fluid (CSF) tau is an excellent surrogate marker for assessing neuropathological changes that occur in Alzheimer's disease (AD) patients. However, whether the elevated tau in AD CSF is just a marker of neurodegeneration or in fact a part of the disease process is uncertain. Moreover, it is unknown how CSF tau relates to the recently described soluble high-molecular-weight (HMW) species that is found in postmortem AD brain and can be taken up by neurons and seed aggregates.

Methods—We have examined seeding and uptake properties of brain extracellular tau from various sources including: interstitial fluid (ISF) and CSF from an AD transgenic mouse model, and postmortem ventricular and antemortem lumbar CSF from AD patients.

Results—We found that brain ISF and CSF tau from the AD mouse model can be taken up by cells and induce intracellular aggregates. Ventricular CSF from AD patients contained a rare HMW tau species that exerted a higher seeding activity. Notably, the HMW tau species was also detected in lumbar CSF from AD patients and its levels were significantly elevated compared with control subjects. HMW tau derived from CSF of AD patients was seed-competent *in vitro*.

Interpretation—These findings suggest that CSF from an AD brain contains potentially bioactive HMW tau species giving new insights into the role of CSF tau and biomarker development for AD.

Introduction

Alzheimer's disease (AD) clinical progression is thought to be driven by the accumulation of tau neurofibrillary tangles (NFT) progressing through the brain affecting increasing numbers of cortical areas^{1,2}. Recent animal model studies suggest that NFT propagation occurs by a cross-synaptic spread of pathological forms of tau³⁻⁶. We previously identified a bioactive high-molecular-weight (HMW) tau species present in the brain extracts from AD transgenic mice (rTg4510) and AD patients, which can be taken up by neurons and synaptically propagated to other neurons⁷. We also demonstrated that tau with similar biochemical characteristics can be identified in the brain interstitial fluid (ISF) of rTg4510 mice⁷.

Cerebrospinal fluid (CSF) tau is the most widely studied and established biomarker for AD^{8,9}. CSF tau is known to be consistently elevated in AD patients compared with non-demented controls¹⁰⁻¹². Measurements of total tau and phosphorylated tau can be used to diagnose AD with both sensitivity and specificity levels above 80%^{9,13}. The elevation of CSF tau is commonly assumed to reflect ongoing neurodegeneration in AD brains during the progression of the disease; however, the origin of CSF tau and mechanisms underlying its increase remain unclear¹⁴. For example, neither total tau nor phospho-tau are elevated in frontotemporal dementias despite equivalent levels of neuronal loss and, often, the presence of tau containing inclusions^{8,15}.

Here, we examined biological and biochemical properties of brain extracellular tau from various sources including brain ISF and CSF from tau-transgenic mouse model (rTg4510), human postmortem ventricular CSF, and lumbar CSF from AD patients. Our findings suggest that bioactive HMW tau species is present in the brain extracellular space of the mouse model and human AD patients. Notably, the HMW tau species was detected in

human lumbar CSF and its concentrations were significantly higher in AD patients than those in control subjects.

Materials and Methods

Animals

Six- to 13-month-old rTg4510 and control animals were used. The rTg4510 mouse is a well-established model of tauopathy, in which the full-length human four-repeat tau (0N4R) is overexpressed with the P301L frontotemporal dementia (FTD) mutation¹⁶. The rTg4510 mice develop mature tangles by four months¹⁶. Both male and female mice were used. All experiments were performed under national (United States National Institutes of Health) and institutional (Massachusetts General Hospital Subcommittee for Research Animal Care and the Institutional Animal Care and Use Committee at Harvard Medical School) guidelines. All animal experiments were approved by the Massachusetts General Hospital and McLaughlin Research Institute Institutional Animal Care and Use Committees.

Mouse CSF collection

CSF was collected from the cisterna magna, as described previously, with modifications⁸. Briefly, the cisterna magna was exposed under isoflurane anesthesia and the dura was punctured with a 30 gauge needle. CSF was collected with a P20 pipettor under a dissection microscope. Body temperature was maintained at normal levels during the procedure using heating pads. CSF was immediately placed on ice in a low protein binding tube (Microresico, #RC9.201.60, Eicom, San Diego, CA, USA) and stored at -80°C before use. Human tau and total protein concentrations in the CSF were measured using the human tau-specific ELISA (1:50 dilution, see below) and BCA assay (1:5 dilution, Pierce, #23225, Rockford, IL, USA).

The AlzBio3 immunoassay

The INNO-BIA AlzBio3 assay (#80584, Fujirebio, Gent, Belgium) was used to measure CSF A β 1-42, total tau, and p-tau181, as described previously¹⁷. CSF samples were thawed on ice and reagents from the kit were allowed to come to room temperature. Microspheres were vortexed, sonicated, and diluted in the buffer provided by the kit. The conjugate 1 working solution was prepared by diluting the provided biotinylated monoclonal antibodies. After prewashing the plate, 100 μ l of diluted beadmix were transferred to each well, followed by vacuum filtration. All measurements were performed with 25 μ l of conjugate 1 and 75 μ l of standard or CSF samples. The plate was incubated overnight at room temperature with vigorous shaking. Following washing three times, the antigen-antibody complexes were then detected by a phycoerythrin-labeled streptavidin in conjugate (60 min at room temperature with shaking). The plate was washed three times and reading solution was added to each well followed by 2 minutes of orbital shaking. The plate was read on a FlexMap3D platform (Luminex Corporation, Austin, Texas).

Subjects

Study participants were recruited from the Clinical Cores of Massachusetts General Hospital and the University of Washington Alzheimer's Disease Research Center (ADRC). All

participants underwent standard neuropsychological evaluation and clinical examination. Informants were also interviewed and a clinical diagnosis was assigned at consensus diagnostic meetings. All control subjects had Mini-Mental State Examination (MMSE) scores of 28, and neither subjects nor informants reported changes in social/occupational functioning suggesting a decline in cognitive function. Diagnosis of probable AD was made based on standard criteria of NINCDS-ADRDA¹⁸. Following written informed consent, lumbar punctures were performed between 9 am and 12:30 pm and the CSF was immediately aliquoted into polypropylene storage tubes and placed on dry ice. Specimens were then transferred and stored at -80°C before use. Subjects were recruited for lumbar punctures at both Massachusetts General Hospital and the University of Washington, and assays were performed at Massachusetts General Hospital. The demographic and clinical characteristics of all study participants are detailed in Tables 2 and S1.

Ethics statement

The study protocols were approved by the Institutional Review Boards at Massachusetts General Hospital and the University of Washington. Written informed consent was obtained from all participants at enrollment. Because AD patients belong to a potentially vulnerable group, a health care surrogate signed the consent forms in addition to the patient (unless the patient was incapable of doing so). Subjects also demonstrated assent by cooperating with and not objecting to any procedures. Participation in this research was strictly voluntary and potential subjects who did not take part in this project were not disadvantaged in any way. Participation in this project had no impact on eligibility for clinical benefits or services. All aspects of this investigation were conducted according to the principles described in the Declaration of Helsinki.

Recombinant tau

Recombinant full-length human tau (441 aa, #T0576, Sigma) was dissolved in PBS (1 mg/ml), then centrifuged at 150,000 g at 4°C for 30 min to remove fibrillary/oligomeric tau. The supernatant was used in the following experiments. The concentration of tau in the supernatant was determined by ELISA (see below).

Centrifugal devices for size fractionation and concentration

The CSF samples were fractionated and concentrated using Amicon Ultra filter units (Millipore). 100 kDa Amicon Ultra-0.5 (#UFC510096) and 10 kDa Amicon Ultra-0.5 (#UFC501096) devices were used for the sequential ultrafiltration of the mouse CSF samples (Fig. 2). 100 kDa Amicon Ultra-0.5 (#UFC510096) and 100 kDa Amicon Ultra-15 (#UFC910024) devices were used to concentrate the human lumbar CSF samples (Fig. 5). The devices were washed with 0.5 ml (Amicon Ultra-0.5) or 10 ml (Amicon Ultra-15) of Opti-MEM prior to use (10,000 g spin at 4°C for 10 min).

The pooled mouse CSF samples (180 µl total) were fractionated and concentrated using 100 kDa Amicon Ultra-0.5 devices (3,000 g spin at 4°C for 20 min), then the filtrates were transferred to 10 kDa Amicon Ultra-0.5 devices and fractionated by 10,000 g spin at 4°C for 20 min. The resultant 100 kDa cut-off concentrate, 10 kDa cut-off concentrate, and 10 kDa cut-off filtrate were used for the tau ELISA and the tau seeding assay. The human lumbar

CSF samples (1.5 or 8.0 ml) were concentrated using 100 kDa Amicon Ultra-0.5 (3,000 g spin at 4°C for 20 min) or 100 kDa Amicon Ultra-15 (3,000 g spin at 4°C for 30 min) devices. The resultant 100 kDa cut-off concentrate was used for the tau seeding assay in 96- or 384-well plates.

Size-exclusion chromatography

Lumbar CSF and postmortem ventricular CSF samples were separated by size-exclusion chromatography (SEC) on single Superdex200 10/300GL columns (#17-5175-01, GE Healthcare) in phosphate buffered saline (#P3813, Sigma-Aldrich, filtered through a 0.2- μ m membrane filter) at a flow rate of 0.5 ml/min, with an AKTA purifier 10 (GE Healthcare). Fractions were collected every 4 min (2 ml/fraction). Approximately 850 μ l of lumbar or ventricular CSF was loaded onto an SEC column. Ventricular CSF samples were filtered through a 0.2- μ m membrane filter prior to SEC to remove debris. SEC fractions were then concentrated by acetone precipitation (see below) prior to ELISA to determine tau levels in each fraction. Four volumes (vol/vol) of cold acetone (20°C) were added to each fraction, followed by vigorous vortex and incubation for 90 min at -20°C. Samples were then centrifuged at 13,000 $\times$ g for 10 min at 4°C. After carefully aspirating the supernatant, the pellets were resuspended in 105 μ l of PBS or ELISA diluent buffer. 100 μ L of samples were used for tau ELISA without further dilution.

We ran two or three samples, selected from each group in a blinded manner to minimize experimental artifact, every day in random order. The column was washed with PBS before loading each patient's sample and with 20% ethanol and distilled water regularly to avoid carryover of residual proteins on the column. We ran negative-control lumbar CSF, which is known to have no HMW tau, on a regular basis to ensure there was no residual HMW tau on the column and that SEC fractionation was working properly. Additionally, we confirmed that residual protein on the column, if any, did not affect the results by running blank PBS after tau-aggregates containing AD brain homogenate (PBS soluble, 10,000 g extract⁷). SEC HMW fraction from a blank PBS did not show detectable tau on ELISA (data not shown). We also confirmed that the post-wash SEC HMW fraction from a blank PBS did not induce tau aggregation in a sensitive FRET-based cell assay (data not shown).

Ventricular CSF

Postmortem ventricular CSF samples were obtained from the Massachusetts Alzheimer's Disease Research Center Brain Bank. The demographic characteristics of the subjects are shown in Table 1. All the study subjects or their next of kin gave informed consent for the brain donation, and the Massachusetts General Hospital Institutional Review Board approved the study protocol. All the AD subjects fulfilled the NIA-Reagan criteria for high likelihood of AD. Ventricular CSF samples were collected by syringe aspiration from the lateral ventricles. Specimens were stored at -80°C before use. The total tau levels in each sample were determined by tau ELISA (#KHB0041, Life Technologies).

Tau ELISA

The concentrations of human tau in the samples (brain ISF and CSF of rTg4510 mice, ventricular CSF of AD patients, and SEC-separated fractions of ventricular/lumbar CSF)

were determined by Tau (total) Human ELISA kit (#KHB0041, Life Technologies), according to the manufacturer's instructions.

Immunodepletion

Immunodepletion of tau from ventricular CSF was performed using Dynabeads Protein G Immunoprecipitation Kit (#10007D, Life Technologies) according to the manufacturer's instructions with minor modifications. 0.75 mg of Dynabeads Protein G was incubated with 2 µg of total tau antibody (HT7, #MN1000, Thermo Scientific), anti-phospho tau antibody (pS396, #44752, Life Technologies), or control IgG for 10 min with rotation at room temperature. After washing with 200 µl of washing buffer, the Dynabeads-antibody complex was incubated with 100 µl of ventricular CSF for 10 min with rotation at room temperature. Dynabeads-antibody-antigen complex was isolated using a magnetic holder and the supernatant was collected for tau uptake assay and ELISA measurement.

Tau uptake and seeding assay in HEK293-tau-biosensor cells

A tau uptake and seeding assay was performed as previously described (Holmes et al, 2014)¹⁹ with some modifications. In the experiments where sample volume was limited (mouse ISF/CSF (Fig. 2) and the human lumbar CSF underwent ultrafiltration via spin column (Fig. 5A-B)), a small volume 384-well plate was used for the assay, and cells were visually quantified for tau aggregates. We used FRET flow cytometry analysis with a 96-well format in the experiment when large volume samples were available (Fig. 5C-E). Stably expressing CFP-/YFP-Tau-RD(P301S) HEK293 cells were plated at 2,500–4,000 cells per well in a 384-well PDL-coated plate. The following day, brain ISF/CSF of rTg4510 or control mice, or ventricular CSF of AD patients were applied with (for tau seeding assay, 1% final concentration) or without (for tau uptake assay) Lipofectamine 2000 transfection reagent (#11668019, Life technologies). Samples were diluted at 1:4 in Opti-MEM (#11058-021, Life technologies), and a total of 20 µL of diluted samples were applied to each well. Confocal images were obtained via FRET channel (excited with a 458 nm laser and fluorescence was captured with 500–550 nm filter) at designated time points. The number of intracellular tau aggregates per well was counted and used for quantification analysis. Each condition was performed at least in triplicate for human ventricular CSF study. Single measurements for each mouse were made for the mouse ISF/CSF study due to the small sample volume. For FRET flow cytometry assay, HEK-tau-biosensor cells were plated at 30,000 cells per well in a 96-well PDL-coated plate. The following day, the lumbar CSF samples were fractionated and concentrated using an ultrafiltration spin column (see above). The concentrates were diluted at 1:2 in Opti-MEM (1% lipofectamine), and 40 µL of the diluted samples was applied to each well.

FRET flow cytometry

FRET flow cytometry was carried out as previously described¹⁹ with minor modifications. Three days after tau seed treatment in 96-well plate, HEK-tau-biosensor cells were harvested with 0.05% trypsin, postfixed in 2% PFA, and resuspended in PBS. The MACSQuant VYB (Miltenyi) was used to perform flow cytometry.

Semi-denaturing detergent agarose gel electrophoresis

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) was carried out as previously described²⁰ with minor modifications. A 1.5% agarose gel was prepared by dissolving agarose in buffer G (20mM Tris-Base, 200mM glycine) and then adding 0.02% SDS. A total of 30 µl of ventricular CSF and recombinant tau (30 ng) was incubated with 0.02% SDS sample buffer for a total of 7 minutes at room temperature just prior to loading. The SDD-AGE was run in Laemmli buffer (Buffer G with 0.1% SDS) at 30V until the dye front reached 1 cm from the end of the gel. Protein was then transferred via capillary action to Immoblin P (Millipore) membrane at 4°C for 24 hours. Membranes were blocked in 5% non-fat dry milk (NFDm) / Tris buffered saline with Tween20 (TBS-T) for 1 hour and then probed for total tau using mouse monoclonal antibody Tau13 (#MMS-520R, Covance, 1:2000) overnight at 4°C. Membranes were washed three times with TBS-T, probed with goat anti-mouse IgG-HRP (#172-1011, Bio-Rad, 1:2000) for 1.5 hours at room temperature, and washed three times with TBS-T. Membranes were developed using ECL kit (Western Lightning, PerkinElmer, USA) and detected on Hyperfilm ECL (GE healthcare, USA).

In vivo microdialysis

In vivo microdialysis sampling of brain interstitial fluid (ISF) tau was performed as described previously^{7,21,22}. The microdialysis probe had a 4 mm shaft with a 3.0 mm, 1000 kDa molecular weight cutoff (MWCO) polyethylene membrane (PEP-4-03, Eicom, Japan). Before use, the probe was conditioned by briefly dipping it in ethanol, and then washed with an artificial cerebrospinal fluid (aCSF) perfusion buffer (in mM: 122 NaCl, 1.3 CaCl₂, 1.2 MgCl₂, 3.0 KH₂PO₄, 25.0 NaHCO₃) that was filtered through a 0.2 µm pore-size membrane. The preconditioned probe's outlet and inlet were connected to a peristaltic pump (ERP-10, Eicom) and a microsyringe pump (ESP-32, Eicom), respectively, using fluorinated ethylene propylene (FEP) tubing (φ 250 µm i.d.).

Probe implantation was performed as previously described²², with slight modifications. Briefly, the animals were anesthetized with isoflurane, while a guide cannula (PEG-4, Eicom) was stereotactically implanted in the hippocampus (bregma -3.1 mm, -2.5 mm lateral to midline, -1.0 mm ventral to dura at a 12° angle). Three days after the implantation of the guide cannula, the mice were placed in a standard microdialysis cage and a probe was inserted through the guide. ISF samples were collected at a flow rate of 0.5 µl/min.

Statistical analysis

All data were expressed as mean ± SEM, unless stated otherwise. Two-group comparisons were performed by Mann-Whitney U-test, unless stated otherwise. In the immunodepletion experiments with ventricular CSF, comparisons among the three groups were made by analysis of variance (ANOVA) and subsequent Dunnett tests. In experiments evaluating tau levels in lumbar CSF, comparisons among the control, AD, and FTD groups were performed by Kruskal-Wallis and Steel-Dwass multiple comparison tests. Correlations were analyzed with a Spearman's rank test. The statistical test used is described in each figure legend. *P* values less than 0.05 were considered significant.

Results

Sensitive detection of a seed-competent tau species in biological samples using a FRET-based cell assay

In this study, we used a FRET-based highly sensitive cell assay (HEK-tau-biosensor cells)¹⁹ to assess the seeding activity of tau from various sources. First, we examined the sensitivity and specificity of this assay using human AD brain extracts and recombinant full-length human tau (Fig. 1). AD brain extract from entorhinal cortex, where tau pathology is consistently found, induced intracellular tau aggregates in a dose-dependent manner, with a lower detection limit of 10 pg/ml tau (Fig. 1A). Cerebellum extract, which is spared from neurofibrillary changes,²³ did not induce tau aggregation in the same time course of experiment (Fig. 1A). This means that the assay highly specifically detects a seed-competent tau species in biological samples. Recombinant human full-length tau induced few aggregates (Fig. 1B), even when applied at 500 times higher concentration (1 vs. 500 ng/ml), further confirming the specificity of the assay to a seed-competent tau.

Brain extracellular tau from an AD transgenic mouse model showed seeding and uptake activity

Seed-competent tau from tau-transgenic mouse brain homogenate is known to be taken up by neurons and to develop tangle-like tau pathology^{24,25}. However, the uptake and seeding properties of extracellular tau remain largely unknown. We first examined the seeding activity of extracellular (ISF and CSF) tau from an AD transgenic mouse model (rTg4510 mice) using HEK-tau-biosensor cells (Fig. 2A). Brain ISF tau was collected using an *in vivo* microdialysis technique with a large-pore (1,000 kDa cut-off) probe that allowed consistent collection of large molecules from the brain of freely moving animals^{21,22} (Fig. 2A). CSF was collected from the cisterna magna (Fig. 2A). Both ISF (Fig. 2B) and CSF (Fig. 2C) tau from rTg4510 mice induced intracellular tau aggregates, and their seeding activities were significantly higher than those of control mice. We then assessed tau uptake activity using the same HEK-tau-biosensor cells. To evaluate tau “uptake” activity, we added the samples to the cells without prior incubation with lipofectamine, which allowed us to assess not only intracellular seeding activity but also the uptake process of extracellular tau into the cells. Tau needs to be actively taken up by the cells to induce intracellular aggregates and FRET positive signals in this assay. Both ISF and CSF tau from rTg4510 mice were taken up by the cells, even without the aid of lipofectamine (Fig. 2D, E). These findings imply that extracellular tau from a diseased brain can be potentially bioactive, and that ISF and CSF can donate tau that can be taken up by cells.

To examine the molecular weight (MW) of seed-competent tau, rTg4510 CSF samples were fractionated by sequential ultrafiltration with 100 kDa and 10 kDa cut-off membranes (Fig. 2F). Tau seeding activities of each fraction (100 kDa cut-off concentrate, 10 kDa cut-off concentrate, and 10 kDa cut-off filtrate) were assessed in HEK-tau-biosensor cells (Fig. 2G–I). The fractionation efficiency of ultrafiltration was analyzed using molecular weight markers (MWs) followed by size-exclusion chromatography (SEC) (Fig. 2F). Most proteins smaller than 75 kDa were excluded by ultrafiltration with the 100 kDa cut-off membrane (Fig. 2F), suggesting that monomer-sized and truncated forms of tau (< 55 kDa)

were separated from the 100 kDa cut-off concentrate. ELISA measurement of tau levels in each fraction showed that approximately 20% of total tau was in the 100 kDa cut-off concentrate (Fig. 2H). Tau seeding assay in HEK-tau-biosensor cells revealed that tau seeding activity was present in the 100 kDa cut-off concentrate but not in other fractions (Fig. 2I), implying that seed-competent tau in rTg4510 CSF is the HMW form.

Postmortem ventricular CSF from AD patients contained bioactive HMW tau species

The results of the mouse CSF experiments suggested that CSF tau may be bioactive. However, limited sample volumes of mouse CSF precluded a detailed biochemical characterization of CSF tau and its relevance to seeding and uptake properties. To conduct a more detailed biochemical analysis, we utilized a large sample volume of human ventricular CSF obtained at autopsy. Postmortem ventricular CSF samples were obtained from five cases of neuropathologically confirmed AD (Braak stage V–VI) and three non-AD cases (Table 1). The total tau levels in each sample were measured using an ELISA (Fig. 3A). SEC analysis of the MW distribution of tau revealed a small peak of HMW tau species (SEC Frc. 1, > 669 kDa), in addition to a dominant low-MW (LMW) tau peak (SEC Frc. 4, 44–150 kDa) (Fig. 3B, C), in ventricular CSF from AD. A similar distribution pattern was previously observed in a mouse model and human AD brain lysates^{7,26}. Although the amount of HMW tau species was relatively low, it was detected with a sensitive ELISA (the lower limit of quantification of the ELISA is < 10 pg/ml, and the lowest value of HMW tau was 58.3 pg/ml (Frc.1 from #1319) (Fig. 3C). The presence of HMW tau in the AD ventricular CSF was also confirmed by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) blot (most evident in the highest samples #1223 and #1226) (Fig. 3D). A HMW tau smear was also visible in two non-AD cases (#1453, FTD and #1477, control), being most evident in #1477.

AD ventricular CSF showed tau seeding and uptake activity in HEK-tau-biosensor cells (Fig. 3E–F), demonstrating that bioactive tau species was present in ventricular CSF. Non-AD cases did not induce tau seeding (Fig. 3E), even though #1453 and #1477 had HMW tau on the SDD-AGE. This implies that not only the MW size of tau but also other post-translational modifications may be important for seeding activity. Notably, the seeding activity of isolated HMW tau species from ventricular CSF (SEC Frc.1) was significantly higher than that of the original ventricular CSF, with the HMW tau exerting seeding activity at a concentration as low as 0.2 ng/ml, whereas the original CSF had no tau seeding at a low concentration (Fig. 3G).

To further characterize the tau species that were capable of seeding, we assessed the tau seeding activity of total or phospho tau immunodepleted ventricular CSF. A phospho-tau specific antibody (pS396) was less efficient at immunodepletion than a total tau antibody (HT7) (Fig. 3H); however, the phospho-tau antibody (pS396) dramatically reduced tau seeding activity (Fig. 3I), and its efficiency was significantly higher than that of the total tau antibody (Fig. 3J). This suggests that the bioactive tau species in ventricular CSF was phosphorylated and that it comprised only a small fraction of total tau. Taken together, these findings imply that phosphorylated HMW tau is present in AD ventricular CSF and, despite its low abundance, is important for the seeding activity.

HMW tau accumulates in lumbar CSF of AD patients

To examine whether the HMW tau species is present in human lumbar CSF, we next assessed the MW size distribution of tau in lumbar CSF from living control, AD, and frontotemporal dementia (FTD) patients. Lumbar CSF samples were obtained from fifteen clinically diagnosed AD patients, ten FTD patients, and nineteen non-demented control subjects (Tables 2 and S1). Total tau, phospho-tau (pT181), and A β 1-42 levels were measured using a multiplex immunoassay system (Fig. 4A-D). The CSF from AD patients showed a typical AD-like pattern: they had significantly higher total and phospho-tau (vs. control and FTD) and lower A β 1-42 levels (vs. control) (Fig. 4A-D). SEC analysis demonstrated that the lumbar CSF from AD patients contained a small peak of HMW tau species, in addition to a dominant LMW tau peak, whereas the CSF from control subjects had only a LMW tau peak and a trace amount of HMW tau species (Fig. 4E, F). FTD cases had only LMW peak with little HMW tau. Interestingly, the difference in the tau levels among the groups was more evident in the HMW SEC fraction (Frc.1) than in the dominant LMW fraction (Frc.4).

Potentially seed-competent HMW tau is present in lumbar CSF of AD patients

We next examined seeding activity of HMW tau in human lumbar CSF. First, we tested eight cases (one control, one non-AD dementia patient, and six AD patients) from which we were able to obtain sufficient CSF (at least 1.5 ml/case) to isolate HMW tau by ultrafiltration with a 100 kDa cut-off membrane (Fig. 5A, B). HMW tau in lumbar CSF was concentrated approximately 50 times (30 μ l of final concentrate from 1.5 ml of original CSF) and applied to HEK-tau-biosensor cells (1% lipofectamine). Intracellular tau aggregates were detected in three AD samples that had high HMW tau on SEC analysis (Fig. 4E, F), but no tau aggregates were detected in low HMW tau AD, non-AD or control CSF samples (Fig. 5B), suggesting that HMW tau is a seed-competent species.

To perform more sensitive and quantitative analysis of tau seeding activity, we measured FRET signals by flow cytometry (Fig. 5C-E) in addition to confocal image analysis. This assay required a 96-well format rather than the 384-well format used for confocal image analysis in other experiments of this study. A much larger volume of the isolated HMW samples (100 kDa cut-off concentrate) was needed to obtain enough material to perform the FRET flow cytometry assay. We were able to obtain a sufficient volume of CSF (at least 8 ml/case) from one non-AD (#V1001) and two AD (#V1018 and #Z09683) patients. HMW tau in these CSF samples was concentrated approximately 40 times (200 μ l of final concentrate from 8.0 ml of original CSF) and applied to HEK-tau-biosensor cells (1% lipofectamine). Intracellular tau aggregates were observed in the high HMW tau CSF of one AD patient (#Z09683), but not in low HMW tau AD (#V1018) or non-AD (#V1001) CSF samples on confocal image analysis (Fig. 5D). Flow cytometry assay detected significantly higher FRET signals in the cells treated with high HMW tau AD CSF (#Z09683) (Fig. 5E). FRET signal values from the other two samples were not significantly different from the no-treatment group (Fig. 5E).

To ensure that the seeding assay results did not occur due to an artificial tau aggregation during ultrafiltration, we performed the same experiment using recombinant human tau. Pre-

and post-ultrafiltration samples (Fig. 5F) were assessed by both SEC (Fig. 5G) and SDD-AGE (Fig. 5H), confirming that ultrafiltration did not induce artificial HMW aggregates from LMW (monomer/dimer) tau. We also confirmed that post-ultrafiltration samples from recombinant tau did not induce tau aggregates in the tau seeding assay during the same timeframe of the experiment (Fig. 5I).

Taken together, these findings suggest that potentially seed-competent HMW tau is present in lumbar CSF of human AD patients, although in amounts that are technically challenging to detect.

Discussion

Elevated CSF tau is found in most patients with AD and is believed to be a well-validated biomarker^{9,11,27}; however, the biological properties of CSF tau and its relevance to AD pathogenesis remain largely unknown. Here, we characterized biochemical and biological properties of brain extracellular tau from various sources: ISF and CSF from a mouse model, human postmortem ventricular CSF, and lumbar CSF from AD patients. We employed a sensitive *in vitro* assay using FRET-based HEK-tau-biosensor cells¹⁹ to assess the seeding/uptake properties of extracellular tau. SEC analysis combined with the subsequent acetone precipitation method and sensitive tau ELISA (see *Materials and Method*) allowed us to demonstrate the presence of the HMW tau species in human lumbar CSF samples (Fig. 4E, F). Notably, HMW tau isolated from lumbar CSF of AD patients is potentially seed-competent (Fig. 5). To the best of our knowledge, this is the first report that has demonstrated the detection and characterization of bioactive HMW tau in human CSF.

The cause of the elevation of CSF tau in AD is uncertain. While it might be a “byproduct” of neurodegeneration in the AD brain, other neurodegenerative diseases such as FTD do not show the same CSF tau signature. Our current data show that the rare HMW species of seed-competent tau that we detected in postmortem human brain⁷ can also be detected using high-sensitive methods in AD CSF, but is absent in FTD CSF (Fig. 4). FTD is known to associate with a variety of neuropathological phenotypes which might influence the results of the CSF tau analysis. In this study, we used lumbar CSF from living patients and the diagnosis of FTD was based on clinical assessments. Further study using a larger number of cases with post-mortem data will provide more specific information regarding the HMW CSF tau signature of FTD patients. Our findings from transgenic mouse CSF and human ventricular and lumbar CSF experiments provide the first direct evidence demonstrating that CSF tau can be potentially bioactive in terms of tau uptake and seeding properties. This suggests that the HMW tau we observed in brain homogenates of rTg4510 mice and in human AD brain homogenates⁷ occurs during the course of human AD, and is not a function of tau over-expression in a mouse model or a postmortem artifact. Moreover, our data suggest that rTg4510 CSF contains a biologically similar species, suggesting that it is a potential model for human AD in this respect.

Taking advantage of the large volume of ventricular CSF, we examined the biochemical characteristics of CSF tau and its relevance to biological properties using multiple approaches such as SEC, SDD-AGE, and immunodepletion by the phospho-tau specific

antibody, combined with HEK-tau-biosensor cell assay. A postmortem ventricular CSF from AD cases contained HMW tau species (> 669 kDa) in addition to a dominant LMW (45–150 kDa) peak (Fig. 3B–D). The HMW tau, though very low in abundance, had significantly higher seeding activity; the isolated HMW tau was bioactive even at a very low concentration (as low as 0.2 ng/ml) (Fig. 3G). Importantly, the result from the IP experiment suggests that the tau species that is competent for seeding is phosphorylated; antibody targeting phospho-tau (pS396), despite pulling down only a small fraction of the tau, dramatically reduced seeding activity (Fig. 3H–J). This is very consistent with our previous observations from transgenic mouse and human AD postmortem brain homogenates⁷. However, it must be noted that we used postmortem ventricular CSF for these experiments, and postmortem changes, such as contamination with intracellular tau released from dead neurons, may have affected our results.

We performed explanatory analyses of the relationship of HMW tau, compared with total tau, with clinical features in this small sample of AD patients (data not shown). We observed a positive correlation between HMW tau levels and age of disease onset (Spearman correlation, $r = 0.544$, $P < 0.05$, $n = 15$). Although total tau CSF is not well correlated with disease state²⁸, we expected, perhaps, that HMW tau would be more closely related to severity of disease. Surprisingly, these preliminary analyses suggested the opposite: a negative correlation of HMW tau and decreased performance in Mini-Mental State Examination (MMSE) (Spearman correlation, $r = 0.574$, $P < 0.05$, $n = 14$). Tau levels in total CSF did not correlate significantly with the age of onset or MMSE scores. An analysis using a larger number of patients' samples, along with prospective follow-up examinations, will be needed to obtain a conclusive interpretation regarding the clinical significance of HMW CSF tau. From a differential diagnostic point of view, further study is warranted to assess the CSF HMW tau signature of other diseases with elevated CSF tau, such as vascular dementia and Creutzfeldt-Jakob disease.

The human lumbar CSF tau level is relatively low even in AD patients (200–1,200 pg/ml), which makes it challenging to detect tau in post-SEC (and therefore diluted) samples even with sensitive ELISA. To overcome this issue, we used an acetone precipitation method to concentrate each SEC fraction and successfully detected rare HMW tau species from lumbar CSF samples. In order to assess seeding activity of the rare HMW tau in lumbar CSF, we fractionated and concentrated HMW tau using an ultrafiltration technique with large-volume lumbar CSF (Fig. 5A, C) and utilized FRET flow cytometry for sensitive and quantitative detection of intracellular tau aggregation (Fig. 5E). We were able to detect tau seeding activity in high HMW tau lumbar CSF of AD patients (Fig. 5B, D, E), suggesting that CSF HMW tau can be potentially bioactive (seed-competent).

The low concentration of the lumbar CSF HMW tau (approximately 40 pg/ml on average in AD, Fig. 4F) precluded detailed biochemical characterization. Nevertheless, the results—that AD patients have a distinct pattern of HMW-size CSF tau compared with control subjects, and CSF HMW tau can be potentially seed-competent—can be of importance for new biomarker development. The results may also give new insight into the biology of extracellular tau in AD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sensitivity and specificity of the FRET-based tau seeding assay *in vitro*

(A) HEK-tau-biosensor cells were treated with AD brain extracts (PBS-soluble, 10,000 g spin) from the entorhinal cortex or cerebellum (1% lipofectamine). Total tau levels were measured with ELISA. The number of tau aggregate-positive cells was quantified at 48 h. ($n = 3$ / group) * $P < 0.05$, Mann-Whitney U-test. (B) HEK-tau-biosensor cells were treated with AD brain extracts (entorhinal cortex or cerebellum) or recombinant full-length human tau (441 aa) at designated tau concentrations (1% lipofectamine). Representative confocal images (FRET channel) of intracellular tau aggregates at 48 h are shown. Scale bar: 10 μ m. The assay was performed in a 384-well plate.

Figure 2. Seeding and uptake activity are present in mouse extracellular tau

(A) The seeding and uptake activity of mouse brain extracellular tau were assessed using FRET-based HEK-tau-biosensor cells. Brain ISF samples were collected from rTg4510 and control mice (6–13 months old) using an *in vivo* microdialysis technique (1,000 kDa cut-off probe that allows the collection of HMW proteins). CSF samples were collected from the cisterna magna. (B–C) Tau seeding assay in HEK-tau-biosensor cells. Mouse ISF (B) or CSF (C) samples were applied to the cells with lipofectamine (1%). Confocal images (FRET channel, left) and the quantification of intracellular tau aggregates (right) at 48 h are shown. ($n = 4–6$) * $P < 0.05$, Mann-Whitney U-test. (D–E) Tau uptake assay in HEK-tau-biosensor cells. Mouse ISF (D) or CSF (E) samples were applied to the cells without lipofectamine. Confocal images (FRET channel, left) at 48 hours are shown. (F) Protein fractionation and concentration using ultrafiltration spin column. (F, top left) Three molecular weight markers (MWs), 669 kDa (thyroglobulin), 75 kDa (conalbumin), and 13.7 kDa (ribonuclease), were fractionated using an ultrafiltration spin column with a 100 kDa cut-off membrane (3,000 g spin, 10 min). The original MWM mixture solution, concentrate, and filtrate were analyzed by using SEC. SEC patterns of the original MWM mixture (F, bottom left), concentrate (top right), and filtrate (bottom right) demonstrate that high-molecular-weight proteins (669 kDa) were retained in the concentrate and most lower-molecular-weight proteins (75 kDa and 13.7 kDa MWs) were fractionated into the filtrate. (G–I) Seed-competent CSF tau was present in the high-molecular-weight fraction. (G) CSF samples from rTg4510 mice were fractionated by sequential ultrafiltration using 100 kDa and 10 kDa cut-off membranes. Pooled CSF samples (180 μ l total volume) were used for each run of fractionation. (H) Human tau levels in each fraction (100 kDa cut-off concentrate, 10 kDa cut-off concentrate, and 10 kDa cut-off filtrate) were measured using human tau-specific ELISA. ($n = 3$) (I) Tau seeding assay in HEK-tau-biosensor cells. Each fraction was applied to the cells with lipofectamine (1%). Confocal images (FRET channel) at day 3 are shown. Scale bar: 10 μ m. Tau seeding/uptake assays were performed in 384-well plates.

Figure 3. Human AD postmortem ventricular CSF contains bioactive HMW tau species (A) Total tau levels in human postmortem ventricular CSF samples (ELISA). (B–D) AD ventricular CSF contains HMW tau species. (B) SEC analysis of ventricular CSF tau. Total tau levels in each SEC fraction were measured using ELISA. (C) Total tau values in HMW SEC fraction (Frc.1). (D) SDD-AGE analysis demonstrates the presence of HMW tau species in postmortem ventricular CSF. Recombinant full-length tau was loaded as a molecular weight marker (30 ng/lane). Total tau antibody (Tau13) was used. (E–F) Tau seeding (E) and uptake (F) assay in HEK-tau-biosensor cells. Ventricular CSF samples were applied to the cells with (seeding assay) or without (uptake assay) lipofectamine in 384-well plates. Confocal images (FRET channel) at 48 or 72 h are shown. White arrowhead indicates intracellular tau aggregate. (G) The ventricular CSF HMW tau displays higher seeding activity. The HMW tau species (SEC Frc.1) was isolated from ventricular CSF using SEC and applied to HEK-tau-biosensor cells (384-well plates) at different concentrations of total tau (0.04, 0.2 and 1.0 ng/ml). (G, left) Representative confocal images of tau aggregates induced by HMW tau (white arrowhead). (G, right) Quantification of intracellular tau aggregates. ($n = 4–7$) $**P < 0.01$, Mann-Whitney U-test. (H–J) The immunodepletion of phospho-tau from ventricular CSF efficiently reduced tau seeding activity. Ventricular CSF (#1319, #1223 and #1226) were immunodepleted with total (HT7) or phospho (pS396) tau specific antibodies. (H) Total tau levels in tau-immunodepleted samples (ELISA). ($n = 3$) $**P < 0.01$, One-way ANOVA and a subsequent Dunnett test. (I) Tau seeding assay in HEK-tau-biosensor cells. (I, left) Representative confocal images of tau aggregates (white arrowhead). (I, right) Quantification of intracellular tau aggregates (384-well plates). ($n = 3$) $**P < 0.01$, One-way ANOVA and a subsequent Dunnett test. (J) Blocking efficiency was defined as the percentage of tau-uptake reduction (vs. control-IgG) multiplied by tau levels

in the immunodepleted ventricular CSF (% control-IgG). ($n = 3$) $*P < 0.05$, Student's t-test. Scale bar: 10 μm . HMW, high molecular weight; LMW, low molecular weight; SDD-AGE, Semi-denaturing detergent agarose gel electrophoresis.

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Figure 4. HMW tau species accumulates in the lumbar CSF of AD patients

(A–C) Lumbar CSF total tau (A), phospho-tau (B) and Aβ1-42 (C) levels in control ($n = 19$), AD ($n = 15$), and FTD ($n = 10$) patients. AD patients had significantly increased total and phospho tau (pT181) levels (vs. control and FTD) and lower Aβ1-42 levels (vs. control). $*P < 0.05$, $**P < 0.01$, Kruskal-Wallis test followed by Steel-Dwass multiple comparison test. Bars indicate mean values. (D) Scatterplot of CSF Aβ1-42 and total tau levels in control, AD, and FTD patients. The cut-points of 133 pg/ml for total tau (red line) and 370 pg/ml for Aβ1-42 (blue line) generate a sensitivity and specificity of $> 80\%$. (E–F) SEC analysis of lumbar CSF tau. (E) The thin dashed lines represent total tau levels for individual subjects, and the thick solid line represents the averaged value for each group. (F) Total tau levels in each SEC fraction. AD patients had significantly higher tau levels in the HMW SEC fraction (Frc.1) compared with control subjects. $*P < 0.05$, Kruskal-Wallis test followed by Steel-Dwass multiple comparison test. HMW, high molecular weight.

Figure 5. Potentially seed-competent HMW tau is present in lumbar CSF of AD patients (A-B) Lumbar CSF samples (1.5 ml) from eight subjects were fractionated and concentrated using an ultrafiltration spin column with a 100 kDa cut-off membrane. Tau seeding activities in the HMW fraction (100 kDa cut-off concentrate) were assessed using HEK-tau-biosensor cells (1% lipofectamine) in 384-well plates. (B) Tau seeding assay in HEK-tau-biosensor cells. Confocal images (FRET channel) at day 3 are shown. (C-E) Sensitive and quantitative detection of tau seeding activity in human lumbar CSF using FRET flow cytometry. (C) Large-volume (8 ml) lumbar CSF samples were fractionated and concentrated using an ultrafiltration spin column with a 100 kDa cut-off membrane. Concentrates were applied to HEK-tau-biosensor cells (1% lipofectamine) in 96-well plates, followed by confocal image analysis and FRET flow cytometry. (D) Confocal images (FRET channel) at day 3. (E) Tau seeding activity measured by FRET flow cytometry. High HMW tau CSF from an AD patient (# Z09683) produced a high FRET signal. ** $P < 0.01$ vs. NT, One-way ANOVA and a subsequent Dunnett test. NT, no treatment. Values in parentheses indicate the levels of HMW tau in SEC Frc.1 measured in Fig. 3. (F-I) Ultrafiltration does not generate a seed-competent HMW tau species from recombinant tau. One point five ml of recombinant full-length human tau (441 aa) at 1 $\mu\text{g/ml}$ was concentrated using an ultrafiltration spin column with a 10 kDa cut-off membrane. (F) Total tau levels of pre- and post-ultrafiltration samples

(ELISA). (G) SEC analysis of recombinant tau. (H) SDD-AGE analysis. Thirty ng of tau from pre-/post-ultrafiltration samples were loaded per lane. (I) Tau seeding assay in the HEK-tau-biosensor cells (384-well plates). Ten ng/ml of tau (pre-/post-ultrafiltration) were applied to the cells with 1% lipofectamine. Confocal images (FRET channel) at day 3 are shown. AD brain extract (PBS-soluble, 10,000 g spin, 10 ng/ml total tau) from entorhinal cortex was used as a positive control. White arrowhead indicates intracellular tau aggregates. Scale bar: 20 μ m. HMW, high molecular weight; SDD-AGE, Semi-denaturing detergent agarose gel electrophoresis.

Table 1

Characteristics of the subjects used in the ventricular CSF study

Case (sample #)	Age at death (years)	Sex	Diagnosis	Braak stage
AD	#1318	Male	AD, CAA	VI
	#1319	Female	AD	VI
	#1362	Male	AD	VI
	#1223	Female	AD	V
	#1226	Male	AD	VI
non-AD	#1453	Female	FTD	I
	#1589	Male	ALS	-
	#1477	Male	Control	II

Table 2
Demographic data of study participants

	AD	Control	FTD	p value
Number	15	19	10	
Age at LP (years), mean (SE)	71.1 ± 6.8	69.7 ± 7.4	63.8 ± 8.0	P = 0.089 (Kruskal-Wallis test)
Gender (male:female)	10:5	8:11	8:2	
MMSE, mean (SE)	24.3 ± 4.3 ^a	29.6 ± 0.6	18.3 ± 9.6 ^a	P < 0.0001 (Kruskal-Wallis test)

Abbreviations: AD, Alzheimer's disease; FTD, frontotemporal dementia; LP, lumbar puncture; MMSE, Mini-Mental State Examination.

^aP < 0.01 compared to control (Steel-Dwass multiple comparison test)