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Isolation and characterization of antibody fragments selective for toxic oligomeric tau

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Abstract

Oligomeric tau species are important in the onset and progression of Alzheimer's Disease (AD) as they are neurotoxic and can propagate tau tangle pathology. Therefore reagents that selectively recognize different key morphologies of tau are needed to help define the role of tau in AD and related diseases. We utilized a biopanning protocol that combines the binding diversity of phage-displayed antibody libraries with the powerful imaging capability of atomic force microscopy (AFM) to isolate single chain antibody fragments (scFvs) that selectively bind toxic oligomeric tau. We isolated three different antibody fragments that bind oligomeric but not monomeric or fibrillar tau. The scFvs differentiate brain tissue homogenates of both 3×TG and tau-AD mice from wild type mice, detecting oligomeric tau at much earlier ages than when neurofibrillary tangles are typically detected. The scFvs also distinguish human post-mortem AD brain tissue from cognitively normal post-mortem human brain tissue demonstrating the potential of this approach for developing biomarkers for early detection and progression of AD.

Keywords

Alzheimer's disease; toxic tau aggregates; phage display; brain tissue; biomarker; scFv; oligomeric tau; antibody fragments; immunotherapy

1. Introduction

Alzheimer's disease (AD) is a devastating progressive neurodegenerative disease that causes brain atrophy, memory deterioration and cognitive loss in affected individuals. It is the sixth leading cause of death in the United States, currently affecting over 5.4 million Americans

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Disclosure statement for authors

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with annual costs of over \$200 billion in medical care (2012). Although AD was first discovered over a hundred years ago, and substantial progress has been made in understanding the etiology of the disease, there are still no effective therapeutic or definitive diagnostic approaches available. AD is characterized by the presence of two hallmark pathologies: extracellular neuritic plaques containing insoluble fibrillar aggregates of amyloid-beta ($A\beta$) and intracellular neurofibrillary tangles (NFTs) containing fibrillar aggregates of tau. Although these insoluble aggregated species have long been considered as the primary toxic elements of AD, increasing evidence indicates that small soluble oligomeric forms of both $A\beta$ and tau play more critical roles in the onset and progression of AD than the fibrillar aggregates (Lambert et al., 1998, Glabe, 2006, Ward et al., 2012). The role of $A\beta$ aggregation in AD in particular has been extensively studied (Ono et al., 2002, Gestwicki et al., 2004, Ono et al., 2004, Ono et al., 2006, Hirohata et al., 2007, Sinha et al., 2011, Sinha et al., 2012), but despite very promising results in animal models, various therapeutic routes to target $A\beta$ aggregation have had only very limited success in clinical trials (Check, 2002, Gilman et al., 2005, Salloway et al., 2009, Sperling et al., 2010). The role of tau in the progression of AD is gaining more attention, including studies to elucidate the roles of different variants and aggregate forms of tau (Berger et al., 2007, Lasagna-Reeves et al., 2010, Lasagna-Reeves et al., 2012b, Ward et al., 2012, Blair et al., 2013, Cohen et al., 2013, Cowan and Mudher, 2013, Gerson and Kaye, 2013, Yanamandra et al., 2013).

Tau is a microtubule-associated protein, generally located in the axons of neurons, where it is involved in the assembly and stabilization of microtubules from tubulin. Although human tau is encoded by a single gene on chromosome 17q21, six major tau isoforms can be formed by alternative posttranscriptional splicing of exons 2, 3 and 10. Tau can also be post-translationally modified by phosphorylation, glycosylation, ubiquitinylation, or glycation among others (Avila et al., 2004, Hernandez and Avila, 2007, Wang et al., 2007) resulting in a wide variety of different tau species that exist in vivo. Since tau hyperphosphorylation is associated with AD, it has been extensively studied, and inhibition of kinases involved in tau phosphorylation has been pursued as a potential therapeutic approach (Schneider et al., 1999, Alonso et al., 2001, Hanger et al., 2009). Levels of phosphorylated variants of tau correlate well with AD and other tauopathies including FTD (Alonso et al., 2004). Hyperphosphorylation of the microtubule-binding domain (MBD) of tau has been suggested to be one of the factors that promotes misfolding and loss of physiological function (Morris et al., 2011). However, phosphorylation of tau may also be required for some cellular functions including adult neurogenesis, as new adult-born granule neurons contain a significant amount of hyperphosphorylated three repeat (3R) tau variants (Bullmann et al., 2007). Therapeutic strategies aimed at regulating kinase activity bear the risk of interrupting normal phosphorylation dependent functions of tau along with other cellular functions. Given the complexity of the many different potential isoforms of tau that can occur in vivo and the uncertainty as to the physiological effects of tau hyperphosphorylation and aggregation, the roles of different hyperphosphorylated and aggregated tau variants in AD require clarification (Schneider et al., 1999, Congdon and Duff, 2008, Cowan and Mudher, 2013) for diagnostic or therapeutic development.

Numerous studies indicate that soluble aggregates of tau play an important role in the pathology of AD (Berger et al., 2007, Yoshiyama et al., 2007). Both brain derived and recombinant oligomeric tau aggregate species disrupt intracellular calcium levels and are toxic to cultured human neuronal cells when added extracellularly (Demuro et al., 2005, Gomez-Ramos et al., 2006, Gomez-Ramos et al., 2008). In animal models expressing human tau, neurodegeneration-related phenotypes including behavioral impairments, neuronal loss, and synapse lesions correlate better with the presence of soluble tau oligomers and pre-filament species than with fibrillar NFT levels (Andorfer et al., 2005, Berger et al., 2007, Brunden et al., 2008, Polydoro et al., 2009). Neuronal loss also precedes NFT formation suggesting involvement of other species such as oligomeric tau variants (Marx, 2007, Brunden et al., 2008, Kaye et al., 2009, Meraz-Rios et al., 2010). In postmortem human brains, high oligomeric tau levels were detected in the frontal lobe cortex at early stages of AD before the presence of NFTs (Maeda et al., 2006, Maeda et al., 2007, Lasagna-Reeves et al., 2012b). Oligomeric tau may also be responsible for transmission of pathology with a prion-like mechanism as NFT tau pathology spreads from brain regions seeded with oligomeric tau into other regions resulting in aggregation of endogenous tau (Clavaguera et al., 2009, de Calignon et al., 2012, Lasagna-Reeves et al., 2012a, Lasagna-Reeves et al., 2012b, Liu et al., 2012, Wu et al., 2013). We have previously shown using recombinant human tau (rhTau) that extracellular trimeric, but not monomeric or dimeric species are toxic to human neuronal cells (Tian et al., 2013).

Here we describe isolation of antibody based reagents that selectively recognize toxic oligomeric tau species. We used an scFv library (Sheets et al., 1998) as a source of binding diversity and an AFM based biopanning protocol (Barkhordarian et al., 2006, Zameer et al., 2006, Emadi et al., 2007, Emadi et al., 2009, Kasturirangan et al., 2012, Kasturirangan et al., 2013) as a selection tool to isolate scFvs that selectively bound the trimeric tau species. We utilized several subtractive panning steps in the selection protocol to ensure the removal of all scFvs cross-reactive with monomeric tau and other off-target proteins. We identified three different scFvs that bound trimeric but not monomeric or fibrillar tau. Since the scFvs bind trimeric, but not monomeric tau, the scFvs do not recognize a linear sequence of tau, but rather an epitope that is at least partially conformation dependent. The scFvs react with oligomeric tau in a mammalian neuronal cell line that overexpresses tau and in brain tissue from two different transgenic AD mouse models, indicating that significant levels of oligomeric tau are present in brain tissue well before NFTs are detected. The scFvs also reacted with oligomeric tau in post-mortem human AD brain tissue. Levels of oligomeric tau in post-mortem human brain tissue were significantly elevated in the AD samples compared to age-matched non-AD brain samples.

2. Material and Methods

2.1 scFv Phage Display Library

The Sheets phage display scFv library (Sheets et al., 1998) with an estimated diversity of 6.7×10^9 was generously provided by Dr. Yu Zhou (Department of Anesthesia, University of San Francisco) and used for biopanning. Phage were produced and purified as previously described (Marks et al., 1991). A final titer of 10^{13} – 10^{14} pfu/mL was used for biopanning.

2.2 Aggregated Tau species

Two isoforms (1N4R and 2N4R) of recombinant human tau (rhTau) species were used for the panning protocols. Monomeric and oligomeric forms of tau were generated as described previously (Tian et al., 2013). A fibrillar 2N4R aggregate stock was prepared following a heparin fibrillation protocol by mixing rhTau 2N4R monomer (final molarity of 4 μ M) and low molecular weight heparin (final molarity of 4 μ M) in final 20 mM Tris-HCl pH 7.4 and final 5 mM DL-Dithiothreitol (DTT) in deionized water (DI water). The mixture was incubated at 37 °C for 2 weeks with occasional mixing.

2.3 Biopanning against rhTau 1N4R trimer

The biopanning process was performed essentially as previously described (Kasturirangan et al., 2013) with the following modifications. The biopanning process was divided into “subtractive panning” and “positive panning” steps (Figure 1). The subtractive panning steps were designed to remove all scFv-displayed phage from the library pool which bound to selected off-target antigens. A control protein bovine serum albumin (BSA) was used to remove non-specific binding phage, and monomeric tau was used to remove all phage binding non-aggregated linear epitopes of tau. The positive panning step recovered any scFv-displayed phage from the remaining phage pool that selectively bound trimeric tau. Each step was monitored by AFM to ensure that essentially all phage binding BSA and monomeric tau were removed and phage binding trimeric tau were recovered.

2.4 Subtractive panning step

A set of high affinity immunotubes were coated with 2 mL/tube of 1 mg/mL BSA in carbonate/bicarbonate coating buffer pH 9.6 and another set with 2 mL of 12 μ g/mL tau 1N4R monomer in the same coating buffer and incubated overnight at 4°C. After antigen immobilization, immunotubes were washed extensively with phosphate buffered saline (PBS) and sealed to keep moist. A total volume of 0.5 mL of the phage display library was added to the first tube coated with BSA. The tube was then sealed and incubated at room temperature for 30 minutes with gentle agitation ensuring that the phage solution did not contact uncoated regions of the immunotube. After incubation, the phage solution was removed and additional unbound phage rinsed off with 100 μ L PBS. The phage and rinse solutions were combined and added to a second tube containing BSA and then to sequential tubes following the same procedure for each tube. The final recovered phage solution volume was approximately 1 mL. A 10 μ L aliquot of phage solution recovered after incubation with each tube was added to mica containing BSA and imaged by AFM to determine whether any phage remaining in the phage pool that could still bind BSA. If no bound phage were observed, the subtractive panning step successfully removed essentially all phage binding to the target antigen, in this case BSA. A second subtractive panning round was performed using immunotubes coated with monomeric 1N4R rhTau to remove all phage binding monomeric tau. The process was performed and monitored as described above. After the second round of subtractive panning, the final remaining phage solution was stored in 100 μ L aliquots at –80°C.

2.5 Positive panning

A 10 μ l aliquot of 60 μ g/mL of positive target antigen, trimeric rhTau 1N4R, was deposited on a piece of freshly cleaved mica, incubated at room temperature for 10 minutes, and then extensively washed with DI water and dried. A 200 μ l aliquot of the remaining phage pool obtained after subtractive panning was added to the mica, incubated at room temperature for 10 minutes, and then washed with 2 mL 0.1% Tween20/DI water and at least 10 mL DI water to remove all unbound phage. The positive panning step was performed in duplicate for analysis by AFM to verify the presence of phage binding trimeric tau. Bound phage were eluted with 1.4% triethylamine (TEA) and neutralized after 5 minutes with an equal volume of 1M Tris-HCl pH 7.4 buffer. The eluted phage stock were recovered as described (Emadi et al., 2009). Single colonies were collected, individually grown and stored at -80°C .

2.6 Atomic force microscope (AFM) imaging

AFM imaging and analyses were performed as described previously (Wang et al., 2009). Aliquots were deposited and incubated for 10 min on freshly cleaved mica at room temperature before the mica surface was washed extensively with DI water and dried with compressed nitrogen flow. To image phage binding specificity for the different tau isoforms, an additional stringent wash with 0.1% Tween20/DI water was performed to remove non-specific binding phage. The coated mica samples were imaged in air using a MultiMode AFM Nanoscope IIIA system (Veeco/Digital instruments, Santa Barbara, CA) operating in tapping mode using silicon AFM probes (VISTAprobes, Nanoscience instruments).

2.7 Single clone screening with AFM

Following positive panning, a phage preparation from each individual recovered clone was analyzed for target binding specificity by AFM. Aliquots of each phage were added to mica coated with either BSA, monomeric or trimeric tau. Samples showing the highest binding levels toward trimeric tau, but no reactivity toward BSA or monomeric tau were selected for further characterization.

2.8 DNA sequence correction

DNA sequences of recovered clones were obtained and compared with other known scFv sequences (Marks et al., 1991). All recovered clones from the positive panning step contained a missing base pair near the amino terminal of the scFv sequence resulting in a shift in the reading frame (Table 1). The frameshift was corrected in selected clones using polymerase chain reaction (PCR) with customized primers (Table 2). The forward primers encompass the NcoI site (5'-CCATGG-3') upstream of scFv sequence and include the missing base, while the reverse primer encompasses the NotI site (5'-GCGGCCGC-3') downstream of scFv sequence. The corrected scFv gene sequences were ligated into the pGEMT plasmid vector for sequencing to confirm the desired DNA sequence, and then ligated into the pIT2 plasmid vector which contains a hexahistidine tag and c-myc tag for protein expression. The pIT2 plasmids were transformed into either *E. coli* strain HB2151 for scFv expression or TG1 for phage expression.

2.9 Phage binding specificity assay

Binding specificities of the sequence corrected phage clones were verified by AFM. Purified phage were deposited and incubated on mica coated with the different tau species and imaged to confirm binding specificity as described above.

2.10 Soluble scFv production and purification

Production and purification of the sequence corrected scFv proteins were performed as described previously (Barkhordarian et al., 2006). Concentrated supernatant, periplasm and cell lysate fractions were prepared separately and tested for presence of scFv. Most of the scFv was located in the periplasmic fraction, with lower amounts excreted to the supernatant as expected (Kipriyanov et al., 1997). All fractions containing scFv were pooled and purified using a Ni-NTA agarose beads column (Qiagen, 5mL beads for 1L expression culture) and imidazole elution essentially as described (Kasturirangan et al., 2010).

2.11 Dot blot assay with human brain tissue

Postmortem pathologically confirmed human brain samples from the middle temporal gyrus (MTG) of Alzheimer's disease (AD) and cognitively normal non-demented (ND) cases were generously provided by Dr. Thomas Beach (Director of Banner/Sun Health Research Institute Brain Bank). Age and gender of the cases are provided in Supplementary Table 1. Brain extracts from the MTG of age-matched ND and AD patients were homogenized in Tris-HCl/EDTA buffer with protease inhibitor. The homogenate was spun down to remove solids and the supernatant containing all the soluble protein was collected and adjusted to a total protein concentration of 3 mg/mL. Aliquots of 2 μ L of 3 mg/mL brain tissue were dotted on gridded nitrocellulose membrane and probed with anti-oligomeric tau scFv essentially as described (Zameer et al., 2008). Samples were analyzed in triplicate using purified scFv. Reactivity of scFv with brain tissue samples was analyzed using ImageJ and recorded in the form of densitometric value (Kasturirangan et al., 2013). Each value was calibrated on a scale of 0 to 1 in which 0 denotes the background and 1 denotes the positive control of anti-phosphorylase b (plb) scFv. We also tested reactivity of just the detection anti-c-myc antibody without any scFv added to ensure that the detection antibody was not reacting directly with the brain tissue, β -actin levels were also determined to ensure that equal protein concentrations were utilized in all samples.

2.12 Mouse brain tissue

Brains from 5, 8, 11 months old wild-type mice and 5, 9 and 13 months old 3 $\times$ transgenic Alzheimer's (3 $\times$ TG-AD) mice overexpressing human tau P301L (Oddo et al., 2003a) were generously provided by Dr. Travis Dunckley (Translational Genomics, Phoenix, AZ). Mouse hippocampus was homogenized as described above for human brain samples.

Brain tissue samples from 3 and 6 months old AD mouse model expressing mutant human tau (4R) in a mouse tau knock-out background (Tau406+/TauKO) and from a negative knock-out control (Tau406-/TauKO) were generously provided by Dr. Ottavio Arancio (Columbia University, NY).

2.13 Phage biotinylation

Phage was biotinylated for enhanced signal detection in ELISA using the EZ-Link Pentylamine-biotin kit (Thermo Scientific) as described (Williams et al., 2014). A 10^{11} pfu/mL aliquot of phage stock (0.729 mg/ml) was incubated with Pentylamine-Biotin (4.86 mM final concentration) and 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) of 0.1M final concentration at room temperature for 2 hours with stirring. Excess Pentylamine-Biotin and EDC were removed with desalting columns.

2.14 Capture ELISA

To validate that scFvs were targeting tau and to quantify oligomeric tau present in mouse brain samples, we utilized a capture ELISA essentially as described (Williams et al., 2014). Basically high affinity polystyrene microtiter 96-well plates were first coated with 100 μ L/well of 0.3 mg/ml purified scFv (the capture antibody) and incubated at 37 °C for two hours. After the unbound scFvs were removed, the plates were washed three times with 0.1% Tween20/PBS. The plates were then blocked with 2% non-fat milk/PBS at 37 °C for one hour. After a Tween20/PBS wash, an aliquot of 100 μ L/well of 0.2 mg/ml mouse brain homogenate (the target analytes) was added, incubated at 37 °C for two hours and then washed with Tween20/PBS. PBS was used as a negative control. A 100 μ L/well aliquot of 10^7 pfu/ml biotinylated phage (the detection antibody) was added at 37 °C for two hours. The wells were washed with Tween20/PBS, and then a 100 μ L/well aliquot of 0.5 μ g/ml avidin-HRP was added and incubated at 37 °C for one hour. The plates were washed again with Tween20/PBS and binding monitored using a chemiluminescent ELISA kit (SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific)). The chemiluminescent signal was read 1 minute after addition to the mixture. The immunoreactivity signals were normalized by dividing the absolute chemiluminescent readings of the samples by that of PBS control. Within each independent experiment, the mean signal obtained from all the wild-type mice samples was used as a baseline to normalize the signals obtained with the transgenic mice samples.

To verify that the isolated scFvs were binding oligomeric tau in the mouse brain tissue samples, we utilized a capture ELISA where the scFvs were used as the capture antibody and a commercially available anti-tau monoclonal antibody, AT8 (Thermo Scientific), was used as the detection antibody. AT8 targets tau forms with hyperphosphorylated Ser202/Thr205 mainly found in tau aggregates. An anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Thermo Scientific) was used to detect bound AT8.

To quantitatively compare the levels of oligomeric tau in the transgenic tau mouse brain tissue samples we used a sandwich ELISA protocol with F9T scFv as the capture antibody and a phage displayed pan-specific tau scFv (F3A) as the detection antibody essentially as described (Williams et al., 2014). The F3A scFv was obtained from the Sheets library by panning against monomeric tau. Signals reported for the transgenic tau mouse samples are relative to the signal obtained for the negative control samples (Tau406-/TauKO) in each age group.

2.15 Size analysis of individual phage target

To determine the size of the target antigen bound by individual phage particles, we deposited a 10 μ l aliquot of a fibrillar tau aggregate mixture (19.5 μ g/ml, 10 $\times$ dilution of the original prepared stock) on mica, followed by a 10 μ l aliquot of 10¹² pfu/ml phage, then incubated and rinsed the sample as described above. The tau aggregate sample contained a mixture of monomeric, oligomeric and fibrillar rhTau 2N4R aggregates. AFM images (5 μ m²) were obtained and processed using Nanoscope Analysis. The diameter of each target antigen particle bound at the tip of the phage was calculated by taking the difference between the maximum height of the particle and the adjusted baseline. At least 6 different antigen particles for each individual clone were measured and averaged to determine the particle height of the target antigen.

2.16 Statistical analysis

Samples were analyzed by one-way ANOVA with $p < 0.05$ standard and LSD post hoc significant differences test. All analyses were performed with SPSS 21.0 (IBM Corp., Armonk, NY).

2.17 Fluorescent immunocytochemistry assay

To test reactivity of the scFvs with tau aggregates generated by human neuronal cells, we cultured the human neuroblastoma cell line SH-SY5Y and its derivative SH-SY5Y-TMHT441 which overexpresses a human mutant tau 2N4R species (Loffler et al., 2012). Cells were cultivated as previously described (Tian et al., 2013) on aseptic glass slides. Growth media for the SH-SY5Y-TMHT441 cells also contains 300 μ g/ml Geneticin G-418. For immunocytochemistry analysis, cells were grown and fixed in 4% formaldehyde in PBS at room temperature for 15 minutes, then treated with 0.2% Triton 100 at room temperature for 15 minutes, washed three times with PBS and blocked for 1 hour with PBS containing 5% goat serum. The cells were incubated overnight with a 0.3 mg/ml aliquot of one of the anti-tau scFvs, F9T or D11C. The rabbit anti-c-Myc antibody (Abcam, 1:1500) was applied for 30 min. The cells were washed 3 times with PBS and incubated with 1/1000 dilution of the secondary antibodies, goat anti-mouse IgG Alexa Fluor 488 (Life Technologies, New York, NY) and DAPI (Sigma), for 1 hour at room temperature. Images were taken using a fluorescent microscope (Observer D1, Zeiss). Polyclonal rabbit antibody against human tau (Catalog: PA1-18272, Thermo-scientific, 1:400) was used as a positive control to detect tau species in SH-SY5Y-TMHT441 neurons.

3. Results

3.1 Isolation of scFvs selectively binding oligomeric tau

We utilized an AFM based panning protocol incorporating sequential subtractive and positive panning steps (Figure 1A) to isolate scFvs that selectively bind a toxic trimeric tau species. We first eliminated from the scFv library pool essentially all phage containing scFvs binding to the control protein (BSA) (Figure 1B) and then those binding to monomeric tau. After the subtractive panning steps, we isolated scFv-displayed phage that selectively bound toxic oligomeric tau employing a single positive panning step using trimeric rhTau 1N4R as the target antigen. We recovered 96 phage clones from the positive

panning step against trimeric tau (Figure 1C). Phage from each of the 96 clones were prepared separately and each sample was assayed for binding specificity to trimeric tau, monomeric tau and BSA by AFM (Figure 2A). The large irregular shaped white particles present in AFM images showing reactivity of phage particles with different antigens in Figures 1, 2 and 3 primarily represent PEG and other contaminants that are present in the phage preparations. We selected twenty clones that selectively recognized trimeric tau for further study. The DNA sequences were obtained to verify the presence of full length scFv (Marks et al., 1991, Sheets et al., 1998), and six distinct full-length scFvs were selected for further analysis (H2, F9, D11, H7, D4 and G12). Although all six clones contained complete scFv sequences, they each lacked a DNA base pair downstream of the N-terminal NcoI site and the methionine start codon (Table 1) resulting in a reading frame shift. Correct protein production despite the presence of frame shifts has been previously observed including in other phage display applications and attributed to ribosomal shift (Spanjaard and van Duin, 1988, Atkins et al., 1990, Jacobsson and Frykberg, 1995, Carcamo et al., 1998, Goldman et al., 2000, Namy et al., 2004, Suzuki et al., 2005). To enhance soluble scFvs expression efficiency in *E. coli* HB2151, we corrected the reading frame shift using PCR. Purified soluble scFv protein produced from both the uncorrected and corrected scFv sequences both had the expected 29 kDa full length scFv molecular mass (Supplemental Figure). We also verified that each sequence corrected scFv maintained the same binding specificity of the original clone using AFM (Figure 2B). Three sequence corrected clones F9T, D11C and H2A were then selected for further studies based on binding specificity and distinctive CDR sequences.

3.2 Verification of binding specificity to tau trimer

To verify that the sequence corrected F9T, D11C and H2A scFvs were selectively binding trimeric tau, we incubated a phage displayed version of each scFv with a sample of aggregated tau and used AFM to determine the average height of the particles bound by each phage particle. We then compared the height of the bound particles to the known height values of different tau aggregate species (Tian et al., 2013). A representative AFM image of an F9T scFv phage (blue arrow) binding to a target rhTau 2N4R aggregate (gray arrow) in the mixed monomeric, oligomeric and fibrillar (black arrow) tau sample is shown (Figure 3A). The average height of at least 6 different bound antigens for each scFv was determined and compared to the size of known oligomeric tau aggregates (Tian et al., 2013). The target antigen size for all three scFvs correspond to the size of a rhTau 2N4R trimer (Tian et al., 2013) (from 2.5 nm to 3.0 nm) providing further evidence that the scFvs selectively target trimeric tau (Figure 3B). The phage did not bind fibrillar tau (Figure 3A black arrow) nor did they bind other protein aggregates such as mixed aggregates of α -synuclein (Figure 3C) and A β (Figure 3D) indicating that the scFvs bind a conformational epitope specific for oligomeric tau and not a common β -sheet structural motif found in multiple protein aggregates.

3.3 Characterization of samples for oligomeric tau

Since the scFvs were isolated against a synthetic oligomeric tau variant, we then verified that the F9T, D11C and H2A scFvs recognized naturally occurring tau aggregates either

produced by cultured neurons or naturally present in brain tissue from an AD mouse model or human AD brain samples.

We tested the F9T and D11C scFvs for reactivity with a neuroblastoma SH-SY5Y cell line over expressing tau441 (SH-SY5Y-TMHT441). The control SH-SY5Y cells did not show any staining for total tau with the polyclonal tau antibodies (Figure 4A) or for oligomeric tau with D11C or F9T scFvs (Figures 4B and 4C). However extensive immunopositive staining of total tau was observed with the tau-expressing cell line (Figure 4D) and limited punctuate staining of oligomeric tau observed with D11C and F9T (Figures 4E and 4F). While fibrillar tau aggregates are generally not produced in overexpressing cell lines (Bandyopadhyay et al., 2007) it is clear from these results (Fig 4) that small oligomeric tau aggregates are selectively generated at modest levels in overexpressing tau cell lines.

The F9T, D11C and H2A scFvs also reacted with oligomeric tau aggregates present in hippocampal tissue homogenates from 9-month old 3×TG-AD mice and but not with tau protein in wild-type mouse samples (Figure 5). We utilized AT8 as a detection antibody to ensure that the scFvs bound tau aggregates.

Since the three selected clones (F9T, D11C and H2A) contain distinctive CDR sequences, we tested whether they bind similar or different epitopes on the tau oligomers. A capture ELISA method was performed using the purified scFvs as capture antibodies and the phage displayed version of each scFv as detection antibodies (Figure 6) with the 3×TG-AD mouse brain homogenates as antigen. When F9T-displayed phage was used as the detection antibody, strong signals were obtained for all three capture antibody types. In contrast, when D11C-displayed phage was used as a detection antibody, lower signals were obtained with either D11C or H2A scFv as capture antibody, and no signal with F9T scFv as capture antibody. Similarly, when H2A-displayed phage was used as a detection antibody, a lower signal was obtained with D11C scFv as the capture antibody and no signal with either F9T or H2A scFv as capture antibody (Figure 6). Since F9T phage produced the strongest immunoreactivity with brain extracts retained by all three scFvs as capture antibody, we used F9T phage as the detection antibody in all further capture ELISAs.

3.4 Time dependent tau oligomer accumulation in AD mouse brain

We analyzed how tau oligomeric levels varied with time in the hippocampus of 3×TG-AD mice (Oddo et al., 2003b). The 3×TG-AD mouse model overexpresses human mutant tau P301L (0N4R), which is prone to aggregation in tauopathies (Nacharaju et al., 1999, Pennanen et al., 2004, von Bergen et al., 2005). Whereas insoluble tau tangles are typically detected around 12–15 months of age in this mouse model (Oddo et al., 2003a), oligomeric tau levels were already quite high at 5-months of age, peaked around 9-months and declined by 13-months (Figure 7). Samples from age-matched wild-type mice did not show significant reactivity with the scFvs (Figure 7). Since the scFvs were isolated against 1N4R trimeric tau and also recognize 2N4R (Figure 3) and 0N4R (Figure 5) oligomeric tau aggregates, the scFvs target a common conformational epitope of the oligomeric tau aggregates attributable to the 4R region of the protein through which tau self-associates. In addition to tau aggregates, the 3×TG-AD mouse model also generates substantial A β aggregates. While we already demonstrated that the scFvs did not bind A β or α -synuclein

aggregates generated in vitro (Figure 3C and Figure 3D), to ensure that the anti-oligomeric tau scFvs were not also binding to other protein aggregates generated in vivo, we also tested reactivity of the F9T scFv with an AD-mouse model overexpressing human tau (Tau406+/TauKO). Similar to the results obtained with the 3×TG-AD mouse model, oligomeric tau is detected in the 6-month Tau406+/TauKO mouse samples, but not in the 3-month Tau406+/TauKO or 3- or 6-month control Tau406-/TauKO mouse brain samples (Figure 8).

Since the scFvs do not react with fibrillar tau, aggregated α -synuclein or A β , they do not recognize the β -sheet structure common to these aggregates but rather recognize epitopes unique to oligomeric tau. Since the concentration of oligomeric tau species increases at early time points (5–9 months) in the 3×TG mouse model before insoluble tau tangles begin to form, and then decreases after neurofibrillary tangles begin to form (12–15 months), the oligomeric tau species may be incorporated into the insoluble tau aggregates. The oligomeric tau aggregates are present already at 5-months well before NFTs are detected indicating that these aggregates have promise as earlier biomarkers for tau aggregation.

3.5 Analysis of post-mortem human brain tissue

Since the scFvs effectively detected oligomeric tau species present in brain tissue from an AD mouse model, we next probed post-mortem extracts of human middle temporal gyrus (MTG) specimens at different Braak stages with F9T for the presence of oligomeric tau (Figure 9). In postmortem examination of severe AD brains and ND brains, the middle temporal lobe displays conspicuous atrophy and weight loss in AD brains (Brun and Englund, 2002). When probed with the anti-oligomeric tau scFv there was only minimal detection of oligomeric tau in the ND samples, but significantly higher levels in the AD samples (Figure 9). All samples were normalized to contain equal total protein concentrations.

4. Discussion

Aggregates of A β and tau are the primary protein constituents of the hallmark senile plaques and neurofibrillary tangles of AD (Goedert, 2004, Gregersen, 2006, Ballatore et al., 2007), respectively. While many studies have focused on accumulation and aggregation of A β as an initiating factor in AD pathogenesis and neuronal death with tau dysfunction considered to be a downstream event following A β aggregation (Hardy and Allsop, 1991, Hardy and Selkoe, 2002), other studies suggest that tau interacts with A β to accelerate the progression of AD, and that reducing aggregated tau levels is also important to ameliorate AD symptoms (Oddo et al., 2006). A β and tau aggregation may be linked by separate mechanisms driven by common upstream causes (Small and Duff, 2008). Numerous studies have implicated the role of soluble oligomeric A β species in mediating toxicity in AD (Roher et al., 1993, Harper et al., 1997, Walsh and Selkoe, 2007), and evidence now suggests that oligomeric tau may also play toxic roles in AD (Berger et al., 2007, Maeda et al., 2007, Roberson et al., 2007, Bretteville and Planel, 2008, Lasagna-Reeves et al., 2011, Tai et al., 2012, Castillo-Carranza et al., 2014). Recent studies indicate that soluble tau species including oligomeric, prefibrillar and immature pre-filament forms play more crucial roles in AD than the hallmark NFTs which instead may rather play an adaptive and protective role (Bretteville and Planel, 2008, Congdon and Duff, 2008, Cowan and Mudher, 2013) Oligomeric tau has

been shown to have prion-like self-propagating features (Lasagna-Reeves et al., 2012a) and can be taken up by endocytosis into neurons where they can induce endogenous tau pathology in vivo (Wu et al., 2013). Therefore the roles of oligomeric and fibrillar tau species in AD progression is drawing increased attention and oligomeric tau is a promising therapeutic target for AD (Yoshiyama et al., 2007, Congdon and Duff, 2008, Honson and Kuret, 2008). Because of the diversity of tau species that can be present in the human brain due to alternative post-transcriptional splicing and post-translational modifications there is a critical need to develop reagents that can selectively identify individual tau aggregate variants to probe the roles of the various forms in disease progression and to assess their value as diagnostic and therapeutic targets.

We previously reported that a trimeric, but not monomeric or dimeric tau species was neurotoxic at low nanomolar levels (Tian et al., 2013). Here we isolated three different scFvs (F9T, D11C and H2A) that selectively recognize this toxic trimeric tau species. The scFvs recognize a conformational epitope specific for oligomeric tau and not a linear sequence epitope since the scFvs do not react with monomeric tau or a common β -sheet structural motif since the scFvs also do not react with fibrillar tau, A β or α -synuclein aggregates. All three scFvs selectively react with oligomeric tau species produced in a human neuronal cell line overexpressing a mutant human tau form prone to aggregation (Figure 4). The scFvs also show the presence of oligomeric tau very early in AD mouse models, by 5-months of age well before the presence of NFTs. Finally, the scFvs also detect oligomeric tau aggregates in post-mortem human AD brain tissue samples. While NFTs are a hallmark feature of AD, oligomeric tau species may play an intermediate role in tau aggregation (Maeda et al., 2007) and have been shown to play an important role in neuronal toxicity. Here we generated scFvs that selectively bind a unique epitope of oligomeric tau and show that these scFv reagents can be used to detect the presence of oligomeric tau in cell and tissue samples. Oligomeric tau aggregates are present much earlier than NFTs in mouse models of AD and levels of these aggregates correlate with AD in human post-mortem tissue. Therefore detection of toxic oligomeric tau aggregates has promise as an early diagnostic for AD (Sahara et al., 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Aβ	amyloid beta
AD	Alzheimer's disease
AFM	atomic force microscopy
ANOVA	analysis of variance
BSA	bovine serum albumin
CDR	complementarity-determining region
DI water	deionized water
DTT	DL-Dithiothreitol
EDC	1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FTD	frontotemporal dementia
HCFR	heavy chain framework region
HRP	horseradish peroxidase
LSD	least significant difference
MBD	microtubule-binding domain
MTG	middle temporal gyrus
ND	non-demented
NFT	neurofibrillary tangle
PBS	phosphate buffered saline
PCR	polymerase chain reaction
plb	phosphorylase b
rhTau	recombinant human tau
scFv	single chain variable fragment
TEA	triethylamine
3$\times$TG-AD mouse	triple transgenic Alzheimer's mouse

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- Isolated antibody fragments that selectively bind toxic oligomeric tau using AFM based biopanning protocols
- Oligomeric tau present much earlier in AD mouse model than tangles
- Oligomeric tau levels distinguish human AD brain tissue from healthy tissue
- Potential value as biomarkers for AD

Figure 1. The novel biopanning process combines subtractive panning and positive panning from phagemid scFv library with screening using AFM

(A) Schematic of the AFM based panning process. (B) Subtractive panning against BSA after 1 (left), 3 (middle) and 5 (right) rounds of negative panning. The absence of phage after 5 rounds of negative panning indicated that the BSA subtractive panning step was complete. The scale bar of 1 μm applies to all three images. (C) Positive panning against tau trimer was performed and imaged with AFM. Left image: purified trimeric tau 1N4R immobilized on mica; Right image: the same sample after addition of the remaining phage

pool from subtractive panning and rinse, and it shows phage bound to target antigen. Large irregular shaped white particles in samples with added phage reflect contaminants present in the phage preparation. The scale bar of 1 μm applies to both images.

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Figure 2. Single clones against trimeric rhTau selection, expression and purification

(A) Representative AFM images of single clone selection based on the specificity to trimeric tau (right) versus monomeric (left) and BSA. The scale bar of 1 μm applies to both images.

(B) Representative AFM images of sequence corrected phage displayed scFv (F9T) incubated with tau 2N4R monomer (left) and trimer (right). The scale bar of 1 μm applies to both images. Large irregular shaped white particles in samples with added phage reflect contaminants present in the phage preparation. (C) SDS-PAGE of F9T scFv periplasmic preparation shows full length 29 kDa protein. Lane 1, wash after scFv loading; lane 2 to lane 4, elution with imidazole of 200 mM, 100 mM and 50 mM respectively; lane 5, periplasm flow through nickel beads column; lane 6, molecular weight standards.

Figure 3. Particle size analysis of oligomeric tau bound to scFv-displayed phage from rhTau 2N4R mixed aggregates

(A) Representative AFM image of F9T phage (blue arrow) interacting with rhTau 2N4R mixed aggregates containing oligomers (gray arrow) and fibrils (black arrow). (B) Size of purified rhTau monomer, dimer and trimer compared to average size of antigens bound by F9T, D11C and H2A phage. The size of each individual particle bound at the tip of a phage particle was measured using the section function in Nanoscope Analysis. The mean value of the particles targeted by each phage is between 2.5 nm and 3.0 nm, consistent with the size of trimeric rhTau 2N4R (Error bar: $\pm$ standard deviation). (C) AFM analysis showing that F9T phage does not react with fibrillar α -synuclein. (D) AFM analysis showing that F9T phage does not react with fibrillar $A\beta$. Large irregular shaped white particles in samples with added phage reflect contaminants present in the phage preparation.

Figure 4. Immunofluorescent detection of oligomeric tau in SH-SY5Y-TMHT441 neuroblastoma cells using D11C and F9T

Oligomeric tau was detected by staining cells with F9T and D11C scFvs. Bound scFv was labeled using a rabbit anti-c-Myc antibody and Alexa Fluor® 488 Goat Anti-Rabbit Mouse IgG (H+L) antibody. Total tau was detected with a nonspecific tau polyclonal antibody. Results showed a negative immunostaining of total Tau (A) and Oligomer Tau with clone D11C (B) and clone F9T (C) in SH-SY5Y cells. However, in SH-SY5Y cells expressing human tau 2N4R, the immunopositive staining were observed with total Tau (D) and oligomer Tau aggregates (arrows in E and F, clone D11C and F9T, respectively).

Figure 5. Selective reactivity of F9T, D11C and H2A scFvs with 9-month 3×TG-AD mouse brain tissue

F9T, D11C or H2A scFv were used as capture antibody. Mouse hippocampus homogenates from 3×TG-AD mice and wild-type mice were added as target analytes. PBS was used as the negative control. The bound tau aggregates were detected using anti-tau antibody AT8 conjugated with HRP. Relative immunoreactivities are presented as the ratio of sample signal to the mean value of PBS control signals. (Error bar: $\pm$ standard deviation)

Figure 6. Capture ELISA for detection of oligomeric tau

Each scFv (F9T, D11C and H2A) was utilized as a capture antibody in combination with each phage displayed scFv as the detection antibody using 9-month 3×TG-AD mice brain homogenates as antigen. All three scFvs produced the strongest signal when F9T phage was used as detection antibody. The mean comparison was performed within each group of the same capture antibody. Relative immunoreactivities are presented as the ratio of sample signal to the mean value of PBS control signals. Relative immunoreactivities are chemiluminescent signal ratio to the mean value of wild type mice. (Error bar: +/- standard deviation)

Figure 7. Oligomeric tau levels in different age 3×TG-AD

The levels of oligomeric tau in the different age 3×TG-AD and wild-type mice were determined using the F9T, D11C and H2A scFvs as the capture antibody and F9T scFv displayed phage as the detection antibody. Two mice for each age group were tested in triplicate. The data were grouped by the age of the mice. The mean comparison was performed within each age matched group. Oligomeric tau levels peak at 9 months and decrease by 13 months. Relative immunoreactivities are chemiluminescent signal ratio to the mean value of wild type mice (Error bar: $\pm$ standard deviation).

Figure 8. Mutant human tau knockin mouse (Tau406+/TauKO) at different ages have a varying oligomeric tau amount compared with negative control (Tau406-/TauKO)

Levels of oligomeric tau in different age (Tau406+/TauKO) and (Tau406-/TauKO) mice were determined using F9T as the capture antibody and a phage displayed version of the pan-specific tau scFv, F3A, as a detection antibody. Three test mice and one control for each age group were tested in triplicate and data was grouped by the age of the mice. The mean comparison was performed within each age matched group. Oligomeric tau levels were detectable at 6 months but not 3 months of age ($p < 0.05$). Relative immunoreactivities are chemiluminescent signal ratio to the mean value of control knockout mice (Error bar: $\pm$ standard deviation).

Figure 9. Oligomeric tau levels in post-mortem human brain samples distinguish AD from controls

Brain homogenates from age-matched human middle temporal gyrus (MTG) of non-demented (ND) and Alzheimer's (AD) samples were blotted on membranes and probed for presence of oligomeric tau using F9T scFv. Oligomeric tau levels determined by reactivity with F9T are significantly higher in AD group cases than in ND group cases. A negative control without scFv gave readings close to the background signal. Signals displayed were scaled between 0 and 1 by ImageJ analysis where 0 is the background signal and 1 is the positive control signal of anti-pLB scFv. Statistical analysis was performed in one-way ANOVA comparing means of two groups.

Table 1
DNA sequences of the N-terminal region and the first heavy chain framework region (HCFR1) of recovered scFvs after panning compared to known scFvs

F9, H7, D4, D11 and G12 were recovered from the panning protocol, others are published scFv sequences. All recovered scFvs contain similar framework regions as published sequences except for a missing nucleotide near the N-terminal.

<u>Sfil</u>	GGCCCAGCCGGCC							
<u>NcoI</u>	<i>CCATGG</i>							
<u>Met start</u>								
			<u>ATG</u>			HCFR1		
α phOx15	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAG		
α BSA3	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGGTGCAGTCTGGGGGAGGCGTGTTTC		
α TEL9	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTACAGCTGCAGCAGTCAGGCTCAGGACTGGTG		
α TEL14	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGCAGGAGTCGGGGCCAGGACTGGTG		
α TEL13	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAA		
α TEL16	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGGTGCAGTCTGGGGCAGAGGTGAAA		
F9	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGCAGGAGTC■GGGGGAGGCGTGTTTC		
D11	GGCCCAGCCGG	CC	<u>ATG</u>	G	■C	CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGTTTA		
H2	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTG■TGGAGTCTGGGGGAGGCGTGTTTC		
D4	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGGTGC■GTCTGGAGGAGACTTGATC		
H7	GGCCCAGCCGG	CC	<u>ATG</u>	G	CC	CAGGTGCAGCTGCAGGAGTC■GGGGGAGACGTGGTC		
G12	GGCCCAGCCGG	CC	<u>ATG</u>	G	■C	CGAGTGCAGCTGGTGGAGTCTGGGGCTGAGGTGACG		
HCFR1 (Cont'd)								
α phOx15	AAGCCTGGGGCCTCAGTGAAGGTCTCCTGCAAGGCTTCTGGTTACACCTTTACC							
α BSA3	CAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTCAGT							
α TEL9	AAGCCTTCACAGACCCTGTCCCTCACCTGCTCTGTCTCTGGTGACTCCATCTCC							
α TEL14	AAGCCTTCGGAGACCCTGTCCCTCGTCTGCACTGTCTCTGGTGGCTCCCTCAGT							
α TEL13	AAGCCCCGGGCAGTCTCTGATGATCTCCTGTGAGGGTTCTGGATACAGCTTTAGC							
α TEL16	AAGCCCCGGGCAGTCTCTGAGGATCTCCTGTAAGGGTGCTGGATACAGCTTTAGC							
F9	CAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATTACCTTTCAGT							
D11	CAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAGC							
H2	CAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTCAGT							
D4	CAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAAT							
H7	CAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTCAGT							
G12	AAGCCTGGGGCCTCAGTAAGGGTCTCCTGCAAGGCTTCTGGATACACCTTCAGC							

Table 2
Designed primers for correction of missing base pair

Forward primers contain the NcoI (5'-CCATGG-3' in italic) site upstream of scFv sequence and the missing base pair (underlined). The reverse primer includes the NotI (5'-GCGGCCGC-3' in italic) site downstream of scFv sequence.

Primer names	DNA sequences
F9T forward primer	5'-ATA TAT <i>CCA TGG</i> CCC AGG TGC AGC TGC AGG AGT <u>CT</u> G GGG GAG GCG TGG TCC-3'
D11C forward primer	5'-ATA TAT <i>CCA TGG</i> <u>CC</u> C AGG TGC AGC TGG TGG AGT CTG GGG GAG GCT TGG TAC-3'
H2A forward primer	5'-GCC GGC <i>CAT GGC</i> CCA GGT ACA GCT <u>GCA</u> GGA GTC TGG GGG AGG CTT GGT-3'
Reverse primer for all three clones	5'-CCC GTG ATG GTG ATG ATG ATG <i>TGC GGC CGC</i> ACG TTT GAT CTC CAG-3'