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Internalization of tau antibody and pathological tau protein detected with a flow cytometry multiplexing approach

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Abstract

INTRODUCTION—Tau immunotherapy has emerged as a promising approach to clear tau aggregates from the brain. Our previous findings suggest that tau antibodies may act outside and within neurons to promote such clearance.

METHODS—We have developed an approach using flow cytometry, a human neuroblastoma cell model overexpressing tau with the P301L mutation, and paired helical filament (PHF)-enriched pathological tau to effectively screen uptake and retention of tau antibodies in conjunction with PHF.

RESULTS—The flow cytometry approach correlates well with Western blot analysis to detect internalized antibodies in naïve and transfected SH-SY5Y cells ($r^2=0.958$, and $r^2=0.968$, $p=0.021$ and $p=0.016$, respectively). In transfected cells, more antibodies are taken up/retained as pathological tau load increases, both under co-treated conditions and when the cells are pre-treated with PHF prior to antibody administration ($r^2=0.999$ and $r^2=0.999$, $p=0.013$ and $p=0.011$, respectively).

DISCUSSION—This approach allows rapid in vitro screening of antibody uptake and retention in conjunction with pathological tau protein before more detailed studies in animals or other more complex model systems.

INTRODUCTION

Antibody immunotherapies are a major focus of treatment approaches for Alzheimer's disease and other neurodegenerative diseases [1–3]. Our laboratory has pioneered targeting pathological tau proteins for clearance with active and passive immunotherapies for

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Alzheimer's disease and other tauopathies [4,5], which have been confirmed and extended by multiple groups over the last several years [6–33]. With numerous potential tau epitopes to target, as well as various antibody isotypes and effector mechanisms to consider, it has become a daunting task to pre-screen potential candidate antibodies to test in animal models.

We have developed an approach using flow cytometry and a human neuroblastoma cell model to detect internalization of tau antibodies and paired helical filament (PHF)-enriched pathological tau protein. Our findings show that this approach correlates very well with Western blot detection of tau antibody uptake, and confirms such uptake observed in different model systems [4,13,14,21,28,34]. This rapid and highly quantitative approach is an effective means to screen antibody internalization with or without pathological tau, which can be detected in a similar manner. Tau antibodies can in theory lead to tau clearance extra- and/or intracellularly. To target both pools of the pathological protein simultaneously is likely more efficacious. Hence, it is important to be able to quickly and quantitatively screen antibody's ability to enter cells, which varies and likely depends primarily on its charge [1].

Overall, such multiplexing approach to detect antibodies and pathological targets in cells has great potential to be used with conventional techniques to better understand the mechanism of action of prospective immunotherapies for neurodegenerative diseases.

METHODS

Paired helical filament (PHF) Protein Preparation

Human brain slices were homogenized and prepared in buffer (pH 6.5; 0.75 M NaCl, 1 mM EGTA, 0.5 mM MgSO₄, and 100 mM 2-(*N*-morpholino) ethanesulfonic acid) along with protease inhibitor cocktail (Roche) and centrifuged at $11,000 \times g$ for 20 min at 4°C. Supernatant was subsequently centrifuged in an ultracentrifuge at $100,000 \times g$ for 60 min at 4°C. The pellet was then re-suspended in PHF extraction buffer containing sucrose (10 mM Tris; 10% sucrose; 0.85 M NaCl; and 1 mM EGTA, pH 7.4) and spun at $15,000 \times g$ for 20 min at 4°C. The pellet was re-extracted in the sucrose buffer at the same low-speed centrifugation. The supernatants from both sucrose extractions were pooled and subjected to 1% sarkosyl solubilization by stirring at ambient temperature, then centrifuged at $100,000 \times g$ for 60 min at 4°C in a Beckman 60 Ti rotor (Beckman Coulter, Fullerton, CA, USA). The resulting pellet was re-suspended in 50 mM Tris-HCl (pH 7.4), using 0.5 µL of buffer for each milligram of initial weight of brain sample protein, and designated the PHF preparation.

Tau Antibody and Fluorescence Labeling

In this study we used the tau antibody, 6B2G12 (6B2), which detects Ser396/404 tau epitope, and has previously been characterized, by our laboratory [14,35]. The antibody and the PHF-enriched brain fraction were tagged with Alexa Fluor 488 and Alexa Fluor 647, respectively, using protein labeling kits, as detailed in the manufacturer's instructions (Invitrogen).

Cell Culture

SH-SY5Y human neuroblastoma cells obtained from ATCC were transfected using Lipofectamine 2000 (Invitrogen) and pcDNA3.1(+) P301L hTau 4R0N with gentomycin (G418) selection. Naive and transfected cells were cultured in complete media (Dulbecco's Modified Eagle Medium (DMEM) with glutamax (Invitrogen), 10% heat inactivated fetal bovine serum (FBS), 10,000 Units/mL Penicillin, and 10,000 µg/mL streptomycin). Transfected cells additionally contained 200 µg/mL gentomycin. Cells were plated at 4×10^2 cells/mm², allowed to recover for 3 days before each experiment, and grown in an incubator with 5% CO₂ at 37°C. For dose response experiments, naive and transfected cells were treated with increasing doses of 6B2 antibody (0–60 µg/mL) for 24 h. For co- and pre-treatments, transfected cells were either pre-treated with increasing levels of tagged PHF (0.1–10 µg/mL) for 24 h, then washed several times to remove remaining extracellular PHF, and subsequently incubated with tagged 6B2 tau antibody (5 µg/mL) for another 24 h or co-incubated with both for 24 h.

Flow Cytometry

All cells analyzed with flow cytometry were incubated with tagged 6B2 antibody and/or tagged PHF. Prior to measurements, cells were washed with PBS, trypsinized, re-suspended in ice-cold complete media, and then spun down and pelleted at $200 \times g$ for 5 minutes. Supernatant was discarded and pelleted cells were then resuspended in ice-cold PBS, and put through a 0.2 micron filter (PARTEC). To quench any surface bound fluorescence, trypan blue was added to each sample at a final concentration of 0.02% (w/v) (Sigma Aldrich) and the sample was placed on ice. Samples were analyzed using flow cytometry (LSRII, Becton Dickinson) to a count of 10,000 cells per sample, and were gated for viable and singlet cell populations. Cells were further analyzed using FlowJo for either Alexa Fluor 488 and/or 647 positive cells. Median fluorescence intensity (MFI) values were obtained for both fluorescent signals.

Western Blot

All cells analyzed using western blot were incubated with non-tagged 6B2 antibodies and/or PHF. All samples were homogenized in RIPA buffer and prepared as described previously [14]. Samples were boiled and loaded on 10% SDS-PAGE gels, electrophoresed, and then transferred to nitrocellulose membranes, which were blocked in 5% milk with 0.1% TBS-T. Blots were then probed for total tau (Dako polyclonal antibody), phospho-tau (PHF-1 monoclonal antibody) or GAPDH (Abcam polyclonal antibody) primary antibodies overnight at 4°C, washed and then probed with anti-horseradish peroxidase (HRP) conjugated rabbit or mouse secondary antibody (Pierce) for 1 h. For antibody uptake detection, membranes were incubated with an anti-mouse IgG1 HRP-conjugated secondary antibody with specificity against the heavy chain (Bethyl Laboratories), and signal was detected with an ECL substrate (Thermo Scientific). Images of immunoreactive bands were then acquired and quantified using the Fuji LAS-4000 imaging system.

Immunocytochemistry

Coverslips were coated with poly-D-lysine. Cells were plated onto coverslips at 4×10^2 cells/mm², allowed to recover for 3 days, then washed, and fixed with 4% paraformaldehyde. After subsequent washing, cells were treated with 0.1% Triton X-100 and blocked with 5% BSA, which were both in PBS. A total tau primary antibody, CP27, was incubated overnight at 4°C at 1:200 dilution. Cells were then washed, incubated in Alexa Fluor 488-conjugated mouse secondary antibody for one h at room temperature, and washed again. Finally before mounting, cells were incubated with Hoechst nuclear stain (Invitrogen). Fluorescent imaging of cells was performed on a Nikon C1 confocal system.

Live Cell Imaging

Chamber glasses (Nunc) were coated with Pluripro protein matrix (Cell Guidance Systems) as directed by the manufacturer. Cells were plated onto these glasses at 4×10^2 cells/mm², allowed to recover for 3 days, then treated with PHF and/or 6B2 tau antibody as described above, followed by washing with complete media. Prior to imaging, cells were incubated with Lysotracker Red, an endosomal-lysosomal dye, and Hoechst nuclear dye as directed by the manufacturer (Invitrogen). Cells were then washed and finally placed into complete media with no phenol red-DMEM and HEPES (Invitrogen). Chamber glasses were then analyzed using an Applied Precision PersonalDV live-cell imaging system in an Olympus IX-71 inverted microscope with a 60× objective, with heating at 37°C. DeltaVision filter setting (DAPI, FITC, TRITC, and Cy5) corresponded to fluorescent signals from the respective dyes (Hoechst, Alexa Fluor 488, Lysotracker Red, and Alexa Fluor 647).

Immuno-Electron Microscopy

PHF preparations were placed on 300 mesh copper or nickel electron microscope grids. Grids were transferred to drops of 1% PBS-BSA and blocked for 30 minutes at room temperature, then incubated in PHF-1 primary antibody with blocking buffer overnight at 4°C. The grid was then washed with PBS, reacted with gold-IgG complex secondary antibody, washed again with PBS, then stained with 2% (w/v) uranyl acetate and washed with sterile distilled water, and blotted dry. Grids were visualized and images were taken with a Phillips CM12 Cryoelectron microscope.

Statistics

All data were analyzed using GraphPad Prism 6. Pearson correlation coefficients were generated for western blot vs. flow cytometry data. Other data sets were analyzed using one-way ANOVA with Bonferroni post hoc test or Student's t-test (two-tailed).

RESULTS

Tau antibody uptake into neuroblastoma cells measured by flow cytometry correlates very well with uptake detected by Western blot

Initial characterization of the transfected SH-SY5Y cells overexpressing P301L mutant human tau revealed a robust overexpression compared to naïve SH-SY5Y cells, which for comparative purposes are underexposed (Figure 1A, B). Furthermore, naïve and transfected

cells were incubated with increasing doses of monoclonal tau antibody, 6B2 (0–60 µg/mL) for 24 h. Tagged samples were analyzed using flow cytometry, which detected a dose-dependent increase in the Alexa Fluor 488 fluorescence intensity (Figure 1C, D), and a dose-dependent increase in Median Fluorescence Intensity (MFI) for both cell lines (Figure 1E, F). Due to the specificity of our anti-mouse IgG1 HRP antibody, western blot analysis showed a single band for the heavy chain of the antibody (Figure 1G, H), and likewise, its quantification showed a dose-dependent increase in antibody uptake (Figure 1I, J). There was a strong positive correlation between the quantified flow cytometry and Western blot values in naïve and transfected cells (Figure 1K, L, $r^2=0.958$, and $r^2=0.968$, $p=0.021$ and $p=0.016$, respectively). In addition, we analyzed our flow cytometry samples for percent Alexa Fluor 488 positive cells (Figure 2A, B). In both naïve and transfected cells, percent positive cells increased dose-dependently and plateaued between the 40 and 60 µg/mL dosage (Figure 2C, D). From these dose-response curves, we chose 5 µg/mL, at the steepest part of the curves, as an ideal dosage for the dynamic experiments.

Time of incubation and serum deprivation alter tau antibody uptake/retention as measured by flow cytometry

Naïve and transfected cells were treated with tagged 6B2 tau antibody for 24, 96, and 144 h in 10% or 1% FBS serum conditions to determine if prolonged incubation and/or enhanced stress associated with serum deprivation would alter antibody uptake. In both cell types, time-dependent increase in antibody uptake was observed, and was more pronounced in serum deprived media (Figure 3).

A sub-population of neuroblastoma cells seeded with increasing doses of PHF and detected by flow cytometry shows a corresponding dose-dependent uptake of 6B2 tau antibody

PHF tau was extracted and purified from the brain of a human AD patient. Purified PHF samples were characterized using electron microscopy and Western blot analyses (Figure 4). Transfected cells were pre-treated with increasing levels of tagged PHF for 24 h and then incubated with tagged 6B2 tau antibody for 24 h or co-treated with both reagents for 24 h. All samples were measured using flow cytometry. This paradigm revealed a sub-population of cells within the Q2 gating quadrant, which contains both PHF and tau antibody (Figure 5A). Quantification of the 6B2-AF488 percent positive cells in 6B2 controls samples (Q1 quadrant) compared to PHF and 6B2 treated cells (Q1+Q2 quadrants) showed a trend for a decrease in percent positive 6B2-AF488 cells as PHF load increased (Figure 5B). Further analyses of the Q2 quadrant region revealed that the MFI of both reagents increased with increasing PHF dose (Figure 5Ci, ii, iii, iv), and showed a strong correlation between PHF and antibody uptake levels ($r^2=0.999$ and $r^2=0.999$, $p=0.011$ and $p=0.013$, respectively, Figure 5Cv, vi). In further support of this finding, live imaging showed co-localization of PHF and tau antibody within the endosomal/lysosomal compartments under both conditions (Figure 6).

DISCUSSION

In this study we have successfully demonstrated that flow cytometry is a convenient method to detect and quantify tau antibodies that are internalized into naïve, and transfected SH-SY5Y neuroblastoma cells. We chose this stable cell line because of SH-SY5Y cells' ability to handle the flow cytometry process under live conditions [36,37]. In addition, we chose to transfect SH-SY5Y with the P301L mutation as this mutation is commonly used for tauopathy studies. Our flow cytometry technique showed a strong positive correlation with Western blot analysis, which has previously been used in our laboratory for this purpose in primary neuronal mouse cultures [13], and are more difficult to study with flow cytometry.

We also demonstrated our ability to detect intracellular PHF material in transfected cells with flow cytometry, and how its uptake influences tau antibody uptake/retention. Measuring cells' capacity to internalize various tau fractions and recombinant tau to monitor the uptake and spread of tau pathology has been reported using more conventional techniques [11,16,38–40], as well as with flow cytometry [29,30,41–43]. However, such measurements have not been previously combined with analysis of tau antibody uptake. Hence, we have gone a step further in our flow cytometry studies, in which we multiplexed both the antibody and tau uptake signals to monitor both at the same time and how they relate.

The degree of tau antibody uptake/retention as detected by flow cytometry depended on antibody dosage, incubation period, and the environmental conditions. As expected, the MFI and percentage of positive cells increased with higher antibody doses. From this data we created a dose response curve to select an ideal antibody dosage for treating the cells, which would allow us to detect changes in uptake under varying conditions. Serum deprivation led to an increase in antibody uptake which may be due to enhanced endocytosis under stress conditions. Likewise, as predicted, longer incubation period increased antibody uptake in naïve cells, and to a greater extent in transfected cells. This manipulation was further exemplified in our pre- and co-treatment of PHF and 6B2 tau antibody in transfected cells for 24 h, which revealed a subpopulation of cells that contained both reagents. These pre- and co-treatment experiments showed a strong correlation between the amount of PHF and tau antibody uptake, in agreement with previous studies in our laboratory, which have indicated that tau antibody uptake correlates with tau pathology load [13,14].

The sub-population of transfected cells that contain both antibody and PHF positive cells was between 1.7 to 5.5% of the total cell population. The percentage of 6B2-AF488 positive cells showed a non-significant trend for a decrease as PHF dosage increased mainly in co-treated cells and only at the highest PHF dosage in pre-treated cells. This trend may be attributed to some of the antibody being sequestered by PHF in the extracellular space, and, therefore, not being able to access the cell for uptake. Another possibility is that some of the cells may have died due to PHF toxicity.

The PHF fraction alone is most likely internalized by bulk-mediated endocytosis [39,43], while the antibody with or without PHF appears to be internalized through Fc-receptor and bulk-mediated processes [13,28]. Since the neuroblastoma cells are non-differentiated and only neuronal-like, these mechanisms may not fully apply as we have seen previously in

actual neurons. In non-differentiated neuroblastoma, the primary vehicle of uptake may be bulk-mediated endocytosis, which is a relatively non-specific process. This phenomenon may explain antibody detection in naïve cells, which is much more limited in wild-type brain slice cultures [13,14]. The extent of antibody uptake in the undifferentiated naïve cells is analogous to Fab uptake in wild-type brain slice culture, which is considerable and occurs via bulk-mediated endocytosis [14]. However, based on our live imaging findings, downstream events are likely similar in different model systems, with tau antibodies entering the endosomal-lysosomal system and co-localizing with pathological tau material, as we and others have seen in primary and brain slice cultures as well as in vivo [4,13,14,21,34]. We are in the process of comparing uptake and efficacy of various tau antibodies in non-differentiated-, as used here, and in differentiated SH-SY5Y cells, which are closer to actual neurons [44].

Overall, this study shows the potential of this multiplexing approach to detect antibody and tau internalization into cells in the same culture assay, and will be an asset to rapidly screen and quantify such events. It can then complement conventional techniques to better understand the mechanism of action of tau antibodies and the uptake and spread of pathological tau proteins.

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Research in Context

Systematic review

Prior literature was reviewed through traditional sources, such as PubMed. Several articles have reported on internalization of tau protein or its antibodies but not in the same study. Furthermore, we are not aware of another approach that can quickly measure and quantify such interaction.

Interpretation

This flow cytometry approach can simultaneously track and multiplex the signals of the internalization of tau protein and its antibodies in cells. It allows quick assessment of antibody uptake and retention in conjunction with pathological tau protein prior to longer studies in animals or other more complex models.

Future directions

To use this high-throughput approach to determine if/how the extent of such internalization and association within the same cells translate into clearance of pathological tau protein. Also, to determine if antibody efficacy in undifferentiated cells corresponds to such effects in differentiated cells.

Figure 1. SH-SY5Y neuroblastoma cells dose-dependently take up tau monoclonal antibody, 6B2 as assessed by flow cytometry and Western blot analyses

Non-transfected naïve SH-SY5Y cells and transfected SH-SY5Y cells expressing P301L tau were grown and maintained. Cells were either lysed for western blot or fixed with paraformaldehyde for confocal imaging. **(A)** Shows a comparison of western blot tau levels in naïve and P301L tau transfected SH-SY5Y cells. Transfected cells show a much greater degree of total (Dako) and phospho-tau (PHF-1) expression than the naïve cells. **(B)** Likewise, confocal imaging of these cells shows greater total tau expression (CP27) in

transfected cells compared to naïve cells under the same fluorescence intensity. The naïve cells express tau, but the level of expression is much lower than in the transfected cells and hardly detectable under these conditions. Furthermore, naïve and transfected SH-SY5Y cells were incubated with different doses (0–60 µg/mL) of 6B2 tau antibody for 24 h. Cells were then collected and examined with flow cytometry or western blot analyses. **(C and D)** Scatter plots show how cells were gated for viable and singlet cells and further selected for Alexa Fluor 488 fluorescence. **(E and F)** Quantification of median fluorescence intensities (MFI) of the Alexa Fluor 488 tagged 6B2 normalized to the 10 µg/mL dosage, which show a dose-dependent increase in antibody uptake/retention. **(G and H)** A representative Western blot probed with an anti-mouse IgG1 HRP-conjugated antibody to detect internalized antibody. **(I and J)** Western blot quantification, which shows a dose-dependent increase in antibody uptake normalized to the 10 µg/mL dosage. **(K and L)** Excellent correlation is observed between flow cytometry and Western blot analyses of tau antibody uptake for both naïve and transfected cells ($r^2=0.958$, and $r^2=0.968$, $p=0.021$, and $p=0.016$, $n=3-8$, and $n=3-5$, respectively). Graphs show mean values + standard error of the mean (SEM).

Naive

A

C

Transfected

B

D

Figure 2. Dose dependent increase in antibody uptake and saturation

Naïve and transfected SH-SY5Y cells were treated with 0–60 µg/mL Alexa Fluor 488 tagged 6B2 tau antibody for 24 h. Cells were then collected and examined with flow cytometry. Cells were gated for viable and singlet cells and further selected for Alexa Fluor 488 fluorescence. Samples were analyzed from previously described data sets in Figure 1 for percent Alexa Fluor 488 positive cells. (**A and B**) Show representative gating parameters after selecting for viable and single cells using a histogram to gate for percent positive cells in the 0 µg/mL 6B2 sample, which was then applied to the other doses. (**C and D**) Shows dose-dependent increase in percent positive cells for both cell lines, which plateaus between 40 and 60 µg/mL. Graphs show mean values + SEM.

Figure 3. Antibody uptake/retention in SH-SY5Y neuroblastoma cells is time-dependent and increases during serum deprivation

Naïve and transfected SH-SY5Y, were treated with 5 μ g/mL Alexa Fluor 488 tagged 6B2 tau antibody for 24 to 144 h at either 10% or 1% FBS serum levels. Cells were then collected and examined with flow cytometry, gated for viable and singlet cells, and further selected for Alexa Fluor 488 fluorescence. Quantification of median fluorescence intensities (MFI) of the Alexa Fluor 488 tagged 6B2 was normalized to the negative control at each time point. Naïve cells at 10% and 1% FBS showed a time-dependent increase in antibody uptake/retention between 24 and 144 h (###, ## 239%, 236%, $p < 0.001$, $p < 0.01$, $n = 3$, respectively). Transfected cells showed a similar but more pronounced increase (####, ### 318%, 305%, $p < 0.0001$, $p < 0.001$, $n = 3$, respectively). In addition, at 24, 96, and 144 h time points, the 1% FBS condition led to a greater antibody uptake/retention compared to 10% FBS condition, which trended mostly in naïve cells (n.s., ***, n.s.; 23%, 46%, 21%, $p = 0.20$, $p < 0.001$, $p = 0.08$, $n = 3$, respectively) and was consistently significant in transfected cells (*, **, *; 31%, 38%, 25%, $p < 0.05$, $p < 0.01$, $p < 0.05$, $n = 3$, respectively). n.s.: non-significant. Graphs show mean values + SEM.

A

B

Figure 4. Characterization of PHF extracted from an Alzheimer's brain

PHF tau was extracted and purified from the brain of a human AD patient. Samples were analyzed using electron microscopy and western blot analysis. **(A)** Shows an immuno-electron microscopy image of PHF-1 tau positive fibril. Arrows indicate areas where antibody binding to fibril occurs. **(B)** Shows a corresponding Western blot of PHF tau sample probed with the CP27 antibody.

Figure 5. SH-SY5Y neuroblastoma cells treated with increasing doses of pathological PHF show a dose-dependent increase in tau antibody uptake, and these co-localize within a subpopulation of cells

(A) Scatter plots of transfected SH-SY5Y cells expressing P301L either pre-treated with increasing levels of tagged PHF (0.1–10 µg/mL) for 24 h and then treated with tagged 6B2 tau antibody (5 µg/mL) for 24 h or co-treated with both for 24 h. Negative controls are untreated cells. The 6B2 control plot shows that within the Q1 quadrant, 7.2% and 3.5% of cells were positive in co-treated- and pre-treated cells, respectively. The plots show in the co- and pre-treated PHF+ 6B2 samples a sub-population in the Q2 quadrant gate, which

contains both tau antibody and PHF, which is 1.7 to 5.5% of the total cell population (2.2% to 5.5% in co-treated cells and 1.7% to 3.0% in pre-treated cells). **(B)** Quantification of percent 6B2-AF488 positive cells from Q1 and Q2 quadrant data in co- and pre-treated cells. As PHF load increases, the percent of positive cells trended towards a decrease. **(C)** MFI (Alexa Fluor 488 and 647) of all PHF+6B2 samples were analyzed from the Q2 quadrants and normalized to the Q2 MFI's of the 0.1 µg/mL PHF+6B2 sample. Quantification shows a dose-dependent increase in antibody and PHF signal in pre- and co--treated samples. Under both conditions, uptake/retention of tau antibody and PHF strongly correlate ($r^2=0.999$ and $r^2=0.999$, $p=0.011$ and $p=0.013$, $n=3$, respectively).

Pre-treatment **Co-treatment**

Figure 6. Tau antibody co-localizes with pathological PHF tau in the endosomal/lysosomal compartments

Representative live images of cells under the pre- and co-treatment conditions depicted in Figure 5, using 1 $\mu\text{g/mL}$ tagged-PHF and 5 $\mu\text{g/mL}$ tagged-6B2 antibody. Black arrows in the Merge+Brightfield panel point at foci with intense triple co-localization of Lysotracker, 6B2 tau antibody, and PHF.