Paired Helical Filaments from Alzheimer Disease Brain Induce Intracellular Accumulation of Tau Protein in Aggresomes*[S]

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Background: Misfolded aggregated tau protein released by degenerating neurons could spread toxicity to neighboring cells.
Results: We found that cultured cells internalized paired helical filaments and developed intracellular tau aggregates with attributes of aggresomes.
Conclusion: Paired helical filaments could mediate spreading of pathological tau aggregation.
Significance: Paired helical filament-mediated formation of aggresome-like bodies may be an important step in neurodegeneration.

Abnormal folding of tau protein leads to the generation of paired helical filaments (PHFs) and neurofibrillary tangles, a key neuropathological feature in Alzheimer disease and tauopathies. A specific anatomical pattern of pathological changes developing in the brain suggests that once tau pathology is initiated it propagates between neighboring neuronal cells, possibly spreading along the axonal network. We studied whether PHFs released from degenerating neurons could be taken up by surrounding cells and promote spreading of tau pathology. Neuronal and non-neuronal cells overexpressing green fluorescent protein-tagged tau (GFP-Tau) were treated with isolated fractions of human Alzheimer disease-derived PHFs for 24 h. We found that cells internalized PHFs through an endocytic mechanism and developed intracellular GFP-Tau aggregates with attributes of aggresomes. This was particularly evident by the perinuclear localization of aggregates and redistribution of the vimentin intermediate filament network and retrograde motor protein dynein.Furthermore, the content of Sarkosyl-insoluble tau, a measure of abnormal tau aggregation, increased 3-fold in PHF-treated cells. An exosome-related mechanism did not appear to be involved in the release of GFP-Tau from untreated cells. The evidence that cells can internalize PHFs, leading to formation of aggresome-like bodies, opens new therapeutic avenues to prevent propagation and spreading of tau pathology.

Misfolding and aggregation of the microtubule-associated protein tau are key pathogenic features shared by different neurodegenerative disorders including Alzheimer disease (AD),[2] corticobasal degeneration, progressive supranuclear palsy, and others collectively known as tauopathies (1, 2). Under physiological conditions, the majority of tau protein is associated with microtubules, stabilizing the microtubule network within axons and facilitating axonal transport of organelles, trophic factors, neurotransmitters, and other cellular constituents (3, 4). Under pathological conditions, the loss of the normal micro- tubule-stabilizing function of tau contributes both to axonal transport deficits (2, 3) and abnormal aggregation of tau into paired helical filaments (PHFs) and neurofibrillary tangles (NFTs).

It has been proposed that extracellular tau aggregates contribute to the propagation of neurodegenerative disease pathogenesis (5–7). In AD, the formation of NFTs follows an unvarying pattern starting in the entorhinal cortex. From there, the pathology spreads to the hippocampus and eventually to most cortical areas (8). In AD, the severity of dementia has been positively related to the degree of NFT deposition (9). A similar spreading pattern has also been described in other tauopathies (10). In damaged areas, an inverse relationship between NFT deposition and cellular survival has been reported (11). During the neurodegenerative process or upon cell death, neurons might release misfolded aggregated tau into the extracellular space, *e.g.* as apoptotic blebs (12), where the released tau could mediate and spread toxicity (13, 14). Importantly, *in vivo* microdialysis studies revealed the presence of tau in interstitial fluid in mouse brain, suggesting a continuous release of tau protein from cells (15).

Several findings point to the role of exosomes, membrane-bound vesicles of endocytic origin, in the release and potential

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[S] This article contains supplemental Figs. S1 and S2.

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2 The abbreviations used are: AD, Alzheimer disease; PHF, paired helical filament; NFT, neurofibrillary tangle; GSE, grape seed polyphenolic extract.

cell-to-cell spread of pathological proteins associated with neurodegeneration (16). Among the proteins secreted via exosomes to the extracellular space are prions (17), ¡3-amyloid peptides (18), and a-synuclein (19). It is unclear, however, whether exosomes contribute to intercellular spreading of pathological tau in tauopathies. Unconventional secretion pathways bypassing the endoplasmic reticulum and Golgi have been suggested for tau (20) using mechanisms similar to other proteins lacking a signaling peptide for transport into the endoplasmic reticulum (21, 22).

Aggresomes are perinuclear and pericentriolar microtubule-dependent inclusion bodies of misfolded proteins (23, 24). Perinuclear aggresome formation is a key mechanism to dispose of misfolded proteins that exceed the degradative capacity of ubiquitin-proteasome and autophagy lysosome systems. Functional blockade of either degradative system leads to an enhanced aggresome formation (24, 25). Formation of aggresomes is considered a cellular protective response in a number of neurodegenerative diseases characterized by accumulation of misfolded proteins (26). Recruitment of tau protein into large spherical inclusions resembling aggresomes has been demonstrated in cell culture studies following proteasome inhibition (27, 28). Aggresomes composed of other proteins such as a-synuclein, aA-crystallin, and mutant huntingtin protein have also been observed (28, 29). A close relationship between aggresome and Lewy body formation has been reported (26).

Compounds capable of reducing misfolding of tau offer attractive strategies for the prevention and/or treatment of neurodegenerative disorders with tauopathy (30). We have shown that a natural product, grape seed polyphenolic extract (GSE), may attenuate misfolding of tau protein into fibrillar polymers by promoting deaggregation of preformed tau aggregates and authentic PHFs in cell-free conditions (31, 32) and in animal studies using tau transgenic mouse models (33, 34). Deaggregation may lead to enhanced proteolytic clearance (35). Cellular effects of GSE have not yet been examined.

In the present studies, we investigated whether PHFs isolated from AD brain were taken into the cells in culture and whether upon uptake from the extracellular medium were capable of promoting intracellular aggregation of tau. We studied events associated with formation of intracellular tau aggregates and examined the possibility that tau aggregates resembled aggresomes. We also explored whether exosomes could contribute to intercellular propagation of tau aggregation and studied the potential role of the bioactive monomer-enriched fraction of GSE (monomeric GSE) in modifying tau aggregation induced by PHFs.

EXPERIMENTAL PROCEDURES

Reagents—GSE was obtained from Polyphenolics, Inc. (Madera, CA). It is a highly purified water-soluble polyphenolic preparation from *Vitis vinifera* seeds that does not contain detectable resveratrol. Typically, GSE contains ~8% monomers, 75% oligomers, and 17% polymers (35). For the present studies, GSE was fractionated into monomer-enriched (monomeric GSE) and polymer-enriched fractions through solvent fractionation followed by chromatography on Toyopearl resin (36). The monomeric GSE fraction was used to treat cultured cells. Sodium salt of *N*-lauroylsarcosine (Sarkosyl) and lactacystin were purchased from Sigma.

Lentiviral Vector and Virus Preparation—Cloning of lentiviral vector pLVGFP-Tau for expression of tau in mammalian cells and production of viral particles was carried out following procedures described previously (37). Briefly, we prepared a lentiviral construct (supplemental Fig. S1*A*) by fusion of full-length tau to green fluorescent protein (GFP-Tau). Full-length tau was a human tau isoform of 441 amino acid residues containing four repeats in the microtubule binding domain and two inserts in the N terminus (4R2N). A lentiviral construct of GFP alone was also prepared.

Isolation and Labeling of PHF Fractions from Human Brains—PHFs were isolated from four AD brains as described previously using our standard procedure (38) with minor modifications. The sucrose gradient fraction of PHFs in 1 M sucrose (fraction A2) containing dispersed filaments up to 500 nm in length was used in the present studies. PHFs were labeled with Cy5 using the Amersham Biosciences FluoroLink™ Multifunctional Dye labeling kit (GE Healthcare) according to the protocol described previously (39). Autopsy material was characterized as follows: case 1, 80-year-old male, 3.5-h post-mortem interval; case 2, 74-year-old male, 21.5-h post-mortem interval; case 3, 82-year-old female, 6-h post-mortem interval, and case 4, 91-year-old female, unknown post-mortem interval. All four cases met diagnostic criteria of AD based on neuropathology examination. The same human material was used in our previous studies (32).

Cell Culture and Various Treatments—We used the human HEK 293T cell line (CRL-11268™, ATCC, Manassas, VA) that stably and constitutively expresses the SV40 large T antigen and facilitates optimal lentivirus production. HEK 293T cells and human neuroblastoma SH-SY5Y cells (40) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. For tau aggregation studies, cells were grown for 24 h before lentiviral transduction and 72 h before PHF treatment. Cells were treated with PHFs or left untreated for 24 h. In some experiments, cells were treated with 5 μM lactacystin for 24 h. In antiaggregation studies, monomeric GSE (25 μM or 12.5 μg/ml) was added 2 h before treatment of cells with PHFs. In some experiments, SH-SY5Y and HEK 293T cells were co-treated for 6 h with PHF-Cy5 and 50 μg/ml Texas Red-dextran (M_r 10,000, pH neu- tral) (Molecular Probes, Invitrogen). Cells were extensively washed (six times) in PBS before use.

Sarkosyl Extraction of Aggregated Tau—Sarkosyl-insoluble tau aggregates were isolated from cells by a modification of the procedure described earlier (41). Cells were homogenized in ice-cold buffer (10 mM Tris-HCl, 1 mM EGTA, 0.8 M NaCl, 10% sucrose, and protease inhibitor mixture, pH 7.4) and centrifuged for 20 min at 20,000 X *g*. The pellet was discarded, and the supernatant fraction designated as the crude extract was incubated with 1% Sarkosyl for 2 h at room temperature with agitation. After centrifugation of the mixture (2 h at 100,000 X *g*), the resultant pellet was resuspended in PBS and designated Sarkosyl-insoluble aggregated tau.

Exosome Isolation—The exosome pellet was prepared from the conditioned medium as described earlier (42). Initially, SH-SY5Y cells were grown in four 100-mm^2 dishes per group in DMEM containing 10% fetal bovine serum as the culture medium. On the day of transduction for the GFP-Tau-express- ing cells and 72 h prior to exosome isolation, the medium was replaced by Neurobasal serum-free medium supplemented with 2% B-27, 0.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Neurobasal serum-free medium was used to eliminate serum-derived exosomes from the prepara- tion. For Western blotting analysis, cells and exosomes were suspended in urea buffer (6 M urea, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 1% ¡3-mercaptoethanol, 2 mM EDTA, and protease inhibitors). For electron microscopy, exosomes were resuspended in sucrose buffer (0.32 M sucrose and 3 mM HEPES, pH 7.4).

Western Blotting Analysis—Samples were prepared by adding Laemmli sample buffer and boiling for 5 min. Proteins were separated by gel electrophoresis using sodium dodecyl sulfate-polyacrylamide gels (10 and 12%) and electrotransferred to nitrocellulose membranes (Bio-Rad). Blocking solution contained 5% nonfat dried milk (Sigma) in Tris-buffered saline. Secondary antibodies conjugated to horseradish peroxidase were obtained from Bio-Rad. Specific protein signals were detected using an enhanced chemiluminescence system (Western Lightning Plus ECL, PerkinElmer Life Sciences) and x-ray film. Immunoreactivity of specific proteins was quantified by densitometric scanning (GS-800, Bio-Rad) and analyzed using Quantity One software (Bio-Rad). Values were adjusted for loading using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoreactivity. Statistical analysis was performed using one-way analysis of variance and a two-tailed Student's *t* test in GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA).

Antibodies—Primary antibodies against total tau were Ab 7.51 (Ser^{316}–Leu^{376}), a generous gift from Claude Wischik (University of Aberdeen, UK), Tau 5 (Ser^{210}–Ser^{241}) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and HT7 (Pro^{159}–Lys^{163}) from Thermo Scientific (Rockford, IL). Anti-tau four-repeat isoform (RD4) and anti-tau three-repeat isoform (RD3) were from Millipore (Temecula, CA). Anti-Ser(P)^{214} tau antibody was from Invitrogen, and PHF-1 (Ser(P)$^{396/404}$) was a generous gift from Peter Davies (Albert Einstein College of Medicine, Bronx, NY). Anti-GAPDH and anti-dynein were obtained from Chemicon (Millipore). Anti-LC3B, anti-Cy5, anti-‑γ-tubulin, and anti-vimentin antibodies were from Sigma. Anti-GFP monoclonal antibody (1E4) was purchased from Stressgen (Enzo Life Sciences, Inc., Farmingdale, NY). Antibodies against alix, flotillin-2, and TGN38 were purchased from Santa Cruz Biotechnology, Inc.

Immunofluorescence—All cells were grown on poly-D-lysine-coated glass coverslips for microscopy. For visualization of intracellular GFP-Tau, cells were transduced with GFP-Tau and allowed at least 48 h for expression of the GFP-Tau construct. The cells were then treated with or without PHFs for 24 h. The GFP construct was expressed following a similar procedure. Cells were washed repeatedly (six times) with PBS, fixed with 4% paraformaldehyde, and immunostained as described previously (43). Fluorescent reagents included secondary antibodies conjugated to Alexa Fluor 594 and Alexa Fluor 647, dextran conjugated to Texas Red, cholera toxin subunit B conjugated to Alexa Fluor 488, and LysoTracker Red DND-99 (Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma). Fixed cells were labeled with 5 μg/ml cholera toxin subunit B-Alexa Fluor 488 for 30 min at 4 °C to enhance plasma membrane labeling and then postfixed in 4% paraformaldehyde for 5 min. Quantitation of cells containing perinuclear aggregates was performed using six arbitrarily selected areas per treatment/group and defined as the percentage of the total number of cells expressing GFP-Tau or GFP. Fluorescence microscopy was performed using an automated upright epifluorescence microscope, Zeiss Axioplan2IE, equipped with a Zeiss AxioCam MRm camera for capturing fluorescent dyes and controlled by Zeiss AxioVision software. Confocal microscopy was performed using a laser point scanning confocal microscope, LSM 700 (Carl Zeiss MicroImaging).

Immunogold Electron Microscopy—Samples were absorbed for 5 min onto Formvar/carbon-coated copper 200 mesh grids (EM Sciences, Fort Washington, PA). The grids were processed as described previously (44) using 10- and 25-nm gold-conjugated secondary antibodies (EM Sciences). Samples were negatively stained with 2% uranyl acetate and viewed in a Hitachi H7000 (Japan) electron microscope operated at 75 kV. The electron microscope was equipped with an Advanced Microscopy Techniques (AMT) Advantage HS digital camera (Danvers, MA), and micrographs were digitally recorded.

RESULTS

Lentiviral Vector-mediated GFP-Tau Expression in Neural and Non-neuronal Cells—Lentiviral vector-mediated GFP-Tau expression within a 0.01– 0.24 range of multiplicity of infection was analyzed by Western blotting using both anti-GFP (1E4) and anti-tau (Ab 7.51) antibodies. In HEK 293T cells, a tau- and GFP-positive band of ~90 kDa was observed at the stepwise increased intensity between 0.03 and 0.24 multiplicity of infection, indicating that cells transduced with GFP-Tau lentivirus were expressing GFP-Tau protein in a dose-dependent manner (supplemental Fig. S1, *A* and *B*). The expression of GFP-Tau was confirmed at the cellular level in co-localization studies using Tau 5 antibody and GFP intrinsic fluorescence (supplemental Fig. S1*C*). Similar data were obtained using SH-SY5Y cells (not shown).

Internalization of PHFs by Cell Cultures—It has been reported that aggregated amyloid-¡3 peptide, prion protein, and expanded polyglutamine can gain entry into cells (45, 46). Furthermore, exogenously derived amyloid-¡3 and prion protein aggregates were found to co-localize with dextran, a marker of fluid-phase endocytosis. Hence, we used Texas Red-labeled dextran to test whether PHFs can undergo uptake into SH-SY5Y cells and HEK 293T cells by means of fluid-phase endocytosis. To visualize PHFs, we labeled PHFs with Cy5 and confirmed the efficiency of labeling by Western blotting and immunogold electron microscopy analyses (Fig. 1*A*). Cy5-labeled PHFs were either added alone or with Texas Red-labeled dextran to SH-SY5Y cells or HEK 293T cells for a 6-h incubation. Confocal microscopy analysis showed that Cy5-labeled

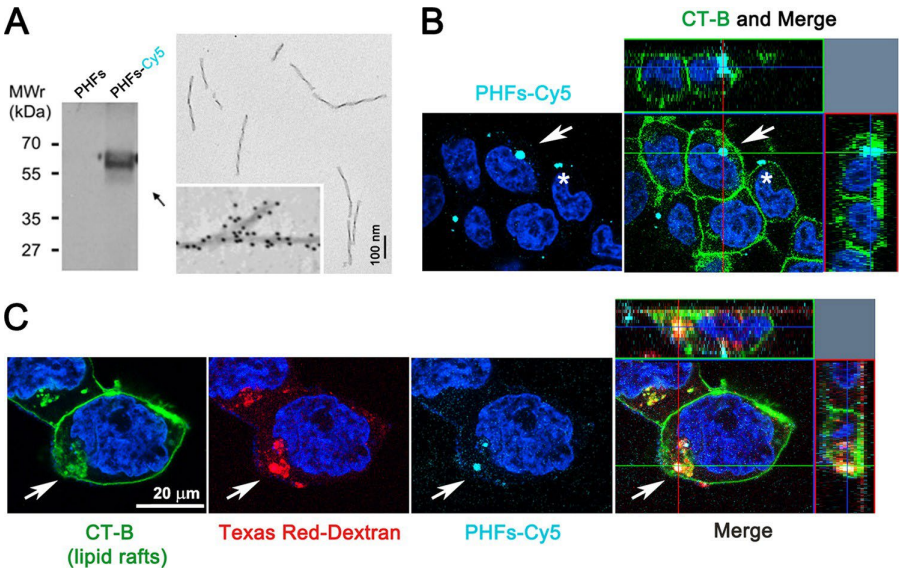

FIGURE 1. **Internalization of PHFs by fluid-phase endocytosis.** *A*, unlabeled PHFs stained with uranyl acetate andexamined byelectron microscopy and PHFs labeled with Cy5 (46) and assessed by Western blotting and 10-nm-immunogold electron microscopy (*inset*) using anti-Cy5 antibody. *B* and *C*, HEK 293T cells treated with PHF-Cy5 alone (*B*) or HEK 293T cells treated with both PHF-Cy5 and Texas Red-dextran (*C*) for 6 h and stained with cholera toxin subunit B (*CT-B*)-Alexa Fluor 488 conjugate to label lipid rafts in the plasma membrane. Cell nuclei were stained with DAPI. *Arrows* indicate internalized PHF-Cy5 aggregates (in *B*) and their co-localization with Texas Red-dextran (in *C*). An *asterisk* denotes an extracellular PHF-Cy5 aggregate. Orthogonal projections in merged panels confirm internalization and co-localization of PHF-Cy5 aggregates. Imaging was performed using a scanning confocal microscope, LSM 700 (Zeiss).

PHFs were able to penetrate the plasma membrane of HEK 293T cells as visualized with cholera toxin subunit B, a marker of lipid rafts (Fig. 1, *B* and *C*, *arrows*). Internalization of Cy5-labeled PHFs was particularly evident in orthogonal projections of confocal images. Cy5-labeled PHFs that did not penetrate the plasma membrane were also evident in the outer layer of the membrane (Fig. 1*B*, *asterisk*). Among internalized Cy5-labeled PHFs, ~30% co-localized with aggregates of Texas Red-labeled dextran inside the cells ($n = 3$; 50 aggregates counted per experiment) (Fig. 1*C*). Our results suggest that PHFs enter the cells by fluid-phase endocytosis.

Extracellular PHFs Accelerate Aggregation of Intracellular GFP-Tau—It has been proposed that tau filaments self-propagate via a seeded polymerization process (47). Furthermore, human recombinant tau protein is able to bind the ends of isolated PHFs, and the sequential binding to PHF fibrils results in an elongation of the original PHFs (39, 48). In the present studies, we determined whether extracellular PHFs upon entering the cell could act as seeds and trigger misfolding of intracellular GFP-Tau. Cultures of HEK 293T and SH-SY5Y cells expressing GFP-Tau were treated with an isolated fraction of authentic human PHFs from AD brain tissue for 24 h. The treatment caused no significant cytotoxicity as judged from lactate production by ELISA ($n = 3$; data not shown). Confocal microscopy examination revealed the presence of distinct GFP-Tau-positive inclusions in PHF-treated HEK 293T (Fig. 2, *A* and *B*) and SH-SY5Y cells (not shown). Inclusions appeared rounded and were located in the perinuclear region of the cell. No more than one inclusion per cell was noted. Although the perinuclear region is enriched in lysosomes, autophagosomes, and Golgi apparatus, we did not detect any significant co-localization of GFP-Tau-positive inclusions with markers for these organelles (LysoTracker, staining for LC3, and staining for TGN38, respectively; data not shown). Occasionally, GFP-Tau inclusions were apparent in cells not treated with PHFs. By quantitative image analysis, we observed a significantly higher percentage of cells bearing GFP-Tau aggregates in the presence of PHFs (43%) as compared with untreated cells (5%) (Fig. 2*E*). We concluded that exogenous PHFs accelerated formation of GFP-Tau aggregates in cultured cells. In comparison, PHF treatment of cells expressing GFP without tau protein sequences resulted in only a minimal increase in the number of GFP-positive aggresomes that was not statistically significant (Fig. 2*E*). Our results suggest that exogenous PHFs may accelerate formation of GFP-Tau aggresomes in a relatively specific manner.

Because formation of similar intracellular tau inclusions has recently been described in cells following inhibition of proteasome activity (27, 28), we performed comparative studies using treatment with 5 µM lactacystin, a proteasome inhibitor. GFP-Tau- and GFP-expressing HEK 293T cells developed intracellular aggregates composed of GFP-Tau (Fig. 2*C*) or GFP (Fig. 2*D*), respectively. The number of lactacystin-stimulated aggregates was slightly higher for cells expressing GFP-Tau (58%) than GFP (40%) (Fig. 2*E*). Because the composition of aggregates depended on the protein that was overexpressed, we concluded that lactacystin induced formation of aggregates in a less specific manner than treatment with PHFs.

To further characterize biochemical effects of PHFs, we examined the solubility of GFP-Tau in cells treated and non-treated with PHFs using fractionation of crude extracts with Sarkosyl. The crude extracts contained overexpressed GFP-Tau with endogenous tau protein too diluted to be detected. Western blotting studies of Sarkosyl-soluble and Sarkosyl-in-

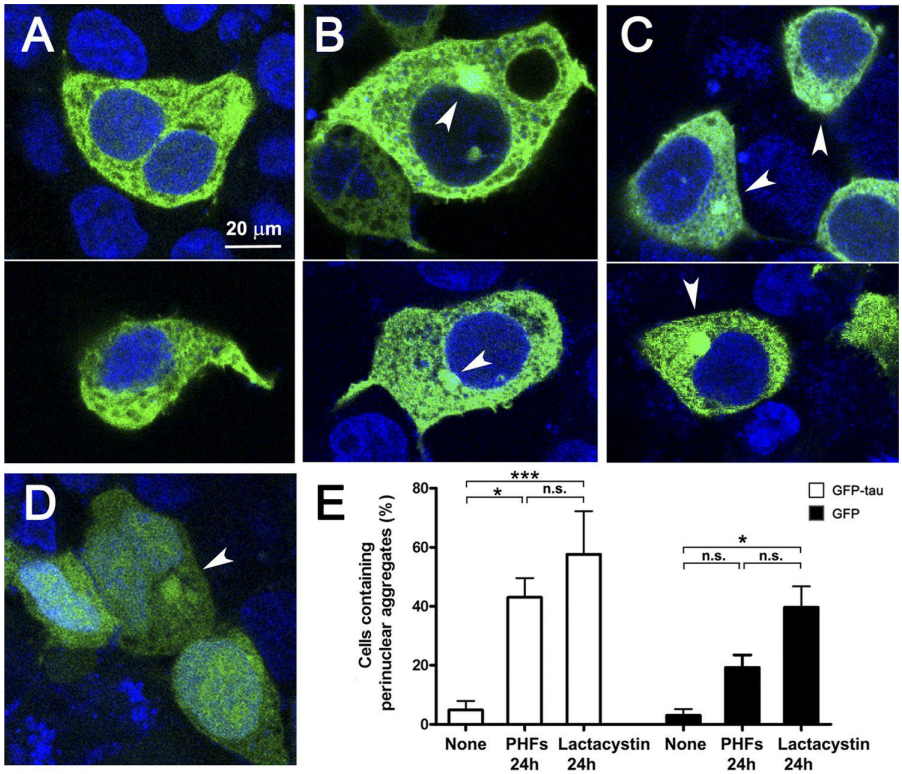

FIGURE 2. **Exogenous PHFs induce intracellular aggregation of tau in HEK 293T cells.** *A*, untreated cells expressing GFP-Tau. *B*, PHF-treated cells showing GFP-Tau-positive aggregates (*arrowheads*). *C*, lactacystin-treated cells showing GFP-Tau-positive aggregates (*arrowheads*). *D*, GFP-expressing cells treated with lactacystin showing GFP-positive inclusions (*arrowhead*). *E*, quantitation of cells containing perinuclear aggregates. Data are means ± S.E.; $n = 6$ ($n = 5$ for GFP + PHFs). *n.s.*, $p > 0.05$; *, $p < 0.05$; and ***, $p < 0.001$ as compared with untreated controls (*None*) using one-way analysis of variance and a post hoc Tukey's test for multiple comparisons among the six groups.

soluble fractions have shown that the treatment of both cell lines with PHFs was associated with a reduced GFP-Tau solubility in Sarkosyl, a distinctive trait for the abnormal aggregation of tau. Quantitatively, SH-SY5Y and HEK 293T cells treated with PHFs displayed ~3-fold increase in the content of Sarkosyl-insoluble GFP-Tau as compared with non-treated cells (Fig. 3, *A* and *B*). Immunogold electron microscopy confirmed the presence of aggregated material in Sarkosyl-insoluble fractions of PHF-treated samples (Fig. 4, *B–E*) and its low abundance in non-treated cells (Fig. 4*A*). Some but not all of the aggregated material consisted of short fibrils, both distinct and in clusters, that were ~20 nm in diameter and appeared twisted. Amorphous material was also detected. Aggregates were labeled for GFP (Fig. 4, *A–E*; 10-nm gold particles) as well as total human tau (HT7) and phosphorylated tau (PHF-1 and Ser(P)^{214}tau) (Fig. 4, *F–H*). Our Western blotting and electron microscopy studies suggest that the treatment with PHFs stimulates the generation of fibrillary and amorphous tau aggregates composed of human GFP-Tau.

Intracellular Aggregates of GFP-Tau Are Aggresomes—The presence of GFP-Tau inclusions in the perinuclear region suggested that they could represent aggresomes known to develop in cells near the centrosome. Using -γ-tubulin as a marker for the centrosome, we confirmed that perinuclear GFP-Tau inclusions formed in close proximity to centrosomes and that their formation was stimulated by PHF treatment (Fig. 5, *A–F*). Next, we examined the occurrence of features associated with aggresome formation. These involved changes in the distribution of cytoskeletal markers including vimentin and the retrograde motor protein dynein in the absence of a gross disruption of the microtubule network (23). In the present studies, vimentin intermediate filaments were distributed evenly throughout the cytoplasm in untreated SH-SY5Y cells (Fig. 6, *A–C*). After 24-h treatment with PHFs, the vimentin filament network appeared to be distorted. There was a redistribution of vimentin filaments to the perinuclear region with the appearance of filament bundles and cagelike structures (Fig. 6, *D–F*, *arrows*) characteristic of aggresomes in cultured cells (23, 24). GFP-Tau aggregates were prominent in the perinuclear region as well (Fig. 6, *D–F*, *asterisks*). Like vimentin, dynein was distributed evenly throughout the cytoplasm in untreated HEK 293T cells (supplemental Fig. S2, *A–C*). Cells expressing GFP-Tau showed more distinct dynein staining than non-transduced cells, which could be due to compensatory dynein expression. After 24-h treatment with PHFs, dynein was clustered in the perinuclear region, most likely around the centrosome, although some dynein was still detected in the cytoplasm (supplemental Fig. S2, *D–F*, arrows). Similar results were obtained using SH-SY5Y cells (not shown). Changes in the distribution of both cytoskeletal markers strongly suggest that PHF treatment accelerated the formation of aggresomes in both non-neural and neuronal cell lines.

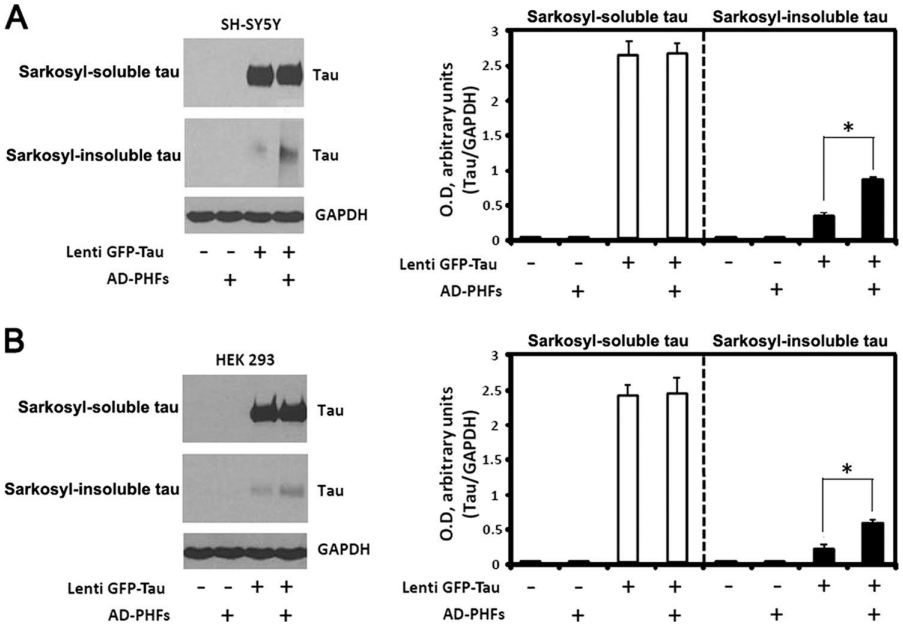

FIGURE 3. **Sarkosyl-soluble and -insoluble tau content with or without extracellular PHFs.** *A*, Western blotting of Sarkosyl-soluble and -insoluble fractions from SH-SY5Y cells with or without expression of GFP-Tau treated or untreated with PHFs. Gels are 10% polyacrylamide. *B*, densitometric quantitation of Sarkosyl-soluble and -insoluble fractions from HEK 293T cells expressing or not expressing GFP-Tau and treated or untreated with PHFs. Antibody 7.51 against total tau was used. Data are means ± S.D.; $n = 3$. *, $p < 0.05$ as compared with untreated controls by Student's *t* test. *Lenti*, lentivirus.

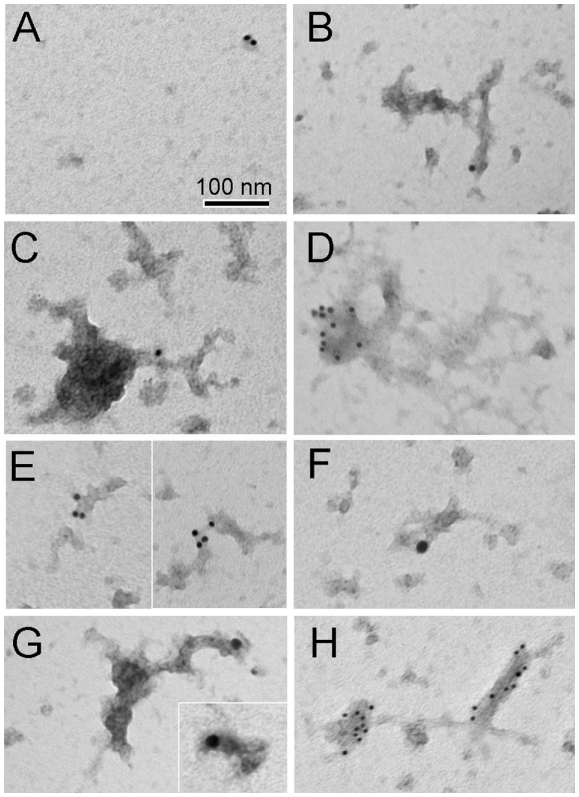

FIGURE 4. **Immunogold electron microscopy of Sarkosyl-insoluble tau aggregates.** Sarkosyl-insoluble fractions from untreated HEK 293T cells (*A*) and HEK 293T cells treated with AD PHFs (*B–H*) for 24 h and labeled with immunogold particles. *A–E*, anti-GFP (10-nm gold); *F*, PHF-1 for phosphorylated tau (25-nm gold); *G*, total human tau (HT7; 25-nm gold); and *H*, Ser(P)^{214}tau (10-nm gold). A Hitachi H7000 electron microscope was used.

Exosomes Released from Cultured Cells Do Not Contain GFP-Tau—Abnormally aggregated tau could be secreted via exosomes in a potential mechanism of pathological spreading among neurons. Therefore, we examined whether exosomes produced and released to the medium by SH-SY5Y cells contained tau protein. As shown by others, expression of tau with the GFP tag was not expected to influence GFP-Tau secretion to the culture medium (49). Results of Western blotting showed that total cell lysates from SH-SY5Y cells expressing endogenous tau (control) and SH-SY5Y cells expressing both endogenous tau and GFP-Tau contained roughly comparable levels of flotillin (Fig. 7*A*, *Cell*, *arrowhead*). Both cells showed the presence of RD3-positive (~55 kDa) and RD4-positive (~55 and ~48 kDa) polypeptides of tau representing endogenous three-repeat and four-repeat tau, respectively (Fig. 7, *B* and *C*, *arrowheads*). Cells expressing GFP-Tau additionally displayed the RD4-positive and RD3-negative band at ~90 kDa, most likely representing the GFP-Tau construct (Fig. 7, *B* and *C*, *asterisk*). Exosome-enriched fractions isolated from the conditioned media of both control and GFP-Tau-expressing SH-SY5Y cells were highly enriched in ~40 –70-nm-diameter exosomes as characterized by electron microscopy (Fig. 7*D*). By Western blotting, exosome-enriched fractions contained the flotillin-positive band of 42 kDa with a marginally higher content in media from GFP-Tau-expressing cells than controls as detected in overexposed blots (Fig. 7*A*, *arrowhead*). A flotillin- positive band of 42 kDa was absent in preparations obtained from unconditioned medium, demonstrating the specificity of the assay. Exosome-enriched fractions had no detectable tau-positive immunoreactivity at 48 –55- or 90-kDa regions even at severely overexposed conditions (up to 1.5 h; *bottom panels*), suggesting that exosomes secreted by SH-SY5Y cells contained

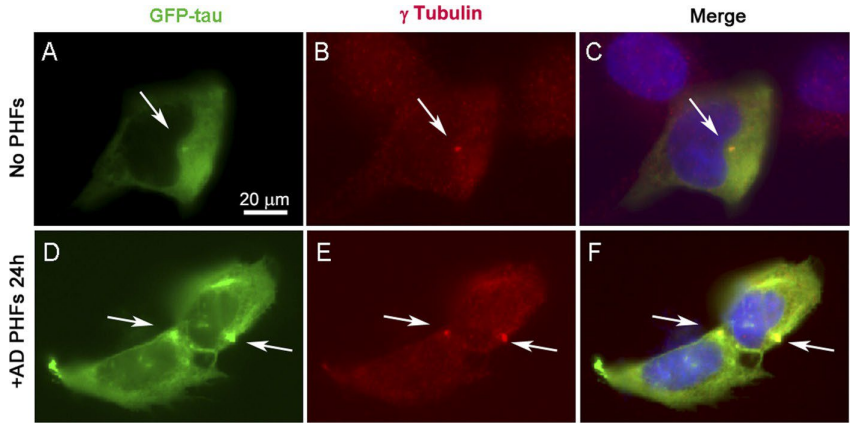

FIGURE 5. **PHFs cause formation of aggresomes co-localized with -γ-tubulin.** Immunocytochemistry of SH-SY5Y cells expressing GFP-Tau (*green*) and either not treated (*No PHFs*) or treated with PHFs (*+AD PHFs*) for 24 h is shown. Cells were fixed and stained for -γ-tubulin (Alexa Fluor 594; *red*). *Arrows* show perinuclear region without (*A–C*) or with aggregated GFP-Tau-positive inclusions (*D–F*) co-localized with the -γ-tubulin-positive centrosome.

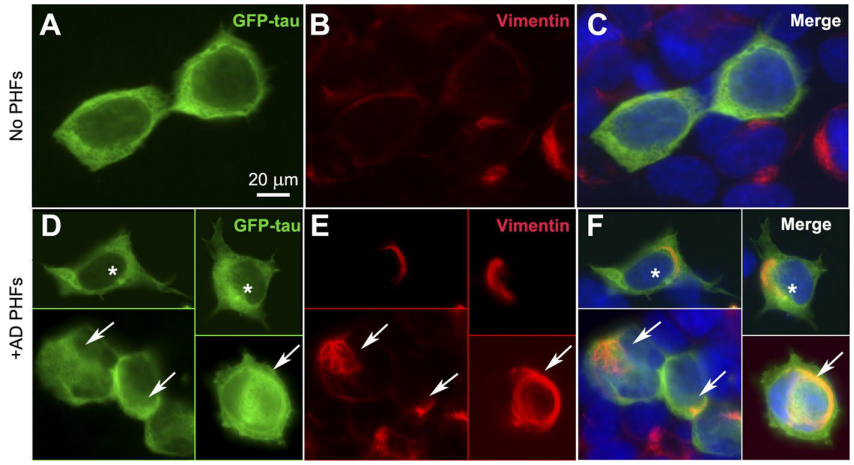

FIGURE 6. **PHFs cause changes in vimentin filament network.** Immunocytochemistry of SH-SY5Y cells expressing GFP-Tau (*green*) and either not treated (*No PHFs*) or treated with PHFs (*+AD PHFs*) for 24 h. Cells were fixed and stained for vimentin (Alexa Fluor 594; *red*). GFP-Tau-positive perinuclear inclusions are marked with *asterisks*. Redistributed vimentin filaments are marked with *arrows*.

little tau or that tau was below the sensitivity of our assay (Fig. 7, *B* and *C*, *bottom panels*, *arrowheads* and *asterisk*). Our studies suggest that it is unlikely that tau is secreted via the exosomal pathway.

In our effort to isolate exosomes secreted by PHF-treated cells, we were unable to adequately separate individual exosomal and PHF fractions by differential centrifugation of the conditioned media. The sucrose gradient centrifugation necessary to isolate each fraction resulted in a significant overlap between preparations. Other techniques and approaches need to be used in our further studies. Therefore, it is still possible that, unlike tau, internalized PHFs could be transported and released via the exosomal pathway.

Monomeric GSE Prevents Intracellular Aggregation of Tau Induced by Extracellular PHFs—Our previous studies have shown that 5–10 μM GSE was able to disrupt fibrillary conformation of PHFs in fractions isolated from AD brain and rapidly within an hour caused a significant disintegration of PHFs *in vitro* (32). Based on those studies, we selected a 25 μM GSE concentration to treat SH-SY5Y cells and explore whether bioactive monomeric GSE may attenuate PHF-dependent misfolding of tau protein in cultured cells. Monomeric GSE was added 2 h prior to PHF treatment for another 24 h. This protocol was well tolerated by the cells as determined by a cytotoxicity assay of lactate production (not significant; $n = 3$; data not shown). GSE and PHF co-treatment resulted in the attenuation of tau aggregation induced by PHF treatment alone. Fractionation with Sarkosyl followed by Western blotting of Sarkosyl-soluble and Sarkosyl-insoluble fractions indicated a significant reduction in the content of intracellular aggregated tau (Sarkosyl-insoluble tau) induced by PHFs (Fig. 8). With monomeric GSE, Sarkosyl-insoluble tau content was reduced approximately from 0.83 to 0.16 arbitrary unit (by 81%), whereas the content of Sarkosyl-soluble tau remained constant (Fig. 8*B*). These studies suggest that monomeric GSE may play a potential role in attenuation of tau aggregation in cell culture models.

DISCUSSION

It has been known for more than 25 years that NFTs have a hierarchical pattern of accumulation reflecting selective vulnerability of neuronal populations in AD brain. Despite such recognition, there is no clear understanding of the mechanism

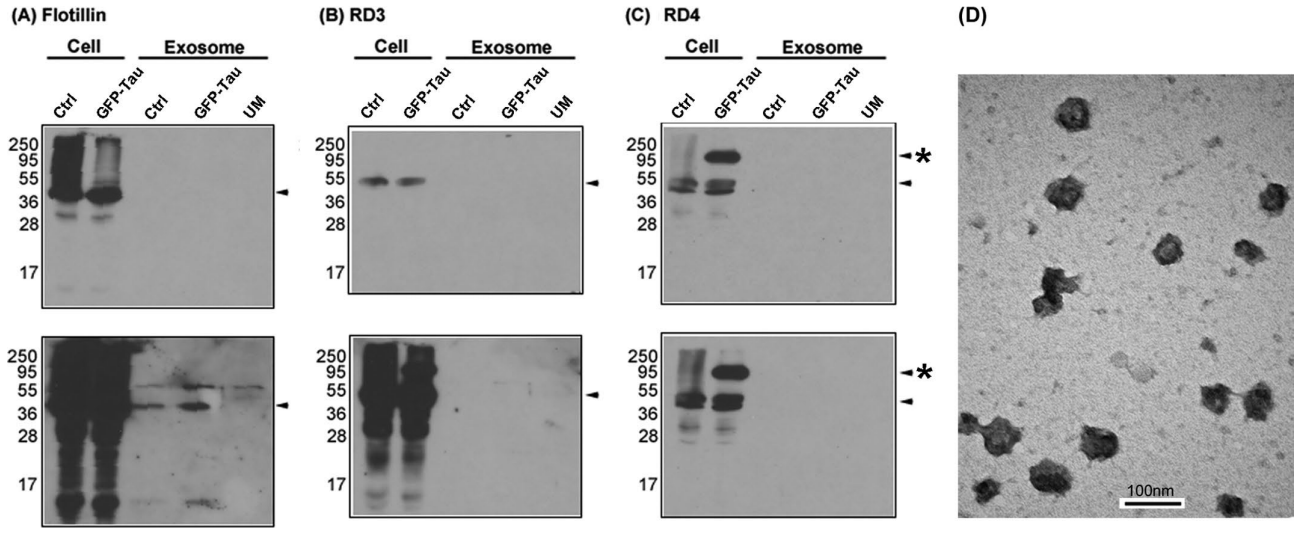

FIGURE 7. **Exosomes secreted by SH-SY5Y cells do not contain GFP-Tau.** Total cell homogenates (*Cell*) and exosomes (*Exosome*) isolated from the conditioned medium of control (*Ctrl*) and GFP-Tau-overexpressing (GFP-Tau) SH-SY5Y cells and unconditioned medium (*UM*) (see "Experimental Procedures"). *A*, staining for flotillin as an exosomal marker protein. *B*, anti-tau clone RD3 binding three-repeat tau at ~55 kDa. *C*, anti-tau clone RD4 binding four-repeat tau at ~48 and ~55 kDa and the GFP-Tau construct (*) at 90 kDa. Respective proteins are indicated by *arrowheads*. The same blots were exposed for 5 min (*upper panels*) or 1–1.5 h (*lower panels*). Gels are 12% polyacrylamide. *D*, electron microscopy of the isolated exosomal fraction (GFP-Tau cells) stained with uranyl acetate. Exosomes measure 40–70 nm in diameter.

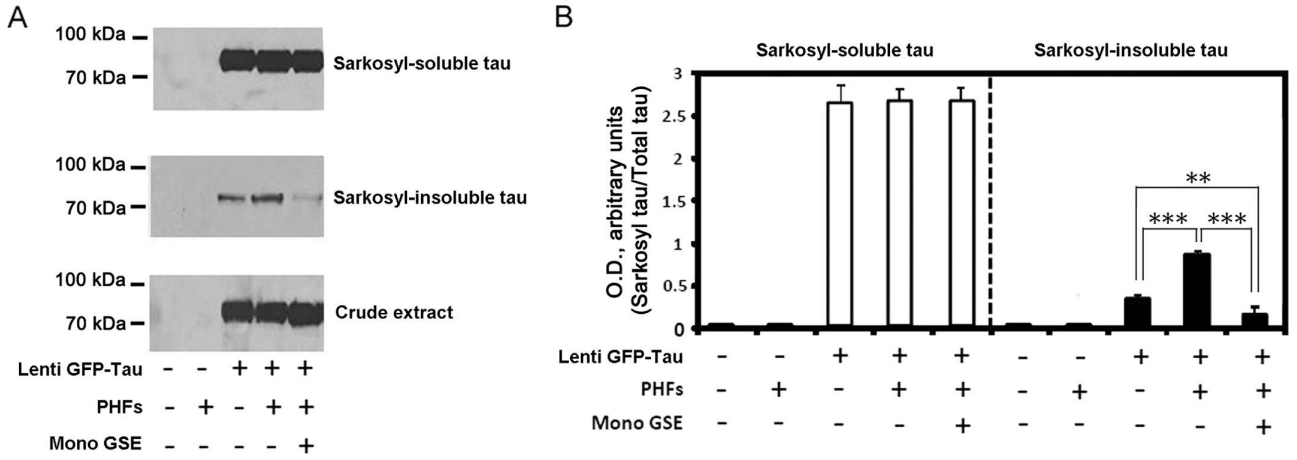

FIGURE 8. **Monomer-enriched fraction of grape seed polyphenolic extract (*Mono-GSE*) prevents formation of Sarkosyl-insoluble tau upon addition of PHFs.** Western blot analysis (*A*) and densitometric quantitation (*B*) of Sarkosyl-soluble and -insoluble fractions from SH-SY5Y cells expressing or not expressing GFP-Tau and treated or not treated with PHFs in the presence of monomeric GSE (25 μM) are shown. Data are means ± S.D.; $n = 3$. **, $p < 0.01$ and ***, $p < 0.001$ as compared with respective controls (no PHFs or no mono-GSE) using one-way analysis of variance and a post hoc Tukey's test for multiple comparisons among the three groups.

of disease progression. In this study, we show for the first time that extracellular human AD PHFs can be internalized by cultured cells and propagate a misfolded state to native soluble intracellular tau protein. This process leads to aggregation of tau into structures resembling aggresomes and may be an initial step in NFT formation and pathological tau protein spreading.

We determined that PHFs entered the cells by fluid-phase endocytosis, a mechanism broadly defined as the endocytic pathway involving engulfment by the cell membrane. In this respect, PHFs appeared to follow many extracellular amyloids and amyloid precursors that could be taken up by a wide variety of cell types. This uptake may occur through phagocytic or endocytic processes that result in delivery to lysosomes (45, 50). However, all of these mechanisms would deliver aggregates to an endomembrane compartment and not to the cytosol where a direct contact between misfolded aggregate and soluble protein might be expected. Further studies are needed to define whether classical or uncommon endocytic pathways are responsible for internalization and cellular transport of PHFs (51). An alternative mechanism has been reported in relation to fibrillar polyglutamine aggregates, which play a role in Huntington disease (46). The fibrillar polyglutamine aggregates were internalized by simply crossing the membrane barrier in a number of cell lines including HEK 293 and N2a cells. Those studies found no evidence for involvement of endosomal compartment, endomembranous structures, or clathrin in the uptake of aggregates and their deposition in cytosol.

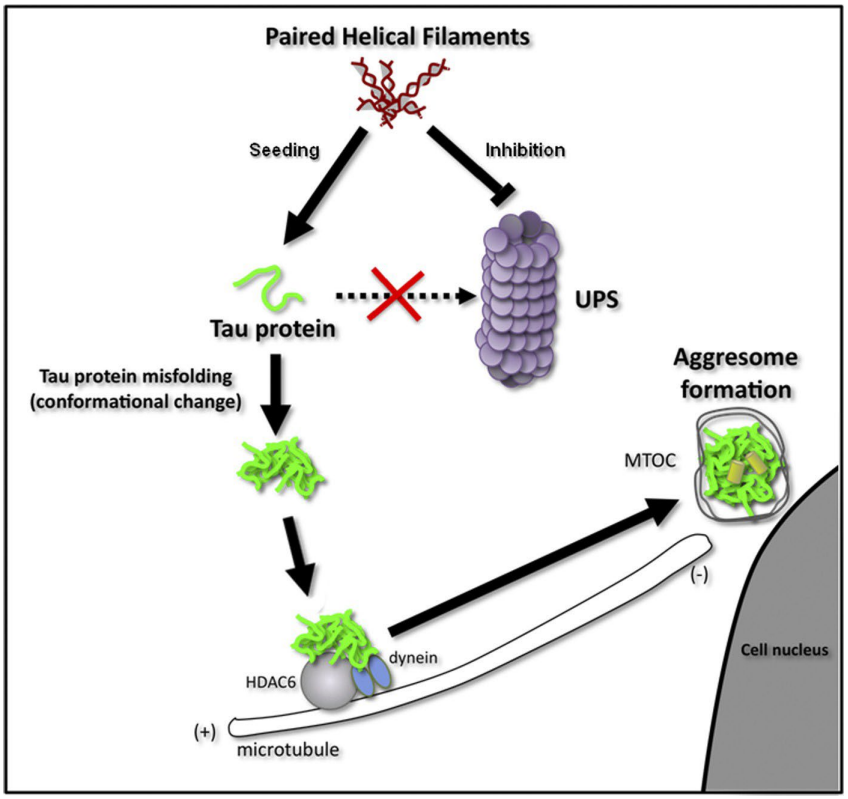

FIGURE 9. **Diagram of PHF-induced misfolding of tau and formation of aggresome.** Extracellular human AD PHFs are endocytosed by cultured cells. Once internalized, PHFs can promote tau protein misfolding and fibrillary changes. PHFs can also inhibit the ubiquitin-proteasome system (*UPS*) (52). Inhibitors of the ubiquitin-proteasome system (*e.g.* lactacystin) block proteasome-dependent degradation of soluble tau protein (53) and cause its misfolding (54). Misfolded tau is sequestered to the aggresome at the microtubule-organizing center (*MTOC*) where it could be recruited to the degradation machinery, *e.g.* autophagy (28, 67). Tau aggresome formation requires microtubule-dependent retrograde transport (motor protein dynein) and histone deacetylase 6 (*HDAC6*) (27).

We observed that highly purified human AD PHFs were internalized within 24 h without any significant cell toxicity. Previous studies also found that 24-h incubations of SH-SY5Y cells with PHFs from AD were well tolerated (13). This is in contrast to extracellular recombinant tau protein or tau protein mutants, which were found to be highly toxic to the cells (13, 14). In the present studies, internalized PHFs were able to decrease the solubility of GFP-Tau in Sarkosyl, a measure of abnormal tau aggregation. In comparison with an insignificant amount of GFP-Tau aggregates in untreated cells, in PHFs-treated cells, the GFP-Tau aggregates were readily apparent by electron microscopy. Some of the isolated fibrils appeared twisted and were ~20 nm in diameter. In others, the fibrillary nature was less prominent, which could be due to the structural modification (GFP fusion) introduced to the N terminus of tau. The intracellular origin of GFP-Tau aggregates was confirmed by their immunogold labeling for GFP. The aggregates were also labeled for total tau (HT7) and phosphorylated tau (PHF-1 and Ser(P)^{214}). Similar phosphorylated tau epitopes characteristic of authentic AD PHFs were found in tau aggresomes and tau aggregates in HEK 293 and other cell lines, indicating that cultured cells express the necessary tau protein kinases (7, 27, 41).

Internalized PHFs were able to accelerate the formation of GFP-Tau-positive inclusions (aggresomes) in a pericentriolar location of the cells. Other hallmarks of aggresome formation were also displayed as seen by redistribution of the vimentin filament network and perinuclear accumulation of the retrograde microtubule motor protein dynein. Both cytoskeletal changes accompanied aggresome formation in other cell culture systems (23, 24).

Based on our observations, we propose that extracellular PHFs are able to transmit fibrillary changes to soluble intracellular GFP-Tau and consequently accelerate the formation of GFP-Tau aggresomes as illustrated in Fig. 9. Such a mechanism could be analogous to that of fibrillar polyglutamine aggregates reported to nucleate the aggregation of otherwise soluble proteins containing polyglutamine tracks in aggresomes (46). In other studies, extracellular tau aggregates prepared in an arachidonic acid-dependent manner were able to induce fibrillization of intracellular full-length tau (4R2N) in C17.2 neural precursor cells (5). Whether the intracellular tau aggregates were aggresomes is not clear because often more than one perinuclear aggregate per cell was detected in that study. To learn more about the underlying mechanisms, it will be important to test whether PHFs originating from other disorders such as corticobasal degeneration, progressive supranuclear palsy, and Pick disease have effects similar to that of PHFs in AD. If fibrillary changes are important for aggresome formation, then similarities among PHFs would be expected.

Because formation of aggresomes is considered a cellular protective response, it is possible that internalized PHFs trig-

gered a common pathway that cells used to dispose of or neutralize aggregated proteins. Isolated PHFs as well as *in vitro* assembled tau fibrils were reported to significantly impair proteasome function *in vitro* (52). Inhibition of the proteasome promoted the formation of large spherical tau-positive inclusions resembling aggresomes in the present studies and by others (27, 28). Such inhibition has been shown to block tau degradation (53) and increase levels of detergent-insoluble tau (27, 54). It is reasonable to conclude that PHFs upon their cellular internalization may not only transmit fibrillary changes to soluble intracellular GFP-Tau but also inhibit proteasome activity with both events leading to aggresome formation as illustrated in Fig. 9. Further studies will determine which event is more prevalent in PHF effects.

Our proposed mechanism of PHF-dependent propagation of tau pathology is consistent with the hypothesis that abnormal tau aggregates propagate misfolding of soluble tau protein within the brain (14) and that AD is associated with the failure of the ubiquitin-proteasome system (55). It is well known that misfolded tau protein accumulates progressively throughout the brain in tauopathies including AD. Following uptake by healthy neurons, the abnormal tau aggregates might stimulate further misfolding of an otherwise stable intracellular tau protein in the manner of prions. This idea has already been tested *in vivo* using transgenic brain extract for spreading its seeding material (6) and in transgenic mouse models evaluating the anatomy of tau pathology spreading (56, 57). Our studies are unique in their use of human PHFs isolated from AD brains for *in vitro* tau pathology spreading applications. We predict that introduction of isolated PHFs into the brain of susceptible animals will induce aggregation of endogenous tau protein and progression of pathology.

Exosomes are considered important organelles for intercellular communication and in the processing of proteins associated with neurodegenerative diseases (12, 16). For example, a-synuclein, which is central in Parkinson disease pathogenesis, was secreted as insoluble oligomeric and monomeric species in the exosomes isolated from conditioned media of SH-SY5Y cells expressing wild-type a-synuclein (19). Exosomes containing prions initiated prion propagation in neighboring cells (58). Exosomes bearing ¡3-amyloid peptides of intracellular origin were released from amyloid precursor protein-stably transfected neuroblastoma N2a cells (18) and from primary cortical neurons (59). Tau protein and microtubule-associated protein 2, however, were absent in an exosomal fraction prepared from conditioned media of rat cortical neurons (60) and a wide variety of primary cells, cell lines, and body fluids (61). The results of our studies showing that tau protein was undetectable in exosomes released by SH-SY5Y cells add to this body of evidence.

However, recent reports showing the inhibitory role of the exon 2 insert in tau secretory activity have shed light on the mechanism of tau secretion via exosomes (49, 62). The importance of this discovery is at least 2-fold: intercellular transfer of certain forms of tau may occur via exosomal release as is known for a-synuclein, ¡3-amyloid, and prions, and heterogeneity in the processing of tau isoforms may be important for neurodegeneration (63). Apparently, tau isoforms expressing exon 2 sequences including the GFP-Tau construct used in the present studies may follow processing and release mechanisms that are independent of exosomes. For these tau species, more relevant intercellular propagation pathways could be exophagy (21), spontaneous intercellular transfer of aggregated tau (5), or budding of membrane vesicles (64). Aggresome formation may represent one of the exosome-independent mechanisms of tau processing and deposition for exon 2-expressing tau species. The present studies and others showing recruitment of 4R1N and 4R2N tau isoforms to aggresomes or aggresome-like aggregates support this conclusion (7, 28).

Extracellular PHFs and intracellular tau aggregates may provide an accessible target for tau-lowering therapies. Grape-derived bioactive GSE preparations comprising proanthocyanidins have been demonstrated to exert a wide scope of neuroprotective biological activities in animal models of tauopathy and neurodegeneration (33–36, 65). More complex proanthocyanidins, however, including dimers, trimers, and oligomers have a reduced bioavailability in the rat brain when compared with monomers (66). Results of the present studies indicate that such a bioavailable monomeric GSE-enriched fraction may interfere with the formation of GFP-Tau aggregates. The interference could be attributed to disruption of fibrillary conformation and seeding potential of PHFs before their contact with the soluble tau pool as shown recently using PHF fractions isolated from AD brains (32). It could also result from direct blocking of the aggregation at the level of soluble GFP-Tau protein as shown in tau peptide studies (31). Both scenarios are equally plausible as they ultimately lead to a reduced formation of GFP-Tau aggregates. Further research is needed to discriminate between these possibilities. The present studies strongly suggest that monomeric GSE may play a potential role in the attenuation of tau pathology associated with extracellularly derived PHFs as a spreading mechanism.

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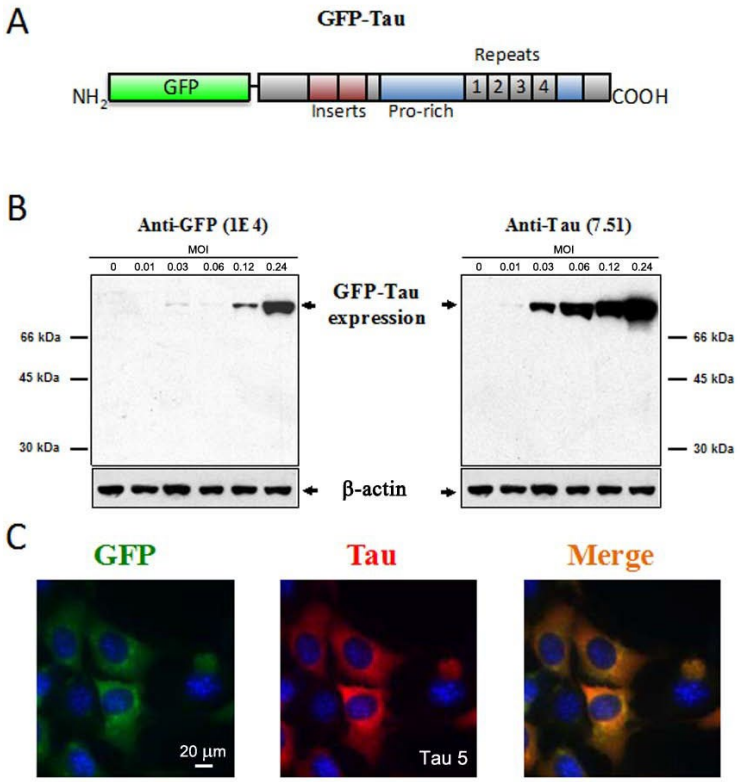
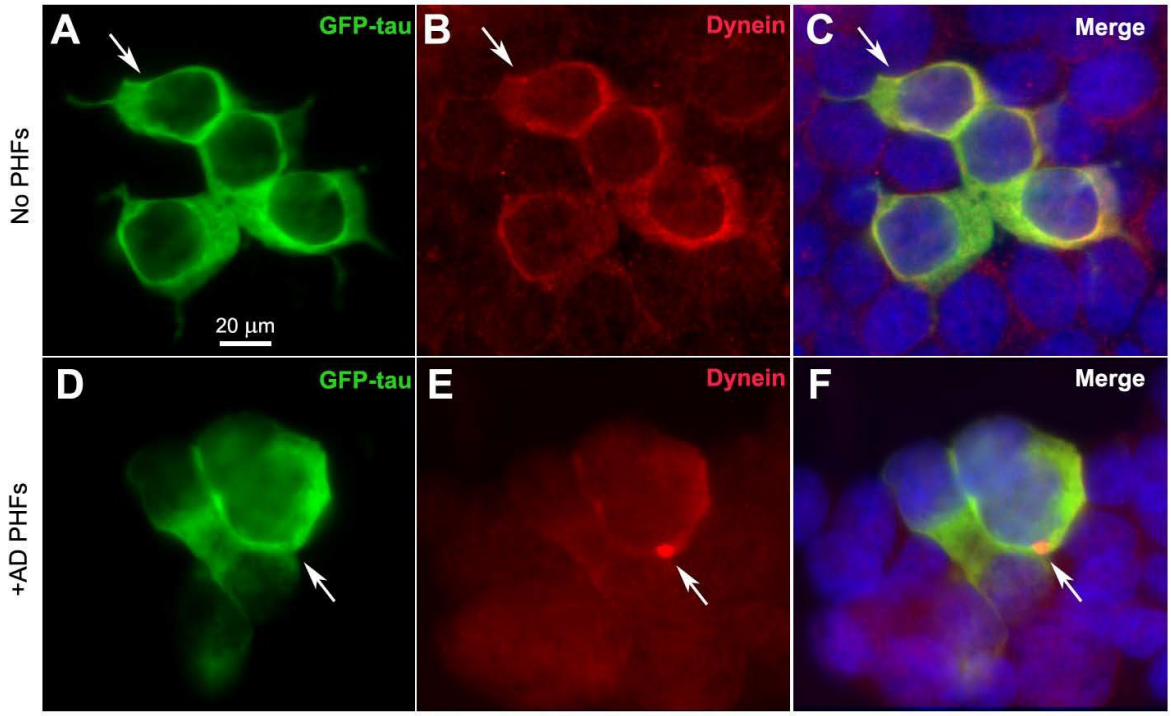
Supplemental Data to Santa-Maria et al.,
"Paired Helical Filaments from Alzheimer's Disease Brain Induce Intracellular Accumulation of Tau in Aggresomes"

Supplemental Figure S1



Supplemental Figure S1. Lentiviral mediated GFP-Tau expression in cell culture. ***A,*** Tau construct: GFP-Tau fusion protein lentivector created for expression in mammalian cells. ***B,*** Western blotting analysis of GFP-Tau protein expression in HEK 293T cells using increasing MOI levels. Beta-actin used as loading control. ***C,*** Immunofluorescence of HEK 293T cells expressing GFP-Tau fusion protein. Antibodies against GFP protein (1E4) and tau (7.51 and Tau 5) as indicated.

Supplemental Figure S2



Supplemental Figure S2. PHFs cause changes in dynein localization. Immunocytochemistry of HEK 293T cells expressing GFP-Tau (green) and either not treated (no PHFs) or treated with PHFs (+AD PHFs) for 24h. Cells were fixed and stained for dynein (Alexa594; red). Arrows show perinuclear region with accumulated dynein. Note a heavy cluster of dynein in PHFs treated but not control (no PHFs) cells.