Down Syndrome Fibroblast Model of Alzheimer-Related Endosome Pathology

Accelerated Endocytosis Promotes Late Endocytic Defects

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Endocytic dysfunction is an early pathological change in Alzheimer's disease (AD) and Down's syndrome (DS). Using primary fibroblasts from DS individuals, we explored the interactions among endocytic compartments that are altered in AD and assessed their functional consequences in AD pathogenesis. We found that, like neurons in both AD and DS brains, DS fibroblasts exhibit increased endocytic uptake, fusion, and recycling, and trafficking of lysosomal hydrolases to rab5-positive early endosomes. Moreover, late endosomes identified using antibodies to rab7 and lysobisphosphatidic acid increased in number and appeared as enlarged, perinuclear vacuoles, resembling those in neurons of both AD and DS brains. In control fibroblasts, similar enlargement of rab5-, rab7-, and lysobisphosphatidic acid-positive endosomes was induced when endocytosis and endosomal fusion were increased by expression of either a rab5 or an active rab5 mutant, suggesting that persistent endocytic activation results in late endocytic dysfunction. Conversely, expression of a rab5 mutant that inhibits endocytic uptake reversed early and late endosomal abnormalities in DS fibroblasts. Our results indicate that DS fibroblasts recapitulate the

neuronal endocytic dysfunction of AD and DS, suggesting that increased trafficking from early endosomes can account, in part, for downstream endocytic perturbations that occur in neurons in both AD and DS brains. (Am J Pathol 2008; 173:370–384; DOI: 10.2353/ajpath.2008.071053)

Morphological alterations of endocytic compartments or perturbations of endocytic trafficking and function have been described in a number of neurodegenerative diseases including Alzheimer's disease (AD),^{1–3} Down's syndrome (DS),^{3,4} Niemann-Pick's disease,^{5–7} amyotrophic lateral sclerosis,^{8,9} and Huntington's disease.^{10–13} In AD, extensive neuronal endocytic pathology is seen in the earliest stages of the disease and before β -amyloid deposition.⁴ In DS, neuronal endocytic abnormalities are seen decades before β -amyloid deposits and tau pathology in brain, which develop in DS individuals in the fourth or fifth decades of life. Evidence has accumulated both from observations made in human tissue and experimental modeling in cell culture that alterations in the endocytic pathway can contribute to β -amyloidogenesis.^{1,2,14–17} AD-related endocytic alterations coincide with the detection of A β within endosomal compartments and the initial rise in soluble A β peptides.^{14,15} The presence of A β in early endosomes is also consistent with the co-localization of its precursor, the amyloid precursor protein (APP) and the β -site APP-cleaving enzyme 1 (BACE1) within the same early endocytic compartments^{18–21} and the degradation of BACE1 within the endosomal-lysosomal system.²² Taken together these data support a close rela-

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tionship between endocytic pathway dysfunction and the amyloidogenic processing of APP and highlight the endocytic pathway and endosomes as important sites for these interactions.

In the widely used segmental trisomy 16 mouse model of DS, the Ts65Dn mouse, early endosomal pathology has been shown in neurons in the basal forebrain, hippocampus, and neocortex²³—areas that exhibit aging-related neuronal atrophy and degenerative changes, particularly of cholinergic neurons.^{24–27} These endocytic changes are believed to lead to dysfunction in endosome-mediated nerve growth factor trophic signaling,²⁸ with this compromise in trophic support resulting in neurodegeneration. In human DS and early onset familial forms of AD, the presence of three copies of the *App* gene is critical to AD development.^{29–31} Similarly, in Ts65Dn mice, lowering the dosage to the diploid level reversed early endosomal pathology²³ and restored trophic signaling by nerve growth factor and abrogated the cholinergic neuron degeneration.²⁸ Taken together, these findings argue for a central role of neuronal endocytic alterations in the pathogenesis of AD by increasing neuronal vulnerability and altering the processing of APP.

For these reasons, we sought to explore in greater detail the nature of altered endocytic interactions seen in neurons at early stages of AD and DS and to assess the functional consequences of the abnormal early endocytic response in AD pathogenesis by using primary skin fibroblasts from individuals with DS. Our results show that the broad endocytic pathway dysfunction seen in AD and DS brain is also exhibited in DS fibroblasts. DS fibroblasts displayed morphological changes in early endosomes resembling those seen in neurons from AD and DS brain, as well as endosome dysfunction reflected in endocytic uptake and fusion and downstream alterations of late endosomes. These perturbations are associated with increased delivery of the mannose 6-phosphate receptor and its ligands, lysosomal hydrolases like cathepsin D (CatD), to endocytic compartments, thereby altering APP processing.

Materials and Methods

Tissue

Postmortem brain tissue from 10 elderly individuals, ranging in age from 62 to 80 years, was examined and diagnosed with neuropathological evidence of early-stage AD according to the guidelines of The Consortium to Establish a Registry for Alzheimer's Disease.^{32–34} We also studied an equal number of age-matched control cases, which were evaluated using the same criteria and found to be normal by neuropathological inspection. Additionally, brain tissue from seven cases of DS and seven age-matched normal controls were examined. The DS and control groups ranged in age from 28 weeks gestation to 12 years and neither group showed evidence of neuritic plaques or neurofibrillary tangles. Fixed AD and control tissue were obtained from the Harvard Brain Tissue Resource Center at McLean Hospital (Belmont, MA),

the Bronx Veteran's Administration (Bronx, NY), and Mt. Sinai Medical Centers (New York, NY). Tissue from DS individuals and age-matched controls were obtained from the Johns Hopkins University Brain Resource Center (Baltimore, MD).

Cell Culture, Western Blot, and Immunolabeling Analyses

Human forearm skin fibroblasts from DS and diploid age-matched controls (2N) were purchased from the Coriell Cell Repositories (Camden, NJ) and grown in minimum essential medium (Life Technologies, Grand Island, NY), supplemented with 15% fetal bovine serum (Hyclone Perbio South, Logan, UT), 2 mmol/L glutamine (BioWhittaker, Walkersville, MD), and penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C and 5% CO₂ and passaged using standard protocols as instructed by Coriell Cell Repositories. For all experiments, cell passage ranged from p4 to p13 and cell confluency was consistently 85 to 90%. For Western blot analyses, cell monolayers initially seeded at 3.5×10^5 cells per 60-mm dish were scraped into cold phosphate-buffered saline (PBS). This cell suspension was centrifuged at 3000 rpm ($1600 \times g$) for 10 minutes at 4°C and the resulting pellet resuspended in lysis buffer containing protease inhibitor cocktail (10 mmol/L NaF, 1 mmol/L ZnCl₂, 1 mmol/L orthovanadate, 5 mmol/L Na pyrophosphate, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 1 µg/ml chymostatin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 µg/ml antipain, and 1 µg/ml leupeptin). Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was done as previously described.^{14,15} Cultures established in a similar manner and grown on glass coverslips were generated for analyses by immunolabeling. Cells on coverslips were washed three times with PBS and fixed for 20 minutes at room temperature with 4% paraformaldehyde, processed for immunofluorescent labeling as previously described,^{14,15} and examined using an epifluorescent microscope (Carl Zeiss, Thornwood, NY).

Antibodies

Immunocytochemical studies were performed using commercially available polyclonal antisera recognizing rab5A, rab4, and rab7 (no. SC-309, no. SC-312, no. SC-10767; all purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA), a myc-tagged epitope (Upstate Inc., Lake Placid, NY), and the lysosomal hydrolase, CatD (rabbit, 1:400, A0561; DAKO, Carpinteria, CA). Commercially available monoclonal antibodies were used that recognize early endosome antigen 1 (EEA1) (BD Biosciences, San Jose, CA), the myc-tag 9E10 (Upstate Inc., Lake Placid, NY) and LAMP1 (1:500, H4A3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IO). A mouse monoclonal antibody, which has been purified and characterized previously^{35–37} was used and directed against lysobisphosphatidic acid (LBPA).

22D4, a monoclonal antibody recognizing the 46-kDa mannose 6-phosphate receptor (MPR46),^{38,39} was a gift from Dr. Jack Rohrer (Department of Physiology, University of Zurich, Zurich, Switzerland). Secondary antibodies for immunocytochemistry were purchased from Invitrogen/Molecular Probes, Inc. (Carlsbad, CA) and for Western blot analyses from Promega (Madison, WI).

Recombinant Viruses

Recombinant herpes simplex virus type 1 (HSV1) amplicons expressing myc-tagged human wild-type rab5, the dominant-positive (GTPase-deficient) rab5Q79L, dominant-negative rab5S34N, or control LacZ were generated as previously described.⁴⁰ Forty-eight hours after seeding, cells were washed with warm PBS and infected for 5 hours with the recombinant viruses in complete media, using 20 μ l of virus per 60-mm dish (multiplicity of infection, 2.0).

Receptor-Mediated and Fluid Phase Uptake

Receptor-mediated uptake studies were performed on three of four DS and age-matched 2N control fibroblasts lines using Alexa 568-tagged transferrin and Alexa 488-tagged epidermal growth factor (EGF) (Molecular Probes Inc., Eugene, OR) as previously described.^{41–43} Briefly, cells were seeded in 100-mm Petri dishes containing glass coverslips and were exposed to ligand at 37°C continuously for up to 60 minutes. To examine the location of ligand at a given time point, coverslips were removed and cooled to prevent further internalization and washed with ice-cold PBS to remove nonreceptor-bound ligand, and immediately fixed at room temperature with 4% paraformaldehyde in PBS containing 5% sucrose, pH 7.4, for 20 minutes, washed with PBS, and mounted with gel-based mounting medium. The amount of transferrin and EGF at a given time point was quantitated within the remaining cells by Western blot analysis. Fluid phase uptake studies were performed using horseradish peroxidase (HRP) type II (Sigma-Aldrich, St. Louis, MO) and processed for electron microscopy as previously described.¹⁵

Morphometric Analysis

Primary fibroblast monolayers were obtained from four DS patients and four age-matched controls (5 months, 2 years, 5 years, and 21 years) and were immunostained in tandem under identical experimental conditions with an antibody against EEA1. Images were taken at $\times 100$ magnification (0.2676 μ m/pixel, AxioVision 4.5 software; Carl Zeiss) with comparable background intensities between 2N and DS cultures. The cross-sectional area, number of EEA1-positive endosomes, average early endosomal area, and total endosomal area per cell area were analyzed for each fibroblast by direct inspection and importing captured images of fibroblasts taken in a single plane of focus to the ImageJ 1.37v morphometry software package (National Institutes of Health, Bethesda, MD). Indi-

vidual fibroblasts and EEA1-positive endosomes were outlined and area measured. For each of the eight cell lines (4 = 2N; 4 = DS), a total of 20 fibroblasts were assessed at random from several fields. In total, 80 fibroblasts from 2N controls and 80 fibroblasts from the DS group were analyzed. For statistical comparisons based on endosome number relative to size, each endosome was assigned to one of three groups on the basis of area (μ m²): 0.01 to 0.54, 0.55 to 1.09, and 1.10 to 6.5 and the numbers of endosomes plotted on a base 10 logarithmic scale. All data sets examined showed nonparametric distributions. Analysis of variance with *F*-test and Student's *t*-test ($P < 0.01$) showed statistically significant differences in total endosomal area and individual endosomal size per cell between DS and 2N control groups. In addition, the data for group comparisons (DS versus 2N fibroblasts) were subjected to log-linear analysis.⁴⁴ Our model included the main effects of group (2N versus DS) and area in addition to the group by area interaction. Results showed this interaction to be highly significant ($P < 5 \times 10^{-12}$).

Results

DS Fibroblasts Exhibit Abnormalities in Early Endosomal Morphology

Given that individuals with DS show early endocytic abnormalities early in life^{1,4} and that mouse models of human DS neuronal endocytic pathology are dependent on *App* gene triplication,^{23,28} we initially sought to determine whether fibroblasts from DS individuals would show similar alterations. As we have done previously in AD and DS brain,^{1,4,45} we used antibodies to rab5, EEA1, and rab4 to identify early endocytic compartments to determine whether DS fibroblasts in culture display early endosomal alterations similar to those seen in DS neurons. Immunolabeling of fibroblasts from control 2N individuals revealed rab5- and EEA1-positive early endosomes that were consistent in size (150 to 250 nm) (Figure 1, A and B) and distributed close to the plasma membrane and cell surface. Occasionally, these compartments were larger (approximately twice the diameter—300 to 400 nm) than the majority of immunoreactive early endosomes and, as reported previously,^{46–48} are consistent with homotypic endosomal fusion. In comparison to all 2N fibroblast lines examined, many of the rab5- and EEA1-positive early endosomes in DS fibroblasts were larger (≤ 400 nm) than those in control cells (Figure 1, C–E)—a finding that was consistent regardless of the age of the individual from whom the fibroblasts were initially obtained. These abnormal early endosomes resembled closely those profiles we previously identified in neurons of early-stage AD and DS brain. In some cells, the lumen of these enlarged early endosomes was visible at the light microscopic level (Figure 1, D and E) and morphological evidence of endosomal budding was often observed. As in 2N, endosomes in the DS cells were concentrated close to the plasmalemma.

Figure 1. Early endocytic uptake, fusion, and recycling are enhanced in DS fibroblasts. Primary skin fibroblasts obtained antemortem from 2N controls (**A** and **B**) exhibit rab5-positive (**A**, arrow), EEA1-positive (**B**, arrow) early endosomes of uniform size. Fibroblasts from DS individuals (**C–E**), displayed a striking difference in early endosomal number and size of rab5-positive (**C**, arrows) and EEA1-positive (**D**, inset, and **E**, arrows) early endosomes. Scale bar = 20 μm .

The number and/or size of early endosomes in many cell types is proportional to the rate of endocytic uptake and endosomal fusion.^{48–50} We used morphometric analysis to determine the number and size of EEA-1-immunoreactive endosomes in each of 20 primary skin fibroblasts selected at random from four 2N control and four DS cell lines (in total, 80 2N fibroblasts and 80 DS fibroblasts). We found that the total number of endosomes of all sizes was increased in the DS fibroblasts compared to 2N controls (Figure 2A). Group comparisons (DS versus 2N) showed statistically significant differences in total number and in the number of EEA1-positive endosomes per cell across all size groups (Figure 2B). Categorizing endosomes within three size ranges and expressing the number in each range as a percentage of the total endosomes per cell revealed that overall the number of larger endosomes represented a higher percentage of the total in the DS fibroblasts compared to 2N cells and that the number of endosomes in the largest size category (1.10 to 6.59 μm) were 2.24-fold more numerous in the DS fibroblasts than in 2N (Figure 2B). Further comparisons (Figure 2C) showed that in DS cells, the number of endosomes per cell ranging in size from 0.55 to 1.09 were increased by 113% whereas the largest endosomes (1.10 to 6.59 μm) were increased by 297% compared to 2N fibroblasts. In the DS fibroblasts, endosomes in the

two largest size categories combined (0.55 to 6.59 μm^2) were increased 141% relative to those in 2N fibroblasts.

Endocytic Uptake Is Increased in DS Fibroblasts

The most likely explanation for the enlargement of early endosomes is either an increase in endocytic uptake by the cell or a reduction in trafficking from early endosomes to late endosomes and more distal or hybrid compartments, both of which have been shown in various systems to increase endosomal volume.^{48–52} Unlike fixed, postmortem brain tissue, fibroblasts in culture have the important benefit of being amenable to experimental manipulations and to time course studies of vesicular trafficking involving the early endosome and its interactions with other compartments of the endocytic pathway. Endocytosis was examined in three of four DS and age-matched 2N fibroblast lines by both receptor-mediated (using fluorophore Alexa 568-tagged transferrin, Tfn, or fluorophore Alexa 488-tagged EGF) and fluid-phase uptake (using horseradish peroxidase, HRP). Ligand receptor internalization and trafficking within the early endocytic pathway, including recycling back to the cell surface, was examined with Tfn and transport from early endosomes to lysosomes with EGF. The cellular distribu-

Figure 2. Morphometric analysis of EEA1-positive endosomes in 2N and DS fibroblasts. **A:** The numbers of EEA1-positive early endosomes in all size groups were increased in the DS fibroblasts compared to 2N controls (total fibroblasts examined: 2N fibroblasts = 80; DS fibroblasts = 80). The number of EEA1-positive endosomes is expressed as a function of endosome size on the logarithmic scale (base 10) for easier visualization. **B:** Group comparisons (DS versus 2N) showed statistically significant differences in total number and in the number of EEA1-positive endosomes/cell in each size category (all p values <0.01, two-sample t -test). **B:** Inspection of the total endosome number/cell revealed a disproportionate and statistically significant increase in larger-sized endosomes among DS fibroblasts relative to the 2N group. **C:** Percent increase in the number of endosomes within each size group of the DS fibroblasts relative to the 2N group.

tion of Tfn and EGF was examined after cell surface-ligand receptor binding and internalization (Figure 3). Both 2N and DS fibroblasts bound Tfn or EGF with no internalization at the cell surface to a similar extent when incubated at 4°C (EGF shown in Figure 3, D and E). After 5 to 15 minutes of internalization, we observed that compared with the number of small, fluorescent Tfn-positive (Figure 3A) and EGF-positive (Figure 3F) vesicles in the 2N controls, the DS fibroblasts showed a qualitative increase in the number of Tfn-positive (Figure 3B) and EGF-positive (Figure 3G and inset) vesicles—a change consistent with increased receptor-mediated endocytosis.⁴¹ Consistent with increased endosomal fusion, DS cells frequently contained large EGF-positive profiles that were located in close proximity to the plasma membrane (Figure 3G, inset). Because Tfn is internalized and recycled quickly within the early endocytic pathway and EGF is a useful marker of endosomal to lysosomal transport,

we went on to follow EGF for up to 60 minutes after internalization. After 30 (Figure 3, H–J) to 60 (Figure 3, K–M) minutes of exposure to EGF, 2N fibroblasts (Figure 3, H and K) displayed fewer numbers of EGF-containing vacuoles (arrows) than the DS fibroblasts (Figure 3, I, J, L, and M). In the DS fibroblasts, these EGF-positive compartments were frequently larger in size compared to 2N controls (Figure 3, compare H with I and K with L). Western blot analysis (Figure 3, C and N) confirmed the morphological findings and the higher levels both of Tfn and EGF in the DS fibroblasts compared to 2N cells after exposure to the ligand at all time points surveyed. To examine whether defects in the downstream endosome to lysosome transport of internalized material contributes to endosomal enlargement in DS fibroblasts, we also performed double labeling using fluorophore-tagged EGF and EEA1 (early endosomes) or EGF and LAMP1 (late endosomes, lysosomes) at 30 minutes and 2 hours after exposure to EGF (Supplemental Figure 1, see <http://ajp.amjpathol.org>). Fibroblasts from individuals with DS showed more frequent co-localization of EGF and EEA1 or LAMP1 after 30 minutes of internalization compared to 2N fibroblasts. After 2 hours of exposure to EGF, little to no detectable co-localization was detected both in DS and 2N fibroblasts between EGF and EEA1 or LAMP1 (data not shown).

Similar results were found in the DS cells with the fluid phase tracer, HRP, which was inspected at the ultrastructural level (Figure 4). Internalization of HRP was examined from 0 to 30 minutes (Figure 4, A–K, and N), to follow its distribution within the endocytic pathway, and then from 6 to 24 hours (Figure 4, L, M, O, and P) to examine movement from the late endocytic pathway toward lysosomes or within the intersecting autophagic pathway. Unlike molecules internalized by receptor-mediated uptake, those endocytosed by fluid phase uptake show no association with the cell surface at 4°C followed by immediate warm-up (Figure 4, A and B). Internalization at 37°C for short incubation times at 3 (Figure 4, C–J), 5 (Figure 4, K and N), and 30 (not shown) minutes revealed numerous enlarged HRP-positive endocytic profiles in the DS fibroblasts (Figure 4, D, G–J, and N) compared with 2N controls that contained significantly fewer and predominantly smaller HRP-positive compartments (Figure 4, C, E, F, and K). After only short 3 to 5 minutes of internalization, HRP clearly labeled a population of surface vesicles consistent with early endocytic recycling/sorting compartments or tubulovesicular profiles of various morphologies or that coalesced to form larger vacuoles that accumulated close to the cell surface in the DS fibroblasts (Figure 4, D, G–J, and N; arrows). These profiles were seen in 2N fibroblasts as well but were fewer in number (Figure 4, C, E, F, and K; arrows). With longer incubations (6 to 24 hours), compartments with the structural features of early-late endosomal hybrids (Figure 4P2), multivesicular bodies (Figure 4P3, arrowhead), autophagic vacuoles (Figure 4, L, O, P1, and P3; arrows), late endosomes, or lysosomes were visible in 2N (Figure 4, L and M; arrows) and DS (Figure 4, O, P, and P1–P3; arrows). DS fibroblasts displayed a modest number of large HRP-positive

Figure 3. Increased receptor-mediated endocytic uptake in DS fibroblasts. Representative images from the examination of receptor-mediated uptake in three of four 2N and DS cell lines using fluorophore-conjugated transferrin (Tfn) (**A** and **B**) or epidermal growth factor (EGF) (**D–M**). Alexa 568-tagged Tfn (not shown) and Alexa 488-tagged EGF showed surface binding but no internalization at the cell surface in 2N (**D**) and DS (**E**) fibroblasts when incubated at 4°C. Uptake of Tfn or EGF after 5 to 15 minutes revealed an increase in the number (5 minutes: **B**, arrows; 15 minutes: **G** and inset) and often size of fluorescent vesicles in the DS fibroblasts (**G** and inset; arrows) compared to 2N controls (5 minutes: **A**, arrow; 15 minutes: **F**, arrow). Endosomal to lysosomal transport was followed with EGF for 30 to 60 minutes after internalization. After 30 (**H–J**) to 60 minutes (**K–M**) of exposure to EGF, the numbers of EGF-containing vacuoles were more numerous in the DS fibroblasts (**I**, **J**, **L**, and **M**; arrows) than in 2N cells (**H** and **K**, arrows) and frequently larger in size. Biochemical confirmation of increased endocytosis in the DS fibroblasts was confirmed by Western blotting using an antibody to Tfn (**C**) and EGF (**N**) and showed increasing amounts of both internalized ligands in the DS cells compared to controls as indicated. Scale bars: 50 μ m (**D–I**); 20 μ m (**A**, **B**, **J–M**, inset).

compartments that were located close to the nucleus (Figure 4, O and P)—the typical pattern seen with late-stage endocytic organelles, ie, late endosomes, or lysosomes, compared to 2N fibroblasts (Figure 4, L and M), which displayed fewer large HRP-positive profiles after longer incubation times.

The activity of the GTPase, rab5, is known to regulate the biogenesis and fusion of early endosomes.⁵⁰ EEA1 is a core rab5 effector protein that associates with rab5 on the endosomal membrane and is required for homotypic endosomal docking, tethering, and fusion and the regulation of endocytic transport.^{47,53,54} Increased rab5 expression increases endocytic uptake and promotes the enlargement of EEA1-positive endosomes.^{15,48,49,55,56} To determine whether the acceleration of receptor-mediated and fluid phase uptake we observed in DS fibroblasts was predictive of an en-

hanced interaction of rab5-EEA1 within morphologically altered early endosomes, we performed dual-immunofluorescence imaging using antibodies to rab5 and its positive effector, EEA1. DS fibroblasts displayed extensive co-localization between rab5 and EEA1, particularly within large early endosomes (Supplemental Figure 2, see <http://ajp.amjpathol.org>). Overlap of rab5-EEA1 was seen in 2N fibroblasts, but to a much lesser degree compared to DS cells.

Immunocytochemical analyses with another marker of the early endocytic pathway, rab4, revealed an increase in the number of rab4-positive recycling compartments in the DS fibroblasts (Figure 5B), compared to 2N control cells (Figure 5A). Overall, rab4 immunoreactivity was associated with small vesicular profiles that were localized close to the plasmalemmal surface. Greater numbers of rab4 compartments observed in the DS fibroblasts are

consistent with increased and rapid endocytic recycling and is an expected compensatory response to increased endocytic uptake.^{57,58}

Redistribution of Lysosomal Hydrolases to Early Endosomes in DS Fibroblasts

We previously showed¹ that CatD was one of several lysosomal hydrolases that is found in higher levels in neuronal endosomes of affected AD brain regions. With trafficking mutants, we established in previous *in vitro* studies that these hydrolases were transported to endosomes by the 46-kDa species of the mannose 6-phosphate receptor (MPR46).¹⁴ Using an antibody to MPR46, we saw that in normal 2N fibroblasts, MPR46 (Figure 6A) was found close to the nucleus in a perinuclear pattern that is consistent with the Golgi apparatus.¹⁴ MPR46 immunolabeling was seen less frequently close to the plasmalemmal surface or detected in small vesicular endosomal profiles near the cell surface. However, DS fibroblasts (Figure 6D), like neurons in AD brain, exhibited both perinuclear and dispersed vesicular localizations with MPR46 antibodies. Double-label immunofluorescence with MPR46 and EEA1 antibodies also revealed a significant overlap in MPR46 and EEA1 profiles in DS fibroblasts (Figure 6F) whereas in the 2N fibroblasts, the punctate labeling pattern of EEA1-positive endosomes rarely coincided with MPR46 immunolabeling (Figure 6C). Control and DS fibroblasts double labeled with CatD and EEA1, showed a higher frequency of CatD localization in EEA1-positive endosomes in the DS fibroblasts than in controls (Supplemental Figure 3, see <http://ajp.amjpathol.org>). The increased presence of CatD in endocytic compartments in the DS fibroblasts seen by immunocytochemistry was supported by Western blot analysis, which showed higher levels of both the immature and mature forms of CatD (Supplemental Figure 4, see <http://ajp.amjpathol.org>).

Late Endosomes Are Abnormally Large in DS Fibroblasts and Prominent in Neurons in AD and DS

Because early endosomes can be in direct contact with late endosomes, and transport material to them, we investigated the consequences of increased early endocytic pathway activation on late-stage endocytic traffic in AD and DS by studying the intracellular distribution of two late endosomal markers, rab7, a small GTPase^{59–61} that regulates transport from early to late endosomes and

homotypic late endosomal fusion, and LBPA, a phospholipid resident of the late endosomal membranes.^{35–37} Immunocytochemistry with antibodies to rab7 or LBPA revealed larger and more numerous immunoreactive late endosomes in DS fibroblasts (Figure 7, H and I) compared with controls (Figure 7, F and G). In fibroblasts obtained from 2N individuals, rab7-positive, LBPA-positive late endosomes were smaller in size and were typically located close to the nucleus (Figure 7).

Using the same antibodies, we also examined the frontal cortices of early-stage sporadic AD, juvenile DS, and an equal number of normal, age-matched control brains (Figure 7). In otherwise normal-appearing cells, abnormally large rab7-positive, LBPA-positive late endosomes were detected in neuronal pyramids in layers III and V of the cortex in AD and DS brain tissue (Figure 7, D and E). The enlarged late endosomal profiles were apparent in the same cells that displayed early endosomal pathology. Neurons in the frontal cortex of control brains showed no evidence of late endosomal enlargement using either rab7 (Figure 7A) or LBPA (Figure 7C), which label late endosomes in a similar manner. Evidence of early and late endocytic pathway activation were confirmed by immunoblot analyses showing higher levels of rab5, rab4, and rab7 immunoreactive proteins relative to tubulin in the DS fibroblasts (Figure 7J) compared with 2N fibroblasts. Double immunolabeling showed that most rab7-positive profiles co-localized with LBPA-positive vacuoles as expected and that rab7 and EEA1 showed a very modest coincidence (data not shown). In 2N cells, little to no coincidence of dual labeling was seen. No detectable co-localization was apparent between rab4- and rab7-positive vesicles in DS and 2N fibroblasts (data not shown).

Late Endosomal Enlargement in DS Fibroblasts Is Dependent on Endocytic Uptake

Our findings suggest that the late endosomal enlargement seen in DS fibroblasts may, at least in part, result from increased endocytic uptake in these cells. To further investigate whether late endocytic pathology in DS fibroblasts could be a consequence of increased uptake and movement of materials through the early endocytic pathway, we manipulated endocytosis by expressing rab5 and rab5 functional mutants using herpes simplex virus (HSV) vectors. We induced increased endocytosis and endosomal fusion by infecting 2N or DS fibroblasts with HSV vectors overexpressing human wild-type rab5 or the GTPase-deficient dominant-active mutant of rab5, rab5Q79L,

Figure 4. Enhanced fluid phase endocytosis in DS fibroblasts. Ultrastructural examination of bulk phase uptake examined using the fluid phase marker, HRP, was performed in three of four fibroblast lines obtained from 2N and DS individuals. Representative micrographs from 2N (**A**) and DS fibroblasts (**B**), showed that no surface ligand binding or internalization occurs at 4°C. After brief (3 to 5 minutes) incubation at 37°C, HRP is widely distributed in early endocytic organelles and, in the DS fibroblasts, is seen in numerous and large, HRP-positive compartments located close to the plasmalemmal surface (3 minutes: **D, G–J**, arrows; 5 minutes: **N**, arrows). Control 2N fibroblasts (3 minutes: **C, E, F**, arrows; 5 minutes: **K**, arrow) contain predominantly smaller and fewer HRP-positive profiles. With longer incubation times (6 to 24 hours), compartments with the structural features of early-late endosomal hybrids (**P2**), multivesicular bodies (**P3**, arrowhead), autophagic vacuoles (**O, P1, P3**, arrows), late endosomes, or lysosomes were visible in 2N (**L, M**, arrows) and DS (**O, P, P1–P3**, arrows). In contrast to 2N fibroblasts, these HRP-positive endosomal-lysosomal compartments were more numerous in the DS fibroblasts and frequently were substantially larger than in 2N fibroblasts. The increase in the number of enlarged and densely labeled vacuoles, probably remnants of endocytosis, identified in the DS fibroblasts suggests a relationship between early endocytic pathway dysfunction and downstream endocytic and proteolytic compartments. Scale bars = 1 μ m.

Figure 5. Markers of endocytic recycling are altered in DS fibroblasts. Immunocytochemistry using rab4, a marker of endosomal recycling, revealed increased numbers of rab4-positive vesicles in DS fibroblasts (**B**, arrows) compared to 2N (**A**, arrow) cells suggesting enhanced early endosomal recycling to the plasmalemma and a change compensatory to increased endocytic uptake in the DS fibroblasts. Scale bar = 20 μ m.

tagged at the N terminus with a myc epitope.⁴⁰ We also inhibited endocytic uptake using myc-tagged HSV vectors expressing the rab5 dominant-negative mutant, rab5S34N.⁴⁰ Efficiency of infection with each of the viral vectors was assessed in multiple experiments by immunolabeling using a monoclonal antibody to the human-specific myc peptide (Supplemental Figure 4, see <http://ajp.amjpathol.org>). Anti-myc did not label HSV/LacZ-expressing 2N or DS fibroblasts or mock-infected counterparts, but consistently labeled 95 to 100% of the cells infected with each of the myc-tagged HSV vectors. Immunocytochemistry with antibodies against EEA1 or another early endosomal marker, rab5 (data not shown), showed that 2N fibroblasts infected with HSV/LacZ or mock-infected with 10% sucrose exhibited the typical early endosomal vesicles of uniform size (Figure 1A and Figure 8A). In contrast, 2N fibroblasts infected with myc-tagged HSV/rab5Q79L (Figure 8, B1–B3) or HSV/wild-type rab5 (not shown), contained numerous enlarged myc- or EEA1-positive vacuoles that resembled the profiles seen in noninfected DS fibroblasts (Figure 1, D and E) and in neurons in AD and DS brains.^{1,4,45} Altered early endosomal morphology was even further exacerbated in the DS fibroblasts infected with rab5Q79L (Figure 8, D1–D3) or wild-type rab 5 (data not shown). Infection of the DS fibroblasts with rab5Q79L enhanced endosomal enlargement (0.6 to 1.0 μ m) and often produced gigantic, myc- or EEA1-positive vacuolar compartments ranging from 5- to 20-fold larger (2 to 8 μ m) than the profiles seen in DS fibroblasts infected with HSV/LacZ (Figure 8C) or mock infected with 10% sucrose (data not shown).

We next sought to rescue the early endosomal pathology and to determine whether blocking upstream endocytic alterations would eliminate the development of late-stage endocytic pathology. Using the GTP-binding defective rab5 mutant, rab5S34N,^{42,62} we found that, compared to DS fibroblasts transduced with HSV/LacZ (Figure 9C) or mock-infected with sucrose, DS fibroblasts transduced with HSV/rab5S34N and immunolabeled with EEA1 showed a significant, but incomplete, reduction in the number and size of EEA1-positive early endosomes (Figure 9D)—a morphological change consistent with a decrease in endocytic uptake and fusion.^{48,62} The inability to completely suppress all enlarged early endosomal

Figure 6. Altered lysosomal hydrolase trafficking in DS fibroblasts. Immunofluorescence studies showed that 2N control fibroblasts exhibited a prominent and typical perinuclear distribution of the hydrolase receptor, MPR46—a distribution consistent with localization to the Golgi apparatus (**A** and **C**, arrows); EEA1 staining is shown in **B**. In the DS fibroblasts (**D–F**), CD-MPR immunolabeling was also associated with vesicular compartments and was frequently located within enlarged EEA1-positive compartments (**E** and **F**, arrows). Significant overlap (yellow) of MPR46 (green) with EEA1 (red) in a representative DS fibroblast (**F**) strongly suggests that delivery of MPR46-tagged hydrolases like CatD (not shown) to endosomes is increased in DS fibroblasts as in AD and DS brain and supports the notion that proteolytic activity within these compartments is increased. **C**: Signal overlap was less frequently seen in 2N cells. Scale bar = 20 μ m.

profiles in the DS fibroblasts within 5 hours after infecting with rab5S34N suggests that the remaining fusion activity is most likely attributable to endogenous rab5 activity. In 2N fibroblasts, the inhibitory effect of the rab5 mutant (Figure 9B) was modest compared to HSV/LacZ (Figure 9A) but was less apparent than that in DS fibroblasts, because they normally undergo less endosomal fusion than DS cells and, therefore, exhibit fewer large early endosomal profiles (Figure 9B). Morphometric analysis of 15 fibroblasts from each of four groups (2N LacZ, 2N HSV/rab5S34N, DS LacZ, DS HSV/S34N; in total, 60 fibroblasts) (Figure 9I) was used to confirm the reduction in size and number of EEA-1-immunoreactive endosomes infected with dominant-negative rab5 (S34N) compared with LacZ-infected cells. We found that relative to LacZ-infected fibroblasts, DS fibroblasts infected with rab5S34N exhibited a 23% decrease in the total number of endosomes (Figure 9I) whereas 2N fibroblasts showed a 7% decrease in total endosome number. For all size groups, the percent decrease in the mean number of endosomes per cell in the S34N-infected fibroblasts relative to the LacZ-infected cells was greater in the DS cells compared to 2N cells (Figure 9J). In DS fibroblasts, transfection with

Figure 7. Late endosomal markers reveal alterations in DS fibroblasts and neurons of AD and DS brain. Immunofluorescence labeling of 2N and DS fibroblasts with rab7, a protein localized to late endosomes, revealed numerous and frequently enlarged rab7-positive (arrows) vesicular compartments in the DS fibroblasts (B, arrow), which accumulated close to the nucleus. By contrast, 2N fibroblasts contained fewer and consistently smaller rab7-positive profiles (A, arrow). Analyses of at-risk neurons in AD and DS brains with rab7 or another frequently used marker of late endosomes, lysobisphosphatidic acid (LBPA), showed increased numbers of LBPA-positive (E, arrow) and rab7-positive (F and inset; arrow) profiles that were found close to the nucleus and were more numerous in the neurons of AD and DS brains than in neurons from control brains (C and D, arrows). Control (G and H, arrows) and DS (I and J, arrows) fibroblasts, labeled with LBPA displayed a pattern similar to rab7 and identified many abnormally large late endosomes in the DS fibroblasts compared to 2N cells. Western blot analyses of cell lysates prepared from 2N and DS fibroblasts (K), probed with antibodies to early and late endosomal markers showed increased levels of early and late endosomal proteins in DS fibroblasts compared to 2N cells, confirming the immunocytochemical results. Equal protein loads are indicated by tubulin immunoreactivity. Molecular weight references are provided to the left of the Western blots. Scale bar = 20 μ m.

S34N reduced the number of the large endosomes disproportionately more than the smaller endosomes. The number of the largest EEA-1-positive endosomes (1.10 to 6.59 μ m²) was dramatically reduced by 66% compared to smaller profiles (0.01 to 0.54 μ m²) that were reduced by 22%. Transfection with the S34N-infected relative to LacZ-infected also decreased mean endosomal size per cell (Figure 9J) to a greater degree in the DS fibroblasts (23%) than in the 2N fibroblasts (13%) (DS and 2N, $P < 0.01$).

Immunocytochemical examination of DS fibroblasts transduced with HSV/rab5S34N using the late endosomal probes, LBPA (Figure 9, G and H) and rab7 (data not shown), revealed a clear reduction in the size and number of LBPA and rab7 late endosomes (Figure 9H) compared to late endosomal profiles labeled in a similar manner in the DS fibroblasts infected with HSV/LacZ (Figure 9G) or mock-infected with sucrose. The rescue of

late endosomal alterations in DS fibroblasts by rab5S34N strongly suggests that inhibition of early endosomal uptake and fusion activity is capable of regulating, at least in part, downstream late-stage endocytic events. Unlike the DS fibroblasts, 2N fibroblasts infected with HSV/rab5S34N (Figure 9F) displayed less striking changes when compared to 2N fibroblasts infected with HSV/LacZ (Figure 9E) in late endosomal size or number compared to the DS cells (data not shown).

Discussion

The dynamic nature of vesicular transport within the endocytic pathway is difficult to appreciate from fixed post-mortem tissue. Although increased levels of early endosomal regulatory proteins^{4,45} are suggestive of altered endocytic function in AD, demonstrating a disease-driven

Figure 8. Rab5Q79L expression in 2N fibroblasts mimics AD/DS-relevant endocytic pathologies and exacerbates endosomal enlargement in DS fibroblasts. After infection with an HSV1 vector that expressed wild-type rab5 (not shown) or the myc-tagged dominant active rab5 mutant, rab5Q79L (**B**), 2N fibroblasts displayed atypically large EEA1-positive early endosomes (**B2**, **arrows**) with transfection of the rab5 construct, that resembled those seen in DS fibroblasts (see Figure 1) (**C–E**), and those seen in neurons from AD and DS brains (not shown). 2N fibroblasts infected with HSV1/LacZ (**A**) exhibited early endosomes of normal size (**arrow**). **B:** HSV1/rab5Q79L-infected 2N fibroblasts labeled with myc (red; **B1**, **arrow**) and EEA1 (green; **B2**, **arrows**) or both (yellow; **B3**, **arrows**), confirming the localization of rab5 in early endosomes. The expression of myc-tagged HSV1/rab5Q79L in DS fibroblasts (**D1** and **D3**, **arrows**) further enhanced the early endosomal enlargement and fusion seen in uninfected cells (**C**) and caused the formation of giant EEA1-positive vacuoles (**D2** and **D3**, **arrows**). DS fibroblasts infected with HSV1/LacZ contained enlarged EEA1-positive compartments (**C**, **arrow**) and were negative for myc immunoreactivity. Scale bars = 20 μ m.

relationship between endosomal morphology and altered endocytic transport and function is difficult using human tissue. Our previous studies of DS brain^{4,45} and additional studies of neurons from mouse models of DS^{23–28} strongly suggest that the early endosomal changes seen in these systems are similar. Given the genetic underpinnings of DS, we thought it reasonable to determine whether nonneuronal DS cells would show similar changes, thus offering us an accessible *in vitro* system in which to directly examine disease-relevant endocytic pathway function. Using primary fibroblasts from individuals with DS, we have found that the endocytic morphological abnormalities seen in neurons from early-stage sporadic AD and DS brain^{4,45} are recapitulated in fibro-

blasts from individuals with DS. Extending these morphological observations to endosomal function in DS fibroblasts, our results provide several lines of evidence of early endocytic pathway activation in the DS cells. These include: 1) increased expression of rab5 and rab4, which regulate endocytic uptake and homotypic fusion and fast endosomal recycling, respectively^{42,46,48,57,62}; 2) early endosomal enlargement suggestive of enhanced early endosomal fusion; 3) an increase in receptor-mediated and fluid phase endocytic uptake; and 4) an increase in the presence of MPR46 (and lysosomal hydrolases) to early endosomes. In DS fibroblasts, we observed abnormally large rab5-positive endosomes by immunocytochemistry and, by Western blot analyses, increased rab5-immunoreac-

Figure 9. Reduced endocytic uptake reduces early and late endosomal pathology in DS fibroblasts. Infection of 2N (A, B) and DS fibroblasts (C, D), with HSV1/myc-tagged rab5S34N (B, D), a dominant-negative rab5 mutant that inhibits endocytosis, significantly reduced the numbers (endosomal uptake) and size (endosomal fusion) of EEA1-positive endosomes (arrows) compared with LacZ-infected cells (A, C; arrows). In 2N fibroblasts, the downstream effects of reduced early endosomal uptake and fusion after rab5S34N infection was accompanied by a decrease in the number and size of LBPA-positive late endosomes (F, arrow) relative to 2N fibroblasts infected with LacZ (E, arrows). The effects of HSV1/rab5S34N infection were more dramatic in DS fibroblasts (H), which showed a significant reduction in both LBPA-positive late endosomal number and size compared to the late endosome pattern (G, arrows). **I:** Morphometric analysis confirmed the reduction in number and size of EEA-1-immunoreactive endosomes/cell in fibroblasts from 2N control and DS cell lines infected with rab5S34N. **J:** Relative to LacZ-infected fibroblasts, DS fibroblasts infected with rab5S34N exhibited a 23% decrease in the total number of endosomes whereas 2N fibroblasts showed a 7% decrease in total endosome number. For individual size groups, the percent decrease in the mean number of endosomes/cell, in the S34N-infected fibroblasts relative to the LacZ-infected cells was greater in the DS cells compared to 2N cells. In the DS group, the number of the larger sized endosomes (0.55 μm² and larger) was disproportionately reduced compared to the smaller profiles (*P* < 0.01 two-sample *t*-test). **J:** Further comparisons of S34N-infected to LacZ-infected fibroblasts between the DS and 2N groups showed that the mean endosomal size/cell also was reduced to a greater degree in the DS fibroblasts compared to 2N fibroblasts (DS S34N/LacZ = 23% reduction, *p* < 0.01; 2N S34N/LacZ = 13% reduction, *p* < 0.01). Scale bar = 20 μm.

tive protein levels—changes suggesting enhanced rab5 activation. Given the requirement of rab5 activity for endocytosis and endosomal fusion, this interpretation is also consistent with our findings showing that enlarged rab5-positive endosomes in DS fibroblasts were immunoreactive for the positive rab5 effector protein, EEA1, which stimulates endosomal fusion^{46–48,63} and with the functional increase in endocytic uptake using receptor-mediated and bulk phase tracers.

These changes in early endosomal function suggest that the increase in early endosomal size seen in AD and DS neurons is likely the result, at least in part, from increased endocytosis and endocytic activity. Nevertheless, a failure of vesicular transport from early to late endosomes or from late endosomes to lysosomes could also contribute to early endosomal swelling.^{51,64} Our examination of late endosomal morphology, however, suggests that this is not the case: in neurons both from AD and DS patients, late endosomes are also abnormally enlarged. Moreover, the DS fibroblasts also have enlarged late endosomes. The increased delivery of endocytosed HRP into late endosomes in the DS cells indicates that early to late transport is apparently not impaired in these cells. Indeed, our conclusions are consistent with extensive evidence showing that late endosomal enlargement can be driven by increased endocytic uptake, as is the case when rab5 is overexpressed.⁶⁵ The even greater late endosomal enlargement seen in DS cells after rab5 expression is additional evidence that vesicular delivery from early to late endosomes is intact in

these cells and capable of mediating more extensive late endosome pathology as the drive on the early endocytic pathway is increased. The increased mRNA expression⁶⁶ and elevated protein levels of rab7, which regulates early to late endosomal transport, in the DS fibroblasts compared to 2N controls would support this notion. Finally, the reversal of late endosomal enlargement by dominant-negative rab5 expression is direct evidence that the late endocytic pathway alterations in the DS cells are dependent on early endocytic activity.

Accompanying endocytic pathway activation in the DS fibroblasts, we found an increase in MPR46 levels within enlarged early endosomes with lesser amounts associated with the trans Golgi network (TGN). MPR46 is involved in the delivery of acid hydrolases from the TGN to early endosomes and/or the cell surface.^{67–69} The distribution of MPR46 in 2N fibroblasts is consistent with previous studies^{67–69} showing that MPR46 displays a predominantly perinuclear distribution at steady state. The elevated level of MPR46 (and hydrolases like CatD) in early endosomes of the DS fibroblasts is consistent with the increased uptake and localization of endocytic tracers in early endosomes of these cells. We believe that this enhancement of hydrolase directed delivery and enrichment within these organelles could be the direct result of accelerated endocytic uptake and delivery of internalized cargo to early endosomes where it is sorted and degraded. Our earlier studies of AD and DS human brain^{4,45} and cell^{14,15} and animal²³ models of DS show the relationship between endocytic compartment pathologies,

increased hydrolase levels within these compartments, and A β production. Consistent with these findings, reduction in the levels of the sortilin-related receptor, SORL1, which has recently been linked to the development of late-onset AD,⁷⁰ affects the processing of APP within endocytic compartments and are associated with increased production of A β .^{71–73} Similar to our findings in AD and DS brain^{1,14} and DS fibroblasts, altered trafficking of MPR-targeted proteins (hydrolases) to endocytic compartments has been observed in Niemann-Pick type C disease. In this disorder, not only are rab5-positive endosomes enlarged but also several other AD-like neuropathological features are present, including neurofibrillary tangles and amyloid deposits.^{3,5}

Cumulatively, our findings strongly indicate that late endosomal pathology results, at least partly, from increased upstream endocytic uptake and the trafficking of early endosomal contents to late endosomes. Our current studies do not rule out the possibility that a partial failure of late endosomes to mature to lysosomes could also contribute to late endosomal enlargement. Given the well characterized changes in lysosomes in neurons in AD brain,^{16,17,74,75} the complex relationship between late endosomes, autophagosomes, and lysosomes,^{76–79} and the growing understanding of the importance of autophagy in neurodegenerative diseases,^{76–79} it seems likely that additional changes in function downstream from the late endosome may contribute to endosomal pathology. The DS fibroblast model is likely to prove valuable in further dissecting alterations in function of the endosomal-autophagic-lysosomal system.

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