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Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease

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ABSTRACT

We present evidence here that exosomes stimulate aggregation of amyloid beta (Aβ)1–42 in vitro and in vivo and interfere with uptake of Aβ by primary cultured astrocytes and microglia in vitro. Exosome secretion is prevented by the inhibition of neutral sphingomyelinase 2 (nSMase2), a key regulatory enzyme generating ceramide from sphingomyelin, with GW4869. Using the 5XFAD mouse, we show that intraperitoneal injection of GW4869 reduces the levels of brain and serum exosomes, brain ceramide, and Aβ1–42 plaque load. Reduction of total Aβ1–42 as well as number of plaques in brain sections was significantly greater (40% reduction) in male than female mice. Our results suggest that GW4869 reduces amyloid plaque formation in vivo by preventing exosome secretion and identifies nSMase2 as a potential drug target in AD by interfering with exosome secretion.

1. Introduction

Alzheimer's disease (AD) is characterized by cognitive decline, alteration of synaptic transmission, and neuronal death brought on by accumulation of neurofibrillary tangles in neurons and amyloid beta (Aβ) in the brain parenchyma. There is no viable therapy to slow or reverse AD progression. The leading hypothesis for the cause of AD is an impairment of clearance of Aβ resulting from the β-cleavage of the amyloid precursor protein (APP) (Bertram and Tanzi, 2008). Strong evidence comes from early-onset familial AD, in which patients carry mutations in genes encoding for APP or the presenilin-1 (PS1) component of the γ-secretase complex that cleaves APP to generate Aβ (Bird, 2008; Selkoe, 2011). Recent studies showing that reducing Aβ load in mouse models improves cognitive function strongly suggest that reducing plaque burden is a potential treatment strategy for AD (Cramer et al., 2012; Geekiyanage et al., 2013; Liu et al., 2013).

Exosomes are ceramide-enriched vesicles, 40–100 nm in diameter, generated by inward budding of the endosomal membrane, and secreted when these multivesicular endosomes fuse with the plasma membrane (Thery, 2011; Trajkovic et al., 2008). Exosomes carry signaling factors and microRNAs that mediate intercellular communication, and there is evidence that exosomes play a role in the progression of AD (Bellingham et al., 2012). Aβ processing has been shown to be associated with endosomal compartments with a fraction of Aβ and C-terminal fragments being secreted in association with exosomes (Haass et al., 1995; Perez-Gonzalez et al., 2012; Rajendran et al., 2006; Sharples et al., 2008; Vassar et al., 1999). It is however unclear what role exosomes play in the aggregation and/or clearance of Aβ. Exosomes have been implicated in the extracellular enzymatic degradation of Aβ (Bulloj et al., 2010) and in contrast, are reported to promote Aβ fibrillization and clearance by microglia (Yuyama et al., 2012). Our laboratory reported that cultured primary astrocytes secrete exosomes in response to Aβ exposure, a process dependent upon ceramide generation by neutral sphingomyelinase 2 (nSMase2) (Wang et al., 2012), which suggests that exosome secretion is upregulated during AD. Other work has shown that glia preferentially take up oligomeric Aβ compared with fibrils (Nielsen et al., 2010), suggesting that Aβ aggregation interferes with efficient clearance. In this study, we show that exosomes promote Aβ aggregation and by extension, plaque formation in vivo. Inhibition of nSMase2 with GW4869 (Luberto et al., 2002) in the 5XFAD mouse blocks exosome secretion to reduce plaque formation and decrease the overall brain amyloid load.

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2. Methods

2.1. Animals and GW4869 administration

All experiments involving animals followed approved protocols by Georgia Regents University's Institutional Animal Care and Use Committee. Mice expressing 5 human mutations in APP and PS1 (B6SJL-Tg[APP*K670N*M671L*I716V*V717I, PSEN1*M146*L286V] 6799Vas/J) under neuron-specific elements of the Thy1 promoter were purchased from The Jackson Laboratory and crossed to wild-type C57Bl/6 mice to generate offspring hemizygous for the APP and PS1 transgenes. These mice predominately generate Aβ1–42 that accumulates in plaques beginning at the age of 2 months. GW4869 (N,N′-Bis[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]-3,3′-p-phenylene-bis-acrylamide dihydrochloride; molecular weight 577.5 g/mol; Cayman Chemical) was maintained in DMSO at 8 mg/mL. Two-month-old mice were injected intraperitoneally with 200 μL of 0.3 mg/mL GW4869 in 0.9% normal saline (60 μg/mouse; 2–2.5 μg/g body weight) or 200 μL of 3.75% DMSO saline control every 48 hours for 6 weeks (21 injections total). Mice were sacrificed 24 hours after the final injection. No obvious behavioral or health problems were observed during treatment.

2.2. Sample preparation

Mice were sacrificed by decapitation following isoflurane sedation. Blood was drained for serum collection and brains were removed and cut mid-sagitally. One half was frozen on dry ice and stored at −80 °C for Aβ1–42 enzyme-linked immunosorbent assay (ELISA) and ceramide analyses and the other was fixed in 4% p-formaldehyde in phosphate-buffered saline (PBS) for cryosectioning. Hemibrains were homogenized in cold NaCl (50 mM, 1 mL/100 mg tissue). To extract soluble Aβ, diethylamine was added to a final concentration of 0.2%, and the samples were centrifuged at 100,000*g* for 1 hour at 4 °C. One hundred microliters of 0.5 M Tris (pH 6.8) was added to 1 mL supernatant to neutralize diethylamine and samples were diluted and used directly for ELISA. To extract total Aβ, 200 μL of homogenate was added to 440 μL cold formic acid (88%, Fisher Scientific), and samples were sonicated for 1 minute on ice and centrifuged at 150,000*g* for 1 hour at 4 °C. Supernatant (100 μL) was diluted into 2 mL of neutralization solution (1 M Tris base, 0.5 M Na_2HPO_4, 0.05% NaN_3) and further diluted for ELISA. ELISA was performed with 50 μL sample according to the manufacturer's instructions (Life Technologies human Aβ1–42 ELISA kit).

2.3. Characterization of ceramide using GC-MS

Fatty acyl composition of ceramide was analyzed as methyl esters while the base composition was characterized as trimethylsilyl-derivative (Dasgupta and Hogan, 2001). Briefly, a defined amount of ceramide solution was transferred to a screw-cap tube, dried under nitrogen, and hydrolyzed with methanol-water-HCl 29:4:3 (vol/vol) (Gaver and Sweeley, 1965) at 80 °C for 18 hours in a sealed tube. The mixture of free fatty acids and fatty acid methyl esters (FAME) was recovered by partitioning with hexane and remethylated using 1 M methanolic HCl for 16–18 hours at 80 °C, and the recovered base from the methanol-HCl layer was analyzed as trimethylsilyl (TMS)-derivative after N-acetylation (Pritchard and Todd, 1977). FAME was analyzed using the GC-MS conditions as described previously but with the temperature program extended to 300 °C to ensure elution and detection through the 2-OH, C26 FAME derivatives (Laine et al., 1974). EI-MS detector acquired 50–500 amu for fatty acid analysis.

2.4. Primary cell culture and exosome isolation

Mixed glial cells were isolated from brains of 1-day-old wild type mouse pups. Brains were dissociated in PBS containing 0.1 M glucose, passed through a 40 μm filter, and plated in T-25 flasks in DMEM (Cellgro) containing 10% fetal bovine serum (Atlanta Biologicals, Premium Select) and GlutaGRO (Cellgro). After 7 days, mixed glial cultures were dissociated, labeled with anti-CD11b microbeads (Miltenyi Biotec) and passed through a magnetic column to remove microglia. Astrocytes were plated on 100 mm dishes and grown to confluency for exosome collection. Treatment of cells with GW4869 (or DMSO control) and/or Aβ25-35 (AnaSpec) to induce exosome secretion and Aβ1-42 uptake experiments were done with phenol red- and serum-free DMEM (Cellgro). Primary neurons were isolated from E16.5 mouse cortices following 30 minutes trypsinization and trituration with a flame-polished Pasteur pipet. Neurons were plated on polyethyleneimine coated T-25 flasks and maintained 7 days in Neurobasal medium with B27 supplement (Life Technologies) before GW4869 treatment. To harvest exosomes, conditioned media were centrifuged (4 °C) at 300*g* for 10 minutes, 1000*g* for 10 minutes, and 20,000*g* for 30 minutes to remove dead cells, cellular debris, and larger microvesicles, respectively. Supernatant was then centrifuged (4 °C) at 110,000*g* for 2 hours. Exosome pellets were resuspended in SDS sample buffer (Western blot analysis), Tris-buffered saline (aggregation assays), or serum-free DMEM (amyloid uptake assays). Serum samples from mice were diluted in PBS and exosomes were isolated as described for culture medium. To isolate exosomes from brain tissues, we followed the method described by Perez-Gonzalez et al. 2012.

2.5. Amyloid aggregation assay

Hexofluoroisopropanol-treated human Aβ1–42 (AnaSpec) was dissolved in 0.1% ammonia, aliquoted, and frozen at −80 °C. Exosomes were suspended in (50 mM Tris, 150 mM NaCl, pH 7.5) to which rabbit IgG or anti-ceramide was added (4 μg/mL). Monomeric Aβ1-42 was added to a final concentration of 20 μM, and the samples were incubated at 37 °C for 18 hours on a shaking platform at 100 rpm. Samples were centrifuged at 20,000*g* to pellet Aβ aggregates and washed 3 times before dissolving pellets in 10-μL cold formic acid. Samples were then processed as described previously for brain Aβ and analyzed by ELISA.

2.6. Amyloid uptake assay

Mixed glial cultures were passed to 12-well plates and grown to confluency. Exosomes were resuspended in serum-free DMEM to which unlabeled Aβ1–42 was added followed by a 1 hour incubation period at 37 °C with varying amounts of exosomes (5–15 μg protein). Exosome-Aβ mixtures or Aβ alone (200 μL) were then added to mixed glial cultures in 800 μL serum-free DMEM for 18 hours. The final Aβ1–42 concentration was 0.5 μM. For ELISA assays, cells were washed 3 times with PBS, and solubilized with 100-μL formic acid and processed as described for brain Aβ. Protein content was measured by the RC-DC assay method (BioRad).

2.7. Exosome injection and amyloid labeling in brain sections

To directly label plaques, brain cryosections were washed for 1 minute each in 70% and 80% ethanol and then incubated with 1% thioflavin S in 80% ethanol for 15 minutes. Slides were then washed for 1 minute each in 80% and 70% ethanol, rinsed in deionized water, and mounted with Fluoroshield containing 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). For exosome injection, donor