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A potential function for neuronal exosomes: Sequestering intracerebral amyloid-β peptide

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A B S T R A C T

Elevated amyloid-β peptide (Aβ) in brain contributes to Alzheimer's disease (AD) pathogenesis. We demonstrated the presence of exosome-associated Aβ in the cerebrospinal fluid (CSF) of cynomolgus monkeys and APP transgenic mice. The levels of exosome-associated Aβ notably decreased in the CSF of aging animals. We also determined that neuronal exosomes, but not glial exosomes, had abundant glycosphingolipids and could capture Aβ. Infusion of neuronal exosomes into brains of APP transgenic mice decreased Aβ and amyloid depositions, similarly to what reported previously on neuroblastoma-derived exosomes. These findings highlight the role of neuronal exosomes in Aβ clearance, and suggest that their downregulation might relate to Aβ accumulation and, ultimately, the development of AD pathology.

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1. Introduction

A pathological feature of Alzheimer's disease (AD) is the presence of senile plaques, extracellular amyloid depositions of amyloid-β peptide (Aβ). Aβ is generated by the processing of amyloid precursor protein (APP), and is maintained at a steady state in normal brain. However, disruption of the balance in Aβ metabolism contributes to the formation of toxic Aβ assemblies and amyloid depositions, which are linked to AD pathogenesis. Recently, exosomes, a subtype of secreted vesicles, were reported to associate with extracellular Aβ in cultures of APP-expressing neuroblastoma cells [1,2]. Similarly, our previous study both in vitro and in vivo demonstrated that exosomes released from neuroblastoma N2a can bind Aβ on their surface glycosphingolipids (GSLs) and these Aβ-bound exosomes are then internalized by microglia for degradation [3,4]. This suggests that N2a-derived exosomes may act for Aβ elimination in brain. However, it remains unclear whether the exosomes, which originate from cells resident in the central nervous system, also contribute to Aβ metabolism.

2. Materials and methods

2.1. Animals

Wild type C57BL/6 mice and mice expressing the human APP bearing the Swedish and Indiana (KM670/671NL, V717F) mutations (J20) were obtained from SLC Inc. (Hamamatsu, Japan) and the Jackson Laboratory (Bar Harbor, ME), respectively. All

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer disease; APP, amyloid-β precursor protein; GSL, glycosphingolipid; CSF, cerebrospinal fluid; N2a, neuro2a; EGCase, endoglycoceramidase

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animal procedures were approved by the Animal Care Committees of Hokkaido University.

2.2. Monkey and murine samples

Cynomolgus monkeys (*Macaca fascicularis*) were housed at the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation (NIBIO), Ibaraki, Japan. Monkey CSF samples were obtained by lumbar puncture. Nine CSF samples were from young monkeys (4–8 years old), 8 from adult monkeys (11–21 years old), and 4 from aged monkeys (24–36 years old). Each CSF sample was used for exosome isolation without prior freezing. The parietal lobes of 20 monkeys were used for Western blotting and ELISA. All animals were bred and maintained in an air-conditioned room at the TPRC with controlled illumination (12 h light/12 h dark), temperature (25 ± 2 °C), humidity (60 ± 5%), and ventilation (10 cycles/h), and were given 70 g of commercial food and 100 g of apples daily, with unlimited access to tap water [5]. The maintenance of animals was conducted according to rules of the TPRC at NIBIO regarding the care, use, and biohazard countermeasures of laboratory animals. All animal experiments were conducted according to the guidelines of the Animal Care and Use Committee of the NIBIO, Japan.

Mouse CSF was sampled from the cisterna magna following protocols previously reported [6]. Each 50 μl of CSF was collected from 2-month-old C57BL/6 mice or APP mice at the indicated ages.

2.3. Cell cultures

Primary neuron cultures were prepared from the cerebral cortices of mouse brains on embryonic day 15 as described previously [3]. Primary glial cultures were prepared from the mouse cortices according to published methods with minor modifications [7,8]. Briefly, the neocortex was removed from each 2-day-old mouse pup, dissociated in a dissociation solution (Sumitomo Bakelite, Tokyo, Japan), and plated in DMEM and 10% fetal bovine serum (FBS). After being cultured for 14 days, the microglia were detached by shaking, and the separate cells were cultured in DMEM/5% FBS. The astrocytes remaining in the flasks were cultured in DMEM/10% FBS. The resultant glial cells were cultured for two days and used for further analysis.

2.4. Exosome isolation

Exosomes were prepared from supernatants of primary cultures as described previously [3]. Briefly, after culture of cells for one day, the culture supernatants were sequentially centrifuged at 2000×*g* for 10 min, and at 10000×*g* for 30 min, and at 100000×*g* for 1 h to obtain exosomes as pellets. Using this same method, exosomes were also isolated from the CSF samples of APP mice or monkeys.

2.5. Electron microscopy

Exosomes were stained with phosphotungstic acid. For immunolabeling, the exosomes were incubated with anti-Aβ antibody (4G8) then 10 nm gold-coupled anti-IgG. Images were taken with JEM-ARM200F (JEOL Ltd., Tokyo, Japan) transmission electron microscope.

2.6. Western blotting

To detect target proteins, we employed monoclonal antibodies against Alix, GM130, Transferrin receptor (BD Biosciences), and Aβ (6E10, Signet, Dedham, MA), and a polyclonal antibody against flottilin-1 (Santa Cruz Biotechnology). Ganglioside GM1, was detected by cholera toxin B subunit (Sigma).

2.7. Fluorescence labeling for the exosomes

Labeling of the exosomes was performed using PKH26 (Sigma) as described previously [3].

2.8. Analysis of particle size and number

The exosomes collected from primary cultures of neurons and CSF samples were suspended in PBS, and a qNano System (Izon Science, Ltd) employed to analyze the particle size and densities. CPC100 was used as the calibration sample.

2.9. Exosome administration into mouse brains

The experiment was performed as previously described [4]. Briefly, mice were continuously treated with exosomes (2 mg protein/ml) or PBS by Alzet minipump at 0.25 μl/h for 14 days. The Brain Infusion Kit was implanted into the right hippocampus using a stereotactic instrument. One hemibrain was fixed for immunohistochemistry, and the other was frozen for use in ELISA.

2.10. Immunohistochemistry and thioflavin-S (ThS) staining

Immunostaining and ThS staining were performed as described previously [4].

2.11. Aβ ELISA

$A\beta_{1-40}$ and $A\beta_{1-42}$ levels were determined using an ELISA kit (Wako, Osaka, Japan) as previously described [4].

2.12. Measurement of glycosphingolipids (GSLs)

Levels of GSLs in the exosomes of primary cultures were measured as described previously [4]. After their extraction, the GSLs were enzymatically digested with EGCase I and II, further purified by glycoblotting, then analyzed by MALDI-TOF MS.

2.13. Endoglycosylceramidase (EGCase)

Exosomes (1 mg protein/ml) were incubated with 0.5 U/ml EGCase II (Takara Bio Inc., Shiga, Japan) at 37 °C for 15 h in PBS.

2.14. Thioflavin assay

Thioflavin-T (ThT) assay was performed as previously published [4].

2.15. Aβ binding assay

Fluorescent $A\beta_{1-42}$ (25 μM) was incubated with the PKH26-labeled exosomes (treated with or without EGCase) in serum-free medium at 37 °C for 5 h. The exosomes and bound Aβ were observed after wash out free Aβ.

3. Results

3.1. Exosomes associate with Aβ in the CSF of non-human primates

The non-human primate cynomolgus monkey is widely used for AD-related preclinical studies [9]. In the monkey brains, Aβ naturally increases in an age-dependent manner (Fig. 1A) as described in the

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