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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 October 2014; accepted: 10 January 2015; published online: 29 January 2015.

Citation: Shimamoto A, Yokote K and Tahara H (2015) Werner Syndrome-specific induced pluripotent stem cells: recovery of telomere function by reprogramming. Front. Genet. 6:10. doi: 10.3389/fgene.2015.00010

This article was submitted to Genetics of Aging, a section of the journal Frontiers in Genetics.

FEBS Letters 589 (2015) 84–88

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

Kohei Yuyama [a,*], Hui Sun [a], Seigo Usuki [a], Shota Sakai [a], Hisatoshi Hanamatsu [a], Tetsuo Mioka [a,b], Nobuyuki Kimura [c], Megumi Okada [d], Hidetoshi Tahara [d], Jun-ichi Furukawa [e], Naoki Fujitani [e], Yasuro Shinohara [e], Yasuyuki Igarashi [a]

[a] Laboratory of Biomembrane and Biofunctional Chemistry, Graduate School of Advanced Life Science, and Frontier Research Center for Post-Genome Science and Technology, Hokkaido University, Sapporo, Japan
[b] Division of Molecular Interaction, Institute for Genetic Medicine, Graduate School of Life Science, Hokkaido University, Sapporo, Japan
[c] Section of Cell Biology and Pathology, Department of Alzheimer's Disease Research, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Obu, Japan
[d] Department of Cellular and Molecular Biology, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan
[e] Laboratory of Medical and Functional Glycomics, Graduate School of Advanced Life Science, and Frontier Research Center for Post-Genome Science and Technology, Hokkaido University, Sapporo, Japan

ARTICLE INFO

Article history:
Received 24 September 2014
Revised 7 November 2014
Accepted 19 November 2014
Available online 29 November 2014

Edited by Jesus Avila

Keywords:
Exosome
Amyloid-β peptide
Alzheimer's disease
Cynomolgus monkey
APP transgenic mouse
Cerebrospinal fluid

ABSTRACT

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.

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

* Corresponding author.
E-mail address: kyuyama@pharm.hokudai.ac.jp (K. Yuyama).

http://dx.doi.org/10.1016/j.febslet.2014.11.027
0014-5793/

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

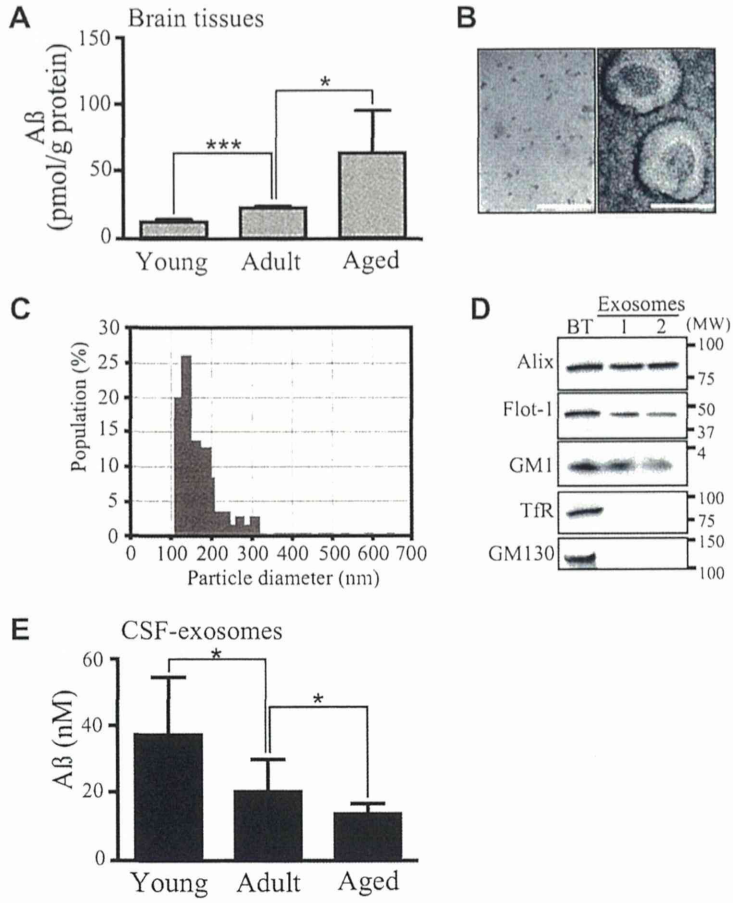

Fig. 1. Exosomes associate with Aβ in the CSF of cynomolgus monkeys. (A) Aβ levels in the brain tissues were analyzed. Values are presented as the mean ± S.D. (Young, $n = 5$; Adult, $n = 6$; Aged, $n = 9$, $^{*}P < 0.05$, $^{***}P < 0.001$). (B) Images of the exosome fraction. Bars: 1 μm in left panel, 100 nm in right panel. (C) Particle size distribution of the exosome fraction. (D) Western blotting of the exosomes. Alix, flotillin-1(Flot-1), and ganglioside GM1 (GM1) as exosome markers; transferrin receptor (TfR) and GM130 as negative markers. BT, brain tissues (E) Aβ levels in the CSF-exosomes were measured. Values are presented as the mean ± S.D. (Young, $n = 9$; Adult, $n = 8$; Aged, $n = 4$, $^{*}P < 0.05$).

previous reports [10,11]. To investigate the effect of exosomes originating from brain cells against Aβ metabolism, we isolated exosomes from monkey CSF, then measured the Aβ levels in the exosome fractions. The CSF-derived exosomes were confirmed by electron microscopy to mainly consist of small membrane vesicles 50–200 nm in diameter (Fig. 1B), similar to previously described [12]. The exosome size was further verified by a nanoparticle analyzer, which found the sizes to range from 100 to 200 nm (Fig. 1C). The exosomal markers were also identified in the CSF-exosome fractions (Fig. 1D). We found that Aβ was detectable in all CSF-derived exosome fractions but that the levels of exosome-associated Aβ were markedly lower in the adult and aged brains compared to the young subjects (Fig. 1E). The level of free Aβ, which was not associated with the exosomes in the CSF, declined only in the aged brains (Supplemental Fig. 1). These results suggest that the function of exosomes transporting Aβ in the central nervous system may deteriorate with age (>11-year-old).

3.2. Age-related alterations in the number of exosomes in primary neuronal cultures and APP transgenic mouse CSF

Neuronal cell cultures are widely used as models to study the molecular mechanisms of aging. We collected the exosomes from primary cultures of cortical neurons at 1, 7, or 14 day in vitro (DIV) and found that there were significant reductions in the number of particles at DIV14 compared to those at DIV1 or DIV7 (Supplemental Fig. 2A). While exosomal GSLs, especially sialylated GSLs, kept rising up to DIV14 (Supplemental Fig. 2B). These suggest that a reduction in exosome release might occur during aging, with maintaining the capacity of individual exosome to bind with Aβ.

To investigate whether the above findings in the non-human primates and in the neuronal cultures are also observed in APP transgenic mice, we collected the exosomes from CSF of 13-month-old APP mice and examined them for the presence of Aβ, using western blot analysis and electron microscopy with immunolabeling (Fig. 2A and B). We then quantified the exosomes isolated from APP mouse CSF at ages 2 to 23 months. Compared to the number of CSF-exosomes isolated from young mice (2–3 months old), the densities of exosomes in mice aged 6–7 months decreased; these low levels were maintained through 12–13 months of age, thereafter drastically declined in 23-month-old mice (Fig. 2C). We also showed marked reduction in Aβ levels in the CSF-exosomes between 2- and 23-month-old APP transgenic mice (2.19 ± 0.36 nM and 0.015 ± 0.012 nM, respectively), in accordance with those in the monkey CSF with age. In contrast, Aβ levels in the brain tissues continued rising in mice from ages 6–7 months to 23 months. The above data raised the possibility that endogenous exosomes released from brain cells may play a role in modulating Aβ metabolism.

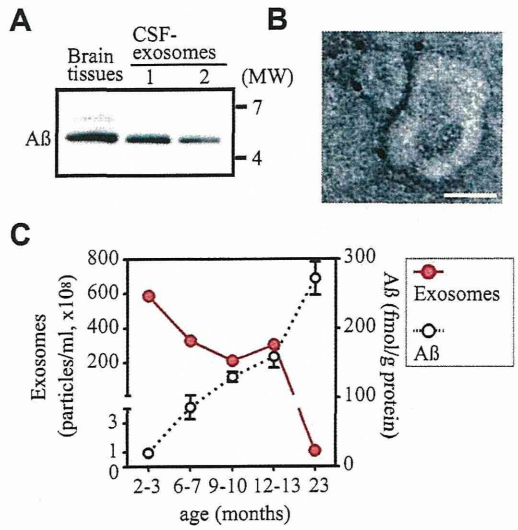

Fig. 2. Age-dependent alterations in the concentrations of exosomes from APP mouse CSF. (A) Aβ in whole brain tissues (5 μg protein) and in exosomes isolated from the CSF (50 μl) of 13-month-old APP mice was detected. (B) Exosomes derived from 13-month-old APP mouse CSF were negatively stained and immunolabeled for Aβ. Scale bar, 50 nm. (C) CSF was collected from APP mice at the indicated ages, and the densities of the exosomes were measured. Aβ levels in whole brains of APP mice were quantified. Values of Aβ levels are presented as the mean ± S.D. ($n = 5$).

3.3. GSL-enriched neuronal exosomes, but not glial exosomes, associate with Aβ

We previously determined that the enriched glycans of the GSLs are essential for Aβ binding and assembly on N2a-exosomes [3]. To examine this in our current model, we collected exosomes from primary cultures of rodent cortical neurons, astrocytes and microglia, and determined the profiles of their GSL-derived glycans and their propensity for trapping Aβ. We found that there were significantly more GSLs in neuronal exosomes than in exosomes from glial cells (Fig. 3A). Sialylated GSLs were also abundant in the neuronal exosomes; in particular, trisialoganglioside GT1 was found in only neuronal exosomes (Supplemental Table 1). Ganglioside GM1, which has been reported to bind Aβ in AD brains, was also highly enriched in neuronal exosomes (Supplemental Fig. 3 and Table 1). Accordingly, neuronal exosomes, but not glial exosomes, associated with Aβ (Fig. 3B). In ThT assays for amyloid fibril detection, fluorescence intensities were enhanced after a 5 h-incubation with Aβ (Fig. 3C). Pretreatment of the neuronal exosomes with EGCase largely prevented the ThT fluorescence (Fig. 3C), indicating that the GSLs abundant in the neuronal exosomes contributed to the potency of the exosomes to bind Aβ.

3.4. Administration of neuronal exosomes decreases Aβ pathology in APP mouse brains

To assess the effect of the exosomes derived from primary neurons on amyloid pathology, we continuously administered the exosomes into the right hippocampus of 13-month-old APP mice. We found that the exosomes induced marked reductions in Aβ immunoreactive burdens (Fig. 4A and B) and ThS-positive plaques (Fig. 4C) in the treated hippocampus. Tissue levels of Aβs were also significantly decreased following the exosome infusion (Fig. 4D). These findings demonstrate that the treatment with neuronal exosomes effectively ameliorates Aβ pathology in APP mice, suggesting a novel role for neuronal exosomes in clearing Aβ in brain.

4. Discussion

Our study shown here demonstrated that exosomes are presented in monkey and murine CSF. The exosomes collected from monkey CSF at all ages contained Aβ (Fig. 1E), demonstrating that endogenous exosomes are also coupled with Aβ. In addition, the numbers of exosomes in the CSF of APP transgenic mice changed in an age dependent manner (Fig. 2C). It remains unclear which age-related factors would modulate exosome numbers, although the impairment of endocytic transport might be a potent possibility. Endocytic disturbances, such as endosome enlargement, are observed in aged monkey brains, with concomitant higher

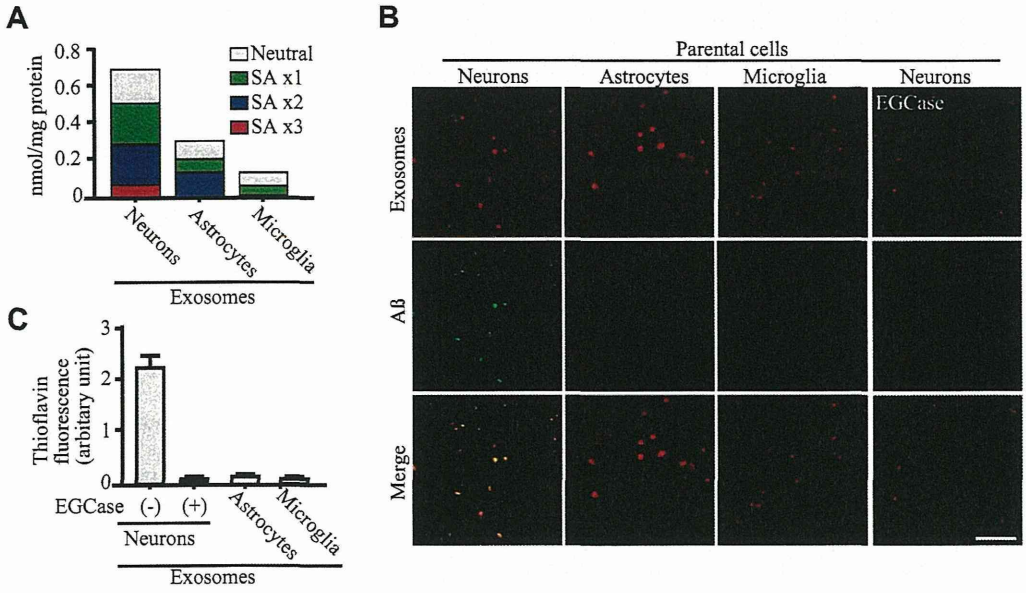

Fig. 3. GSL-enriched neuronal exosomes associate with Aβ. Exosomes were collected from primary cultures of cortical neurons (DIV7), astrocytes, and microglia. (A) Total amounts of GSL-glycans and the number of sialic acid moieties in the exosomes were measured. (B) Representative images of fluorescent Aβ binding on exosomes after a 5 h exposure. Bar, 100 μm. (C) After a 5 h incubation, ThT fluorescence intensities were detected in solutions containing Aβ and exosomes, untreated or pretreated with EGCase. Each column represents the average ± S.D. of four values.

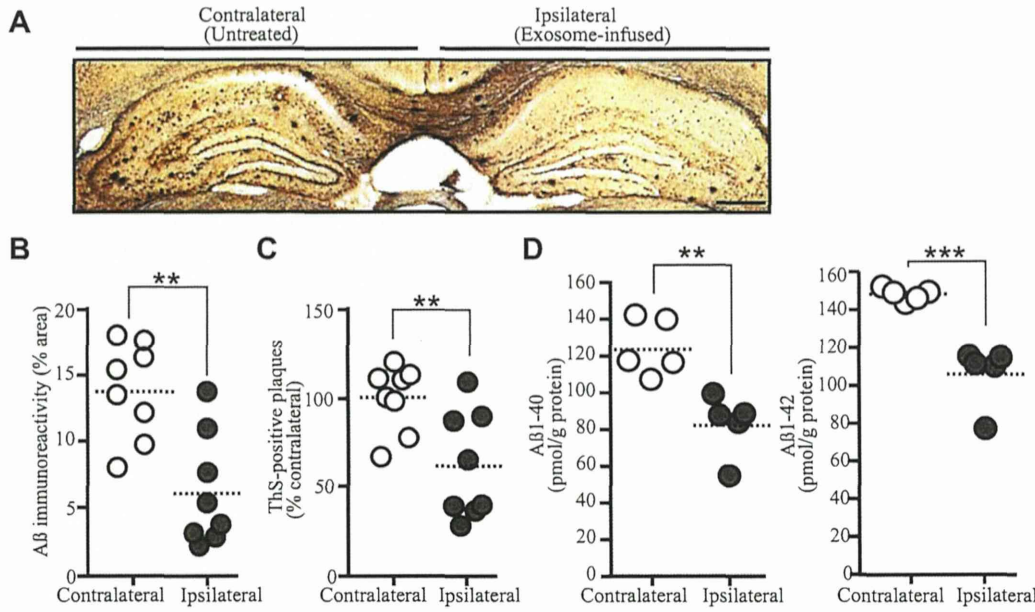

Fig. 4. Neuronal exosomes decreases Aβ pathology in APP mouse brains. Neuronal exosomes were infused into the hippocampus of 13-month-old APP mice for 14 days. (A) Image of Aβ-stained hippocampal section. Bar, 200 μm. (B and C) Aβ-immunopositive areas (B) and the numbers of ThS-positive plaques (C) in each hippocampal region were measured ($n = 4$ animals, 2 sections per brain; $^{**}P < 0.01$). (D) Hippocampal Aβs were analyzed by ELISA. ($n = 5$ animals; $^{**}P < 0.01$, $^{***}P < 0.001$).

expression of Rab GTPases, which are responsible for the endosome transport [13,14]. In addition, the dysfunction of dynein, a microtubule-associated protein active in endosome trafficking, has also been observed in aged monkey brains [13]. Notably, knockdown of dynein reduced exosome release from N2a cells [13]. Considering the appearance of endocytic pathology in early AD pathogenesis [15,16], disturbance of intracellular transport might mediate the reduction of exosomes, eventually leading to Aβ accumulation in the aged brains.

Our study reported here clearly demonstrated that intracerebral infusion of neuronal exosomes results in decreases in Aβ levels and amyloid deposition in the brains of APP transgenic mice (Fig. 4). It is noteworthy that another pathological agent of AD, tau, can be collected from the CSF-exosomes of early AD patients [17], raising the possibility that exosomes modulate multiple factors associated with AD pathogenesis. The improvement of endogenous exosome generation might serve as a novel approach for treating or preserving AD.

Acknowledgements

This work was supported by Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program, Ministry of Education, Culture, Sports, Science and Technology, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.11.027.

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Journal of Extracellular Vesicles

COACTION

EDITORIAL

Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles

Secreted membrane-enclosed vesicles, collectively called extracellular vesicles (EVs), which include exosomes, ectosomes, microvesicles, microparticles, apoptotic bodies and other EV subsets, encompass a very rapidly growing scientific field in biology and medicine. Importantly, it is currently technically challenging to obtain a totally pure EV fraction free from non-vesicular components for functional studies, and therefore there is a need to establish guidelines for analyses of these vesicles and reporting of scientific studies on EV biology. Here, the International Society for Extracellular Vesicles (ISEV) provides researchers with a minimal set of biochemical, biophysical and functional standards that should be used to attribute any specific biological cargo or functions to EVs.

Keywords: *extracellular vesicles*; *microvesicles*; *microparticles*; *exosomes*; *ectosomes*; *extracellular RNA*

Over the past decade, there has been a rapid growth in studies of secreted membrane vesicles, collectively called extracellular vesicles (EVs). Publications in high-impact journals have proposed exciting functional roles of EVs. In particular, the knowledge that EVs can shuttle functional nucleic acids between cells (mRNA, miRNA or other RNA species) has fundamentally changed the thinking about gene regulation, as the EVs can regulate the recipient cell at a post-transcriptional level (1–3).

However, the extracellular milieu is more complex as several body fluids (especially serum/plasma) harbour extracellular RNA (exRNA) in other non-EV carriers, including protein complexes (AGO2) (4) and lipoproteins [HDL and LDL (5)]. Separation of these non-vesicular entities from EV is not fully achieved by common EV isolation protocols, including centrifugation protocols or commercial kits that claim EV or "exosome" isolation/purification. Also, the composition of recovered EVs vary vastly according to the protocols used (6–8). In particular, polymer-based methods to precipitate EVs (used by some commercial kits) do not exclusively isolate EVs, and are likely to co-isolate other molecules, including RNA–protein complexes. Consequently, there is a need to determine the distinct contribution of EVs in any experiment that describes the molecular content or the functional consequences of the isolated material.

We recognize that different experimental systems, sources of biological specimens, investigator's experience and instrumentation used contribute to the heterogeneity of published protocols and the interpretation of results. A framework for providing data and attributing functions to EVs was discussed by the Executive Committee of the International Society for Extracellular Vesicles (ISEV), a group of scientists with collective long-term expertise in the field of EV biology. Here, we propose a series of criteria, based on current best-practice, that represent the minimal characterization of EVs that should be reported by investigators. Adoption of these criteria should aid researchers in planning studies as well as reporting their results. In addition, we suggest appropriate controls that should be included in EV-related functional studies. These controls should support conclusions regarding the functions of EVs and their relationship to physiologic and pathologic mechanisms.

The term "exosomes" is the most commonly used word to designate any type of EV (Fig. 1), and this has become a "buzz term" for EV-related science. The actual meaning of this word, however, is not universally accepted [see letter by Gould and Raposo (9)]. Many publications specify that exosomes are formed in endosomal multivesicular compartments and are secreted when these compartments fuse with the plasma membrane. However, the isolated material generally studied contains a mixture of EVs. Unfortunately, the field of EV research has not matured to the point that we can propose a list of EV-specific "markers" that distinguish subsets of EVs from each other, for example, EVs produced via budding from the cell membrane or produced via endosomal compartments.

The criteria we provide here can be used by researchers to guide them in discriminating EV from non-EV components. These criteria will be updated with improvements

Citation: Journal of Extracellular Vesicles 2014, **3**: 26913 - http://dx.doi.org/10.3402/jev.v3.26913

(page number not for citation purpose)

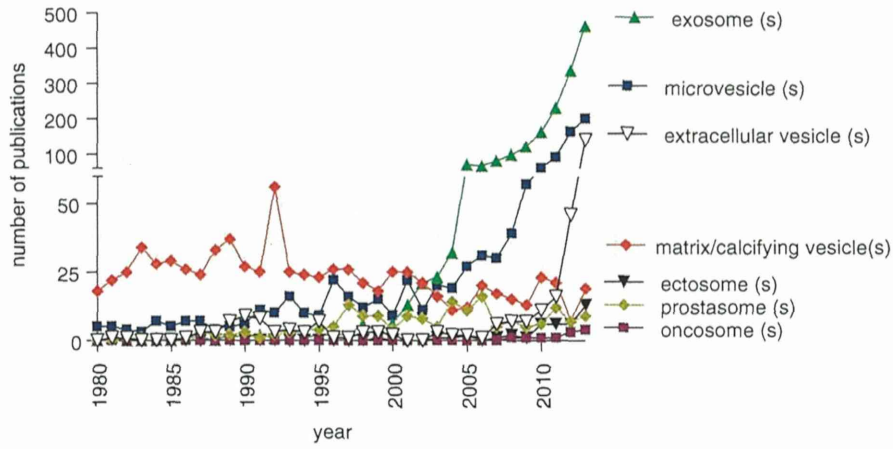

Fig. 1. Comparative evolution of the use of different terms for EVs in the literature. An advanced search was performed in PubMed at the end of December 2013 to find, for each year of publication, all articles using the given term (singular or plural) as text word: exosome(s), microvesicles, oncosome(s), ectosome(s), prostasome(s), matrix/calcifying vesicle(s). Year of final publication (and not advanced online date) of articles in English (and not other languages) was taken into account. Manual elimination of articles describing non-EV-related work was performed for exosome(s) (RNA-excision machinery) and microvesicle(s) (intracellular secretory vesicles). Use of the term microparticle(s) could not be reliably evaluated, since it is massively used to refer to non-vesicle-related particles. Notably, from 2004 onwards, the term "exosome" has become the most often used in published articles describing EVs, whereas the term "extracellular vesicles," chosen as generic term at creation of ISEV in September 2011, is steadily growing. This figure is not intended to show expansion of the EV field as compared to other fields, since numbers are not normalized to the total number of scientific medico-biological publications per year.

in the "state of the art," and we hope to eventually be able to provide specific markers and characteristics of EV subtypes. In the meantime, readers can also refer to 2 detailed Position Papers of ISEV published in 2013, listing recommendations on EV isolation (10) and EV/exRNA analysis (11).

Minimal requirements to claim the presence of EVs in isolates

One of the first criteria to define EVs is that they are isolated from extracellular fluids, that is, from conditioned cell culture medium or body fluids. Importantly, collection of the EV-containing fluid must be gentle, limiting cell disruption. Mechanical disruption of cells or tissues can result in isolation of vesicles that originate from the intracellular compartments, which obviously would reduce the purity of EVs. Therefore, the term "EVs" may not be appropriate for materials isolated in such ways.

Since there is currently no consensus on a "gold-standard" method to isolate and/or purify EVs, it cannot be claimed that there is an "optimal" method that should be uniformly used. The reader should be aware that the methods that are most efficient probably depend on (a) the specific scientific question asked and (b) on the downstream applications used. However, we urge researchers to describe in detail the methods used for EV isolation, to allow interpretation and replication by other researchers. Further, we also suggest a format of characteristics of EVs that should be analyzed and then provided in publication.

General characterization of EVs

A general overview of the protein composition of each EV preparation should be provided, at least in a first publication, including description or quantitation of components not necessarily expected to be present on or in EVs (see Table I). Although numerous proteomic analyses have highlighted proteins commonly found in exosome preparations, it is becoming clear that these do not represent "exosome-specific" markers but rather "exosome-enriched" proteins, as different subsets of secreted EVs contain many common markers. However, the relative proportions of different proteins seem to vary in the different types of EVs. Therefore, we suggest that investigators report the amount of *several* proteins (3 or more) in at least a *semi-quantitative* manner in any EV preparation, including EV isolates from body fluids or obtained from secreting cells *in vitro*. The proteins described and characterized should be proteins *expected* to be present in the EVs of interest, especially transmembrane proteins and cytosolic proteins with membrane-binding capacity (Table I, groups 1 and 2). In addition, the level of presence of proteins *not expected* to be enriched in EVs of endosomal origin should also be determined (Table I, group 3). This description will cast light on the extent of co-isolation of EVs of different intracellular origins and nature in the isolates (Table I). Furthermore, investigators can compare their protein isolates with those described in other EVs, by searches within databases [EVpedia and Vesiclepedia (12,13)].

Citation: Journal of Extracellular Vesicles 2014, **3**: 26913 - http://dx.doi.org/10.3402/jev.v3.26913

Table I. Different categories of proteins and their expected presence in EV isolates, including some examples (non-exclusive)

1. Transmembrane or lipid-bound extracellular proteins	2. Cytosolic proteins	3. Intracellular proteins	4. Extracellular proteins
Argues presence of a membrane in the isolate	With membrane- or receptor-binding capacity	Associated with compartments other than plasma membrane or endosomes	Binding specifically or non-specifically to membranes, co-isolating with EVs
Present or enriched in EVs/ exosomes	Present or enriched in EVs/ exosomes	Absent or under-represented in EVs/exosomes, but present in other types of EVs	Variable association with EVs
Examples: Tetraspanins (*CD9*, *CD63*, *CD81*) Integrins (*ITG***) or cell adhesion molecules (*CAM**) Growth factor receptors Heterotrimeric G proteins (*GNA***) Phosphatidylserine-binding *MFGE8*/lactadherin	Examples: Endosome or membrane-binding proteins (*TSG101*, annexins = *ANXA**, Rabs = *RAB**) Signal transduction or scaffolding proteins (syntenin)	Examples: Endoplasmic reticulum (Grp94 = *HSP90B1*, calnexin = *CANX*) Golgi (*GM130*) Mitochondria (cytochrome C = *CYC1*) Nucleus (histones = *HIST*H**) Argonaute/RISC complex (*AGO**)	Examples: Acetylcholinesterase (*ACHE*) Serum albumin Extracellular matrix (fibronectin = *FN1*, collagen = *COL*A**) Soluble secreted proteins (cytokines, growth factors, matrix metalloproteinases = *MMP**)

At least one protein of each category 1, 2 and 3 should be quantified in the EV preparations. EV association of proteins of category 4 should be demonstrated by other means.
Italics: official gene names; *, ** denotes different possible family members.

Analytic approaches can include Western blots (WB), (high resolution) flow cytometry (FACS) or global proteomic analysis using mass spectrometry techniques to identify e.g. transmembrane proteins. We recommend that analyses should be performed in a semi-quantitative manner, for example, using intensity analysis of Western blot signals or specific mean fluorescence intensity as compared to isotype control in FACS. When EVs secreted *in vitro* by cultured cells are analyzed, their composition should ideally be *compared with that of the secreting cells*, to determine level of enrichment of the EV components. This is *not possible for biological fluid-derived EVs*, as these are produced by a vast array of cells in the tissues. In that case, we recommend that reports include the relative proportion of different EV-associated proteins.

Table I lists the different categories and examples of proteins whose presence/absence should be simultaneously analyzed. Caution should be taken when using the enzymatic activity of proteins to indirectly determine the concentration of vesicles in any sample. An example of this is acetylcholinesterase (ACHE), a GPI-anchored protein localized in the membrane of reticulocytes, which is present in multiple membrane-anchored and non-membrane-anchored secreted forms also in other cells (14). While the activity of ACHE has been used as a marker of EVs released by reticulocytes, the use of this (or any other proteins in which activity can be measured) requires confirmation of the presence of the protein by Western blotting or functional inhibition by a specific enzyme inhibitor, as well as the recognition that these do not represent specific markers of EVs or exosomes (Table I, group 4). Therefore, their use should be restricted to cases where it is not possible to use other quantitative measures as described above, and the reasons for using them should be clearly justified.

Given the variable quality of commercial and home-made antibodies used for quantitation studies, appropriate *negative controls* should also be used and their results should be presented. These controls are best provided in the first reports using these antibodies. Ideally, the signal obtained in EVs should be compared to signals obtained from the biological fluid or conditioned medium depleted of EVs (i.e. recovered after the isolation procedure) and/or from complete medium non-conditioned by cells but processed for EV purification as conditioned medium. The reader should be aware that the supernatant, for example, after a 70-minute post-ultracentrifugation, still contains significant quantities of remaining EVs (15). The Methods section of reports should also contain details of the antibodies used (source, catalogue number and dilution) and conditions of preparations of the samples (e.g. reducing/non-reducing conditions for Western blot, an important issue to analyze some tetraspanins).

Characterization of single vesicles

We recommend characterization of single vesicles within a mixture to be performed, to provide an *indication of the heterogeneity* of the EV preparation studied. As a general rule, at least 2 different technologies should be used to characterize individual EVs. For electron microscopy

(Transmission EM) or atomic force microscopy (AFM), images should show a *wide field* encompassing multiple vesicles in addition to close-up images of single vesicles. For larger vesicles such as apoptotic bodies, cytospins and/or immunofluorescent images may be presented to provide an overview of vesicles isolated, again not focusing on a single vesicle. *Size distribution* measurements of EVs, such as nanoparticle-tracking analysis, dynamic light scattering, or resistive pulse sensing provide diameters of a large number of vesicles. However, the values acquired with these techniques should be compared with TEM, AFM or other microscopy techniques, since they do not distinguish membrane vesicles from co-isolated non-membranous particles of similar size.

Studies of the functional activity of EVs: recommendations for controls

When *in vitro* functional studies are performed with isolated EVs, a *quantitative analysis* of the dose–function relationship should be presented. This dose–response curve should be supplemented by data on the volume of starting fluid and/or the number of producing cells used to isolate the range of functional EVs.

It is important to make use of systematic *negative controls* which should exhibit minimal functional effects. These may include "mock" EVs obtained from culture medium that has not been conditioned by the cells of interest (but incubated at 37°C as if used in culture) or the fluid remaining after the EV isolation (for body fluids and conditioned medium). These controls provide insights into the "background" functional activity or signal and possibly the proportion of functional "activity" present in the soluble versus EV-associated components of the isolated fluid. Clearly, there is value to negative controls being performed at concentrations of negative EVs approximating those of functional EVs. Foetal calf serum EVs and their protein and RNA cargo can influence measurements (16,17). Thus, there should be efforts to perform studies in the absence of the serum-derived EVs. In this regard, it should be noted that 70 minutes of high speed centrifugation is insufficient to remove EV RNA cargo in foetal calf serum (18).

The ISEV Executive Committee remains concerned about the future reporting of functional changes ascribed to specific single or small clusters of molecules (protein, RNA or other) associated with EVs. This will increasingly be important, as EV biomarkers, EV therapeutics and fundamental mechanisms of EV function are brought to clinical utility or claimed in patent protection drafts. *Demonstration of association* of these molecules to EVs should therefore be provided for such use. Some proteins (Table I, group 4), but also different RNA species (5), have been variably described as co-isolated with EVs, but may not necessarily be harboured in EVs. For instance, MMP9 has been described as secreted with EVs (19,20) or, conversely, as a soluble non-EV-associated molecules (21).

A direct approach to prove association of these molecules to EVs can be fractionation of the EV preparation using density gradients. Separation of EVs from other particulate material can be guaranteed only by floatation (=upward displacement). However, for some other separations, sedimentation (=downward displacement) may be more appropriate. Such separation should be followed by qPCR or other biochemical detection methods, and the functional moiety and/or biomarker cluster should be co-fractionated with the transmembrane or EV-enriched cytosolic protein used to characterize EVs (Table I). Thus, the functional activity should be resident within defined density gradient fractions specifically containing the EV proteins. Importantly, we are aware that some density gradients often used may alter or impede functional tests performed.

An alternative approach to link functional activity, or specific molecules, with isolated EVs may be based upon antibody-mediated capture or depletion of EVs from the biofluid or conditioned medium. The antibodies used should be specific to the transmembrane protein of the characterized EVs. In these studies, depleted preparations will have lost functional activity, whereas the antibody-captured EVs should retain it (if proper and non-destructive elution from the antibody-coated beads used for capture is technically possible). We realize that EVs with functional activity but without the transmembrane protein also exist, and thus would not be depleted nor captured with this approach.

Another approach would include the use of fluorescent labels of EVs incubated with target cells. Unstained EVs and non-EV dye materials and aggregates must be eliminated with appropriate technology when this method is used. As EVs elicit their function by binding to, fusing with or being uptaken into recipient cells, it could be possible to determine a functional activity in fluorescent cells (EV-associated cells) versus non-fluorescent cells.

In the absence of any of the above proposed controls, investigators may still conclude that an extracellular functional activity exists and affects recipient cells, but the specific EV nature of this function should not be claimed.

Conclusion

The EV field is rapidly expanding and becoming increasingly complex, especially as it overlaps with the even newer field of exRNA-mediated communication. A generic biological standard of EVs, or of "exosomes," would be very useful as a baseline to compare EV preparations obtained by individual laboratories, and we are aware that European and US networks of researchers are working towards establishing such standards. When available, these standards may provide comparative EV preparative data

Citation: Journal of Extracellular Vesicles 2014, **3**: 26913 - http://dx.doi.org/10.3402/jev.v3.26913