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Spingolipid-enriched extracellular vesicles and Alzheimer's disease: A decade of research

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Abstract

Extracellular vesicles (EVs), particularly exosomes, have emerged in the last 10 years as a new player in the progression of Alzheimer's disease (AD) with high potential for being useful as a diagnostic and treatment tool. Exosomes and other EVs are enriched with the sphingolipid ceramide as well as other more complex glycosphingolipids such as gangliosides. At least a subpopulation of exosomes requires neutral sphingomyelinase activity for their biogenesis and secretion. As ceramide is often elevated in AD, exosome secretion may be affected as well. Here, we review the available data showing that exosomes regulate the aggregation and clearance of amyloid-beta ($A\beta$) and discuss the differences in data from laboratories regarding $A\beta$ binding, induction of aggregation, and glial clearance. We also summarize available data on the role of exosomes in extracellular tau propagation, AD-related exosomal mRNA/miRNA cargo, and the use of exosomes as biomarker and gene therapy vehicles for diagnosis and potential treatment.

Keywords

Alzheimer's; exosome; vesicle; ceramide; sphingomyelinase; amyloid; tau; miRNA; biomarker

Alzheimer's disease (AD) is a neurodegenerative disease and most common form of age-related dementia with three major gross pathological hallmarks: 1) accumulation of insoluble fibrillary amyloid-beta ($A\beta$) peptide, proteolytically processed from the amyloid precursor protein (APP), in the brain parenchyma, 2) aggregation of the microtubule-associated protein tau into neurofibrillary tangles, and 3) loss of connections essential for neural communication and ultimately neuronal cell death. Even though AD was first described in 1906 by Dr. Alois Alzheimer, practically everything we currently understand about the molecular basis of AD has been uncovered in the last twenty years [1–5]. Mounting data from published work indicate that blocking or reducing the $A\beta$ and tau aggregation would be among the best possible therapies for AD [6]. The current success rate in AD therapy is dismal (i.e., >99% failure rate of clinical trials in the last 10 years), and there are only five palliative FDA-approved drugs available, one NMDA receptor antagonist and four cholinesterase inhibitors [7]. One potential new category of biostructures that has

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emerged as a major research topic across disciplines in the last ten years is extracellular vesicles (EVs), in particular, exosomes. Recent evidence implicates these EVs in A β and tau aggregation and spreading and induction of apoptosis, which makes exosomes a potential therapeutic target. However, exosomes may also participate in amyloid clearance, be a valuable diagnostic tool for AD and possibly a means of drug delivery or gene targeting. In this review, we will discuss the data from published literature on the role of exosomes in AD as well as their potential for use as a (pre)clinical biomarker.

Exosomes are vesicles with a lipid bilayer that are generated through the inward budding of the limiting membrane of the multivesicular endosome (MVE) to form intraluminal vesicles (ILVs). The MVE can either fuse with lysosomes or the plasma membrane, in which case the ILVs are released into the extracellular space as exosomes. Exosomes have gained considerable attention in the past 10 years with the discovery that these vesicles carry intraluminal messenger RNA (mRNA) and micro RNA (miRNA) along with proteins and lipids with the potential to act as mediators of intercellular communication in both normal physiology and disease [8, 9]. Under the most stringent definition, exosomes can only be identified as such based on evidence of an intracellular origin in the MVE. Depending on the source, these vesicles generally range from 50–150 nm in diameter, although size alone is not sufficient to determine a population of vesicles [10, 11]. However, cells also release vesicles directly from the plasma membrane (PM), and in the literature, these vesicles are often referred to as microvesicles (MVs), microparticles, shed vesicles, or ectosomes. For a review that summarizes these distinctions in detail, see [12]. These PM-derived vesicles are on average larger in diameter (even up to μ m scale) and pellet at lower centrifugation speeds (10,000 vs 100,000 xg) than secreted vesicles although there is overlap. EVs from a single cell type, tissue, or biological fluid can differ greatly in composition [13–15]. Moreover, a recent study using differential gradient ultracentrifugation and proteomic profiling showed at least four distinct populations of EVs from dendritic cells based on density and unique protein signatures [16]. In the AD field, investigators including ourselves have tended to refer to EV populations as exosomes or microvesicles based on ultracentrifugation isolation conditions, particle size, and protein markers.

Exosome biogenesis: relevance of the sphingolipid ceramide

The formation of the MVE, and thus exosome biogenesis, has been a subject of intense study in various models. There are two categories by which exosomes are now thought to arise in the MVE, one being dependent on the Endosomal Sorting Complex Required for Transport (ESCRT) and the other being ESCRT-independent [17]. In multiple cell types, silencing of ESCRT and ESCRT-accessory proteins reduced the amount of exosomes secreted (reviewed by [18]). However, depletion of multiple ESCRT proteins involved in exosome biogenesis in mammalian cells did not eliminate exosome secretion [19]. While other modes of ESCRT-independent exosomes biogenesis have been reported [18] and references within), there appears to be a common mode of ESCRT-independent exosome formation among neural cells.

While investigating the mechanism of membrane sorting into exosomes, Trajkovic et al. observed that proteolipid protein (PLP) expressed in an oligodendrocyte cell line was sorted

into exosomes independently of ESCRT [17]. They also found that total ceramide was enriched more than 3-fold in these exosomes, leading to the discovery that neutral sphingomyelinase (nSMase) activity was required for the formation and release of exosomes through the generation of ceramide [17]. Ceramides are membrane sphingolipids found in all known eukaryotic cells that consist of a sphingosine long-chain base in an amide linkage to a fatty acid. Ceramides can be formed *de novo* in the endoplasmic reticulum or generated by hydrolysis of sphingomyelin (yields ceramide + phosphorylcholine) by sphingomyelinases.

Several lipidomics studies have been conducted on exosomes from various cell types. With the currently available data taken together, exosomes are enriched with ceramide, sphingomyelin, glycosphingolipids (GSLs, e.g., hexosylceramides, GM1), and phosphatidylserine but have relatively less of phosphatidylcholine (PC) compared to parent cells. The degree of fatty acid saturation is also a factor in exosomal enrichment of lipids (i.e., saturated vs polyunsaturated PC). Cholesterol enrichment in exosomes is variable, depending on cell type and has been used to determine the ratio of enrichment of other lipids in exosomes [17, 20–23]. Since the discovery that generation of ceramide on-demand was critical for exosome biogenesis and secretion, ours and a number of other laboratories have targeted nSMase2 by small interfering RNA (siRNA) or with the inhibitor GW4869 to block exosome secretion [24–28]. Conversely, the sphingomyelin synthase 2 inhibitor D609, which blocks the conversion of ceramide to sphingomyelin (which often exceeds ceramide by 100-fold) has been used to promote the generation and secretion of exosomes through the elevation of ceramide [25, 28, 29].

A study from our laboratory found that mice expressing a mutation in the γ -secretase component presenilin-1 (PS1 M146V) had elevated long-chain ceramides and the pro-apoptotic protein prostate apoptosis response 4 (PAR4) [30], known to be involved in AD-related neuronal apoptosis [31, 32]. Astrocytes from PS1 but not wildtype mice underwent apoptosis when incubated with long-chain ceramides *in vitro* due to the elevated expression of PAR-4 [30]. Taking this study further, we found that wildtype astrocytes cultured *in vitro* released exosomes in response to A β treatment [33]. These exosomes were not only enriched with C18:0 and C24:1 ceramides but also contained PAR-4 protein. When exosomes from A β -treated astrocytes were added to naive astrocyte cultures, the cells underwent apoptosis, which could be blocked by antibodies against ceramide or PAR-4 but not control IgG [33]. Furthermore, exosome secretion was blocked in A β -treated astrocytes lacking functional nSMase2, which were from the *fragilitas ossium* (*fro/fro*) mouse line [34, 35], but exosome secretion could be rescued by providing *fro/fro* astrocytes with exogenous C18:0 ceramide [33].

Exosomes and APP processing

The first study of exosomes in the context of AD pathology was published 10 years ago [36]. While exploring the subcellular location of APP β -cleavage, they noticed accumulation of soluble APP β C-terminal fragments (CTFs) in early and late endosomes in addition to A β being routed to the MVEs where exosomes are generated. Further examination of N2a neuroblastoma cell exosomes revealed an association of the EVs with monomeric A β (identified by western blot) following ultracentrifugation. In addition, the ESCRT accessory

protein Alix (also known as programmed cell death 6-interacting protein; PDCD6IP), which is a common exosome marker, was co-localized in plaques from human AD brain sections [36]. This study suggested the possibility that exosomes may indeed act as seeds for A β aggregation and plaque formation, considering that lipid rafts enriched in GSLs (including GM1 ganglioside) were already known to bind A β and promote its aggregation [37–39]. Further, SY5Y neuroblastoma cells accumulated APP CTFs and amyloid intracellular domain (AICD) in the MVE when treated with chloroquine or bafilomycin A1 and secreted these proteins with exosomes [40]. Similar findings were reported with Chinese hamster ovary (CHO) cells showing the presence of APP-CTFs and A β in exosomes [41]. Also contained in these vesicles were enzymes that process APP into A β : γ -secretase components PS1 and presenilin-2, and β -secretase (BACE1) [41]. Exosomes were also isolated from human and wildtype and APP (Tg2576) transgenic mouse brains [42]. Both β - and γ -secretase enzymes as well as full-length APP, APP-CTFs, and A β were found associated with these exosomes [42]. Another study showed that lactadherin (also called SED1/MFG8) was able to bind A β ₄₂ dependent on an RGD motif known for its interaction with a class of integrins [43]. Lactadherin was enriched in astrocyte exosomes from both humans and mice, suggesting a role for possible exosome-based clearance (i.e., A β uptake by macrophages was blocked by an anti-lactadherin antibody). However, in AD patients, both mRNA expression and protein of lactadherin was reduced compared to normal controls [43]. Another report speculated that disruption of dynein-dependent endosomal traffic in aged monkeys may play a role in intracellular A β accumulation by reducing exosome secretion [44].

Exosomes and A β binding/aggregation and clearance

Exosomes were first reported to be associated with A β fibril formation after it was found that GM1 ganglioside was recovered with an exosomal marker from PC12 cell conditioned medium [45]. The exosomal fraction stimulated the aggregation of A β , which was blocked by an antibody recognizing a GM1-A β complex [45]. This study was carried further with exosomes isolated from both N2a and primary neurons promoting fibrillization of A β ₄₀ and A β ₄₂ with A β ₄₂ aggregation occurring more rapidly [28]. This process was reduced by blocking GM1 with the cholera toxin b subunit and eliminated by enzymatic removal of oligosaccharides from all GSLs with an endoglycoceramidase [28]. Given this result, an interesting finding in this study was that N2a exosomes prevented the formation of A β oligomers from monomers, even after short incubation times prior to the emergence of fibrils [28]. The authors found that N2a exosomes were able to reduce A β -induced apoptosis in primary neuron cultures and increase the amount of A β cleared by microglia and degraded in lysosomes. It is worth noting that 25 μ M of A β ₄₂ in the above apoptosis and clearance experiments, which exceeds soluble A β concentrations in injured human and AD-model mouse brain interstitial fluid by >10-1000-fold [46, 47].

Our laboratory, which had been studying astrocyte responses to A β challenge [30, 33], hypothesized that astrocyte-derived exosomes would interact with A β , possibly acting as a seed for plaque formation. Incubation of exosomes isolated from D609-treated astrocytes with A β ₄₂ resulted in rapid accumulation of high molecular weight aggregates, which were easily pelleted by centrifugation [25]. This process was completely blocked by the addition

of an antibody raised against C18:0 ceramide that recognizes various ceramides but not ceramide derivatives [25, 48, 49]. In contrast to Yuyama et al. [28], we found that astrocyte-derived exosomes reduced the amount of A β ₄₂ cleared *in vitro* by glial cultures containing astrocytes and microglia [25], suggesting there may be differences with respect to glial A β clearance between neuronal (N2a neuroblastoma) and astrocyte-derived exosomes.

With respect to *in vivo* studies, it was first shown that exosomes could bind and sequester small A β oligomers (trimers, tetramers) [50]. Intracerebroventricularly (i.c.v.) administered A β oligomers could inhibit long-term potentiation (LTP) following high-frequency stimulation in rats, as expected, but infusion of N2a cell-derived exosomes prevented LTP inhibition by A β [50]. The authors demonstrated this effect was largely due to the presence of cellular prion protein (PrP^c) in neuronal-derived exosomes [50, 51]. Wildtype hippocampal cell exosomes prevented A β -stimulated LTP inhibition (field excitatory post-synaptic potential traces similar to buffer alone) while PrP^c-null hippocampal cell exosomes was intermediate between wildtype exosomes and A β oligomers. These results demonstrate PrP^c is a critical factor in binding A β oligomers, effectively preventing them from interfering with neuronal function [50]. Our laboratory published a study in 2014 where 5XFAD mice were administered 2–2.5 μ g/g of the nSMase2 inhibitor GW4869 over 6 weeks beginning at 2 months [25], when plaques first appear in this model [52]. We found that males had significantly lower total (but not soluble) A β levels, which were accompanied by reduced plaque load and brain and serum exosomes. Moreover, injection of isolated astrocyte exosomes into 10 day-old 5XFAD mouse brain promoted the aggregation of A β as observed by immunohistochemistry using a human-specific A β ₄₂ antibody. Female 5XFAD mice did not respond to GW4869 treatment [25]. Our results suggested that nSMase2-dependent secretion of exosomes contributed to aggregation of early-forming plaques. In contrast, Yuyama et al. published that continuous i.c.v. infusion of N2a-derived exosomes reduced hippocampal A β and plaque load, again suggesting differences between neuronal-type exosomes (N2a) and total exosome populations [53, 54].

Our own further *in vivo* studies using the *fro/fro* mice (nSMase2-deficient) crossed into the 5XFAD transgenic line (*fro*;5XFAD) confirmed our previous results using the nSMase inhibitor GW4869 [25]. Since nSMase2 has been shown by multiple groups to play a crucial role in exosome formation in neural cells, we postulated that these mice would have reduced exosome secretion in the brain. Compared to 5XFAD control mice, the *fro*;5XFAD mice had reduced overall brain ceramide, reduced brain exosomes, and reduced levels, plaque load, and phospho-tau levels in older mice (10 months) [55]. Additionally, *fro*;5XFAD mice performed no differently than wildtype, non-transgenic littermates in a fear conditioning assessment while 5XFAD control littermates [55] displayed behavioral deficits as previously described [56]. While Yuyama et al. put forward that exosome secretion decreases with age, leading to plaque formation [54], our results would indicate that an overall reduction in exosomes over the life of the organism protects against classical AD pathology and improves cognition [25, 55].

Consistent with reports from our laboratory [25, 55] and An et al. [50], a recently published study respectively confirmed that wildtype exosomes from N2a and SH-SY5Y cells reduced the amount of A β cleared *in vitro* and that the presence of PrP^c was critical for exosome

binding and aggregation of A β [57]. The authors discuss that PrP^c may work in concert with GSLs such as GM1 to promote aggregation [57]. Exosomes have also been implicated in the formation of amyloid fibrils of other proteins such as promelanosome protein [58, 59]. Overall, the research to date has shown there to be an acceleration of A β aggregation in the presence of neuroblastoma exosomes bearing GSLs and PrP^c. However, a separate study showed that A β fibrillization is accelerated merely in the presence of small artificially-generated, chemically-defined phospholipid vesicles close to 30 nm in diameter [60]. The extent to which this aggregation is comparable to that by exosomes is not reported.

Exosomes and proteolytic A β degradation

In contrast to the evidence that exosomes are capable of binding A β , promoting its aggregation, and acting as a seed for plaque formation, other lines of evidence indicate that exosomes may participate in the proteolytic degradation of A β . Exosomes are secreted from various cell types associated with extracellular proteases (for review, see [61]). In the context of AD, two enzymes in particular are important for extracellular degradation of A β : insulin degrading enzyme (IDE) and neprilysin [62, 63]. IDE was found to be sorted into and secreted in association with exosomes from N2a cells [64]. Conditions under which exosome-associated IDE secretion was increased were associated with A β extracellular degradation. However, the authors did not definitely demonstrate this degradation was due to IDE protease activity on A β [64]. An independent study showed that statins (which inhibits the rate-limiting step in cholesterol synthesis) stimulated microglia to release exosome-associated IDE [65]. Conditioned medium from statin-treated microglia was able to degrade A β , and A β degradation was prevented by addition of EDTA (metalloproteinase inhibitor), bacitracin A (IDE inhibitor), or insulin but not the neprilysin inhibitor thiorphan [65]. In contrast, exosomes from primary cultured, A β -treated astrocytes in our laboratory did not contain IDE at a detectable level by western blot although we did not treat cells with statin drugs (unpublished observation). Neprilysin, a major A β -degrading protease, was found to be secreted in association with exosomes from mesenchymal stem cells derived from adipose tissue [66]. Exosomes from these cells could incorporate into N2a cells and, in transwell experiments, were able to reduce both secreted and intracellular A β with loss of activity in the presence of the inhibitor thiorphan [66]. Another recent study corroborated the above findings that statins induce secretion of neprilysin by primary astrocytes [67]. However, the authors did not examine whether the enzyme was associated with EVs or that the A β -degrading activity in conditioned medium of statin-treated activity was due specifically to neprilysin activity [67].

Exosomes and tau pathology

The second major hallmark of AD are intraneuronal neurofibrillary tangles resulting from the buildup of the aggregated, hyperphosphorylated microtubule-associated protein tau. However, tau is also found in brain interstitial fluid and CSF and was previously thought to be only released as a result of cell death [68, 69]. As evidence emerged that tau, which lacks a secretory pathway signal sequence, exhibited intercellular transfer *in vivo* [70, 71], exosomes were speculated to be a vehicle for spreading tau [72]. Tau was demonstrated to be associated with exosomes from M1C neuroblastoma cell and in CSF [73]. Several

proteins known to interact with tau including kinases that promote tau aggregation were found associated with M1C exosomes. Importantly, this study demonstrated that exosomal-associated tau (relative to free tau) is elevated in CSF during mild AD, prior to massive cell death occurring at later stages [73]. The authors continued this line of work, finding that proteins from mitochondria and others involved in axonogenesis are recruited to MVEs and exosomes by the overexpression of tau in neuroblastoma cell [74]. These findings are supported by a study from another group that showed tau was secreted in association with EVs after reaching a critical intracellular level following its overexpression [75].

The most significant study illustrating that exosomes indeed propagate the spreading of extracellular tau was conducted *in vivo*. Virally transduced human tau (h-tau) in the median entorhinal cortex of mice spread to the germinal cell layer (GCL) of the dentate gyrus over a 4-week period [24]. After determination that microglia phagocytosed tau *in vivo*, microglia were depleted by either i.c.v. infused clodronate liposomes or feeding (in the chow) an inhibitor of colony stimulating factor 1 receptor. Mice depleted of microglia showed dramatic reduction in the propagation of h-tau to the GCL [24]. Further, injection of h-tau-containing exosomes into the outer molecular layer led to the appearance of h-tau in the GCL after 3-weeks. Most importantly, treatment of mice injected with the nSMase2 inhibitor GW4869 significantly reduced the amount of h-tau in the GCL weeks after its viral transduction in the entorhinal cortex [24]. These data present the strongest evidence to date for an *in vivo* role for exosomes in the pathogenesis of AD. Recently, another study was published showing that exosomes isolated from brain tissue of mice expressing the aggregation-prone P301L mutation of h-tau contained phosphorylated tau (AT270, pS262, and pS422) [76]. These exosomes were shown to possess the ability to aggregate tau in recipient cells but with a minimum threshold requirement of exosome uptake into cells containing a FRET tau biosensor [76].

Exosomal miRNAs and mRNAs involved in AD

One of the most explored biological properties of exosomes and other EVs is the ability to carry and transfer mRNA, miRNA, and other biomolecules to recipient cells [8, 9, 23]. Several miRNAs from brain, serum/plasma, urine, and CSF have been reported to be dysregulated at various stages of AD. For a comprehensive list of these miRNAs, see [77–79]. In a study of immune response, mRNA for IL-6, IL-12, and TNF α were all elevated in exosomes secreted by macrophages isolated from older (65+ years) patients following *in vitro* A β treatment [80]. In comparison, macrophage exosomes isolated from younger patients (ages 21–45) did not show the same degree of increase of these pro-inflammatory factors [80]. These results point to an age-related difference in potential exosomal intercellular communication with respect to transfer of pro-inflammatory cytokines to other cell types [80].

Several laboratories have conducted screens for differentially expressed miRNAs between normal and AD patients. Of those specifically examining exosomal miRNAs, two in particular identified signatures between 16 (serum) and 20 (plasma) miRNAs [81, 82]. In a study of CSF exosomal miRNA, there were a smaller number of miRNAs differentially expressed in AD patients compared to normal (7 with p-values <0.05 with an additional 5

scoring between $p=0.052$ and 0.092) [83]. However, only one of these miRNAs was reported in at least two of these studies. miR-342-3p was identified in both the serum and plasma screens [81, 82] with no overlap in the miRNAs identified in the CSF as differentially expressed in AD. A different microRNA, miR-34a, was found to be upregulated in the temporal lobe of AD patients and AD model transgenic mice [84]. Exosomes from neurons that overexpressed miR-34a could transfer this miRNA to neighboring neurons. Although it was not definitely shown that exosomal miRNA transfer affected gene expression in recipient neurons, miR-34a expression in AD was shown to affect a plethora of genes known to be altered in AD (glucose metabolism, oxidative phosphorylation, and synaptic plasticity) [84]. However, whether miRNAs spread *in vivo* by exosomes have the potential to regulate gene expression remains unclear.

Because exosomes carry miRNAs as part of their normal function, Alvarez-Erviti et al. sought to harness exosomes for a gene-targeting therapeutic strategy under the idea that exosomes could cross the blood-brain barrier (BBB) [85]. They expressed a fusion protein of Lamp2b and rabies virus glycoprotein (RVG) in dendritic cells, which is incorporated into exosomes. siRNA against BACE1 was electroporated into Lamp2b-RVG exosomes and injected intravenously into mice. The RVG specifically targeted the exosomes to neurons (along with microglia and oligodendrocytes), and the BACE1 siRNA was capable of reducing expression of BACE1 mRNA and protein by 60% [85], demonstrating that exosomes could be used to target specific cell types for gene therapy.

As an aside, we must recognize there is some controversy regarding the *in vivo* relevance of naturally occurring exosomal miRNAs. For multiple sources of exosomes, it was calculated that there was approximately 1 molecule of a given miRNA for every 125 exosomes, thus calling into question the functional significance of exosomes to affect gene expression in recipient cells [86].

Exosomes as biomarker vehicles for AD

Because exosomes are abundant in biological fluids such as blood (serum, plasma), urine, and CSF, they are potentially valuable as vehicles for biomarkers of disease. As noted above, for example, there are various signatures of differentially expressed miRNAs at various stages of AD progression, which can be packaged into exosomes that eventually end up in the CSF or blood. In a disease such as AD, the BBB can be compromised at least in a subset of patients, and a recent study identified in humans that the BBB becomes compromised in the hippocampus rather early during normal brain aging and worsens under mild cognitive impairment [87]. A subpopulation of neural-derived EVs were isolated from patient serum [88]. Significant differences were found with respect to EV-associated tau (pT181 and pS396) and $A\beta_{42}$ between cognitively normal control patients and AD patients at the time of diagnosis as well as up to 10 years prior with a 96% correct classification [88]. These results suggest that EV-associated tau and $A\beta$ in the blood are an excellent predictors of whether an individual will develop AD (or frontotemporal dementia). It should be noted that the authors of this study used the ExoQuick reagent to isolate EVs from serum samples but the methods did not report removal of larger microvesicles by centrifugation at least 10,000 xg prior to EV isolation. ExoQuick is a polymer-based reagent that can co-precipitate non-exosomal

contaminants including microvesicles and lipoproteins [89]. As such, the results may not necessarily reflect contents of a *bona fide* exosome population but include other lipid vesicles as well. In contrast, an independent group recently reported that tau was not found to be associated at higher levels with neural-derived EVs isolated from plasma of AD patients [90]. Using isolation of neural-derived EVs from human plasma, it was also found that the abundance of several proteins was altered up to 10 years prior to AD diagnosis [91]. There were decreases in low-density lipoprotein receptor related protein 6, heat-shock factor 1, and repressor element 1-silencing transcription factor [91] as well as increases in cathepsin D, Lamp1, and various ubiquitinated proteins [92]. Differences were also found in plasma neural-derived EV-associated phosphorylated type 1 insulin receptor substrate (IRS): increases in pS312-IRS and decreases in pY(pan)-IRS [93]. These results indicate that several neural-derived exosome-associated proteins can be used to predict AD up to 10 years in advance [88, 91–94].

An additional AD exosomal miRNA biomarker was also identified [95]. miR-193b transfection of SH-SY5H cells was shown to downregulate mRNA and protein expression of APP with the 3' untranslated region of APP mRNA containing a target for miR-193b. In APP/PS1 transgenic mice and human AD patients, miR-193b was decreased compared to wildtype mice and cognitively normal patients, respectively, in both CSF and serum exosomes [95]. However, miR-193b was not identified as a differentially expressed exosomal miRNA by the studies noted above [81–83].

Microvesicles/ectosome involvement in AD pathology

Another type of EV, 100–1000 nm in size, released from the plasma membrane upon surface bending and budding as a consequence of lipid scrambling and cytoskeleton remodeling, are called microvesicles (MVs) or ectosomes. Depending on their cell origin, MVs may contain proteins, mRNAs, miRNAs, and/or DNA, which carry out functions of intercellular communication and cellular homeostasis [96, 97]. MVs are produced by various cell types, including neurons, astrocytes, microglia, and tumor cell lines. Furthermore, they are also found in several body fluids such as serum, plasma, and CSF [98–101].

MVs are closely related to AD. The production of myeloid MVs, likely of microglial origin, is high in patients with AD or mild cognitive impairment, when compared to healthy controls [102, 103]. Functional studies show that MVs derived from AD microglia are toxic for cultured neurons [103]. A recent study from an independent group found that both the MV levels and calpain activity are significantly higher in the AD patient CSF. More importantly, combined assessment of calpain activity and A β ₄₂ levels in CSF improve diagnostic accuracy of the disease [104].

The molecular mechanisms for MV neurotoxicity is defined using MVs produced by primary microglia *in vitro*. Using the thioflavin T dye-binding assay, Joshi et al showed that MVs from microglia promote the solubilization of larger A β ₄₂ aggregates/fibrils into neurotoxic soluble species. Interestingly, lipids are identified as the active components of MV A β -solubilizing activity [103]. This finding is consistent with evidence that natural lipids shift the equilibrium from insoluble toward soluble highly toxic A β species. This

study confirms the critical involvement of lipids in extracellular vesicle-A β interaction. In addition, a fraction of soluble A β species associate with MVs as indicated by increased A β level on sucrose gradient upon addition of MVs [105, 106]. In addition to soluble A β , MVs contain hyperphosphorylated oligomeric tau, the neurotoxic tau in AD [73, 75]. These findings support the notion that reactive microglia shed MVs, which propagate damage to surrounding neurons, oligodendrocytes, and astrocytes. However, it is still unclear whether increased secretion of microglial MVs contributes to AD progression or are a response to the disease. Indeed, microglia surrounding plaques may overproduce neurotoxic MVs in response to excessive A β phagocytosis when degradative pathways are saturated [103]. Interestingly, high concentration of myeloid MVs in CSF may lower A β ₄₂ levels in CSF by sequestering the peptides, which represents an early biomarker of AD [102, 107].

Just as neuron-derived exosomes may provide a vehicle by which to eliminate A β [28], microglia-derived MVs could represent a means for microglia to eliminate neurotoxic A β or tau when their degradative pathways are overpowered by the disease conditions. Ironically, A β containing MVs are toxic for neurons and oligodendrocytes by furthering nucleation and propagation of neurotoxic amyloids throughout the brain, possibly representing a mechanism behind transsynaptic spread of A β in AD. Further investigations are needed to better define the interaction of distinct EVs populations with different A β and tau forms and their impact on A β assembly and cell-to-cell spreading [103, 108].

Conclusion

There is sufficient evidence available from the last 10 years to implicate exosomes and other EVs in the progression of AD. While multiple laboratories are in agreement that exosomes promote the aggregation/fibrillization of A β and tau, there is still some controversy to whether this phenomenon is universal among all exosomes and also to what extent exosomes promote or prevent clearance of A β peptides. While our laboratory has used the *fro/fro* mouse model to examine AD pathology, there is no means available to date to block all exosome or other EV production. There may be differences with respect to aggregation and clearance between exosomes originating from different sources (e.g. neurons vs. astrocytes), and to date there is no information on relative contribution of different neural cell types to the overall EV populations in the brain. While it has been shown for tau, it has not yet been demonstrated that endogenous exosomes can propagate the spreading of A β throughout the brain or whether these exosomes act locally to seed plaques. There are also differences as to whether exosomes can proteolytically degrade A β with some laboratories showing exosomes being secreted with IDE and neprilysin while others show A β aggregation properties rather than proteolysis. Exosomes have the potential to be biomarkers, stemming from the work showing that the relative abundance of a number of proteins are altered as early as 10 years prior to AD diagnosis in some patients. However, differentially-expressed miRNA profiles have not been consistent among laboratories to be efficient in AD prediction at this time.

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