Exosomes – Potential for Blood-Based Marker in Alzheimer's Disease

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Abstract

Exosomes are believed to be secreted from multivesicular endosomes and containing proteins and nucleic acids, including mRNA and microRNAs, which have been implicated to play a role in neurodegenerative diseases. Neuron-derived exosomes at the circulation provide a unique potential as biomarkers towards assessment of Alzheimer's disease (AD), even at the pre-clinical stage. This review briefly discusses their biogenesis and transport, exosomal protein verses soluble protein, evidence for their role in AD, isolation of exosomes, and challenges and future directions to realize reliable blood-based biomarkers to meet phenomenal unmet clinical and pre-clinical need of AD.

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INTRODUCTION

Extracellular vesicles (EVs) are secreted from cellular membrane during the cyto-physiological process. EVs can be found in various body fluids, e.g. blood, urine, cerebrospinal fluid, semen, pleural fluid and even breast milk [(1)]. Three major subpopulations of EVs have been described based on their size and biogenesis: exosomes, microvesicles, and apoptotic bodies. The last two types of EVs are considered to be larger than 100nm and are released directly from the plasma membrane of cell. This article will focus on the smallest of the EVs family, exosome, with 30-150 nm in diameter [(1)].

Release of exosome into the extracellular space is commenced in a three-steps process: exosome biogenesis, transport of microvesicular bodies (MVBs) to the plasma membrane, and fusion of MVBs with the plasma membrane. Figure 1 illustrates the whole process commencing at the endosomal system [(2)].

Exosome biogenesis

When early endosomes mature into late endosome or MVBs, the endosomal membrane generates the intraluminal vesicles (ILV) in the organelles' lumen [(3)]. The ESCRT machinery plays a vital role in this process. The ESCRT component four different proteins: ESCRT-0, -I, -II, -III, and other associated protein such as Vps4 complex [(4)]. Many evidences have shown that if knockdown of the ESCRTs gene will reduce the secretion of exosomes. They conjecture the reason was ESCRTs participated in the process of early endosomes become MVBs [(5)]. Notably, TSG101, which belongs ESCRT-0 family, and ALEX, ESCRT associated protein, are commonly used as exosomal internal markers.

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Several studies imply that MVBs biogenesis can happen without ESCRTs. It has been shown that if silencing of a critical subunit of all four ESCRT-complexes, the ILVs are still generated in MVBs, indicating the existence of ESCRT-independent mechanisms [6]. One group of transmembrane protein, tetraspanins, has been confirmed to participate in this process [7]. Therefore, some tetraspanins, like CD9, CD63, and CD81, are usually used as exosomal membrane biomarkers.

Here, in a brief summary, an ESCRT-dependent or ESCRT-independent mechanism might work synergistically rather than independently. And different subpopulations of exosomes could be generated from different types of machinery [8].

Transport of MVBs to the plasma membrane and exosomal release

There are two pathways that MVBs can choose to continue their journey: directed to lysosomes to be degraded or transported to the plasma membrane for exosomal release [2]. However, the regulating mechanism of the two pathways is still not clear. This review will focus on release of exosomes to the extracellular environment.

Actin and microtubule cytoskeleton are two significant components to transport MVBs to the plasma membrane [9]. Besides, Rab GTPases, known as the largest family of small GTPases, are also involved in many steps of membrane trafficking, which contain transport of MVBs, membrane fusion, and exosome budding. And pieces of evidence have shown that silencing the Rab GTPases-related gene will dramatically decrease the exosomes' secretion [10].

Many molecules have been confirmed that participate in exosome biogenesis and release. However, little is known about some of their mechanisms, and this exciting field needs to be explored further. Besides, current understanding dictates that exosomes consist of almost all elements representative of the cell of origin, e.g. protein, miRNA, mRNA, and DNA in small portions [11]. Understanding of EV opens a wide range of potential applications pertinent to cancer management, neurodegenerative disease, and even therapeutic delivery [12].

Exosomal protein verses soluble protein

It is well known that aggregation of misfolded proteins is the characteristic feature of most neurodegenerative diseases [13]. Examples include amyloid-β and tau in AD, α-synuclein in Parkinson disease, and TAR DNA-binding protein 43 (TDP-43) in amyotrophic lateral sclerosis. There is reason to believe that exosomal protein is unique due to the role of the lipid membrane to enable stability of the protein cargo from proteolytic activities where soluble proteins are most vulnerable.

The distinction of exosomal protein from soluble protein is demonstrated definitively by considering application in PD-L1 immunotherapy [14]. Blood plasma from melanoma patients prior to PD-L1 treatment were tested from three sources of PD-L1 protein origins: exosomes, microvesicles and soluble form. The goal was to assess the ability of each source of protein to predict PD-L1 immuno-response prior to treatment. Results showed strong statistical difference between the responding and the non-responding cohorts for exosomes ($p=0.0001$), but weak for the other forms of protein: microvesicles ($p=0.1887$), soluble ($p=0.1890$), and the sum of the three sources ($p=0.0815$). Hence, it is clear that exosomal PD-L1 far outperforms the other two sources of protein as biomarker to predict immuno-response which constitutes a vital unmet clinical need in immunotherapy.

EXOSOMES in NEURODEGENERATION

Notably, exosomes are believed to play a prominent role in neurological diseases. Exosomes secreted by several brain-related cells are involved in signal transduction between neural cells and the peripheral nervous system. For example, one critical aspect of neurophysiology and neurological disorders may well be EV-mediated communication between neurons and glial cells [15]. With the entire spectrum of neurodegenerative diseases resulted in some form of synaptic dysfunction, the role of exosomes in synaptic signal transmission has also been postulated [16]. In Parkinson's disease (PD), neuronal exosomes can serve as culprit of disease spread via transferring α-synuclein toxic forms between neuronal and non-neuronal cells, e.g. microglia and astrocytes [17]. The exosomal characteristic (i.e., size and ability to encapsulate causative proteins) is known to cause their spread to CSF and blood, thereby potentially an ideal

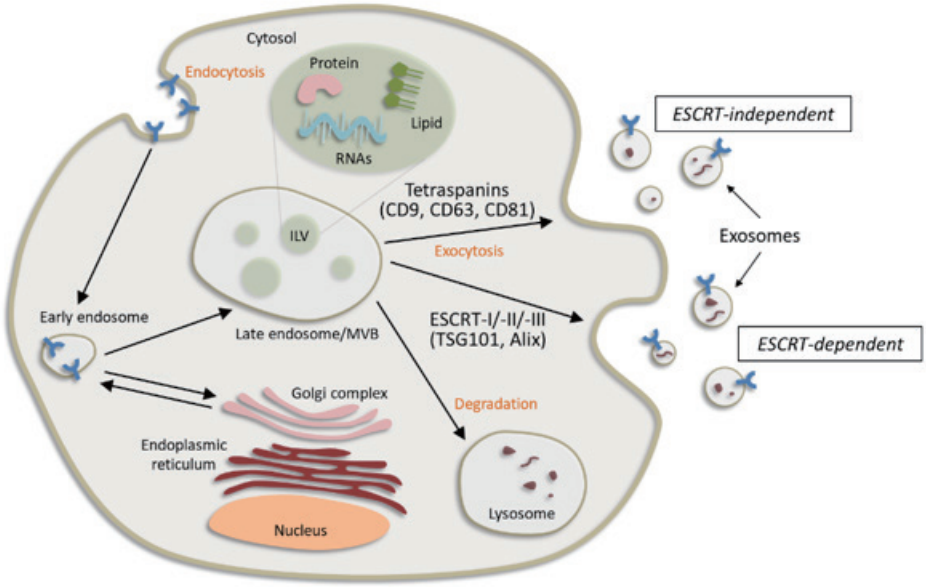

Figure 1. Release of exosomes into the extracellular space requires a three-step process. First, exosome biogenesis begins with early endosomes becoming mature with continuing fusion process into late endosomes or MVBs. Second, transport to the plasma membrane via the ESCRT-independent or the ESCRT-dependent pathway. Third, exosome release via fusion with the plasma membrane.

biomarker [18].

Micro-RNA is known to be involved in RNA post-transcriptional regulation of gene expression. Brain-derived EVs were found to contain an upregulation of disease-associated miRNA [19]. Importantly, this study also correlated changes miRNA in the brain with those in peripheral EVs from AD patients which revealed a subset of miRNA might be viable for liquid brain biopsy.

Extracellular vesicles in neurodegenerative disease has been reviewed elsewhere [20].

ISOLATION of EXOSOMES

Isolation of exosomes with high fidelity and reproducibility are not only essential for downstream analyses but, more importantly, for routine clinical applications. One major challenge in isolation of exosomes lies with their physical properties. With diameter of 30 to 120nm and roughly 10 billion per mL of blood plasma, order of magnitude calculation suggests all exosomes constitute only one part per million in volume fraction in the blood plasma. This suggests that although exosomes are substantial in quantity, their isolation still possesses substantial challenge due to their rarity.

The isolation process usually depends on their physical properties (size, density, molecular weight, etc.) in a label-free manner or immuno-separation using specific antibodies or probes. Currently, much progress has been made in exosome isolation techniques of which ultracentrifugation (UC), density-gradient centrifugation, size-exclusion chromatography (SEC), polymer precipitation, immunological separation, and microfluidic technology are commonly used. Ultracentrifugation was first proposed by Johnstone et al. [21] to harvest exosomes from in vitro culture of sheep reticulocytes. For decades, the UC protocol is being optimized and is often considered as gold standard for exosome isolation [22-24]. The UC techniques, often refers to differential centrifugation (DC), typically requires serial steps of centrifugation starting from low speed centrifugation (300 x g, 200 x g, 10,000 x g) to remove cells and debris, afterwards, the supernatant is centrifuged at higher speed (>100,000 x g) for pelletizing exosomes [25]. Studied have found

that exosomes isolated with UC have better purity but repeated ultracentrifugation can damage exosomes that results in low yield [26]. Besides, UC is operator-dependent, laborious, time-consuming (>10h) and is susceptible to centrifugation parameters – such as force, time, rotor type, etc. [27] – which makes it difficult to apply in clinical settings.

On the other hands, density-gradient centrifugation by adding multi-density of medium like sucrose or iodixanol is an alternative strategy, yet it requires even longer process (>16h) than UC with limited improvement of yield [28]. SEC separates molecules based on their size. By use of gel pores of a specific size distribution, small particles can easily enter the pores and being eluted slowly through column, while large molecules move faster without enter the pores. Further, SEC is faster and do not require specific equipment, but it is difficult to differentiate exosome size due to low resolution [29]. Polymer-based precipitation method can rapidly isolate exosome by reducing the aqueous solubility of exosome membrane in the presence of aqueous polymer (e.g., polyethylene glycol, PEG). While polymer precipitation presents superior exosome yield with relatively simple protocol, the co-precipitated biomolecules are inevitable that results in low purity, hindering subsequent downstream experiment [30].

Immunological separation capture exosomes based on the specific binding of antibodies or ligands on the exosomes such as tetraspanin family proteins, i.e. CD9, CD63 and CD81. Although antibody-based method is possible to produce high purity exosomes, commercially available of good quality antibodies are limited. Furthermore, exosomal surface expression is also heterogeneous, rendering surface marker-based approach challenging. Considering the heterogeneity of exosomes and coexistence of biomolecules with similar characteristics, combinatorial approach consists of different methods could enable their comprehensive isolation.

Moreover, multiple promising technologies such as microfluidics have been developed. Wang et al. reported a microfluidic devices consisting of nanowire-on-micropillar structure to trap vesicles, while proteins and cell debris are filtering out simultaneously. Trapped vesicles can be further recovered in PBS by dissolving nanowires. Lee et al. [31] also proposed an acoustic nano-filter device that utilizes ultrasound standing waves to separate microvesicles (MVs) from cell culture media as well as MVs in stored in red blood cells product. By exerting differential acoustic force on MVs, the device is able to separate MVs with high yield and resolution. Wu et al. [32] further developed an integrated acoustic microfluidic platform that is capable of isolating exosomes directly from undiluted blood samples. The platform contains two separation modules which erythrocytes, leukocytes and thrombocytes are removed at first interdigitated transducer (IDT) electrodes, exosomes then can be isolated from apoptotic bodies and large MVs at second IDT. He et al. [33] demonstrated a microfluidic platform integrates on-chip immunomagnetic isolation of exosomes, chemical lysis, immunomagnetic capture and identify the protein of interest from 30 μL plasma samples within ~100min. Hinestrosa et al. [34] demonstrated a platform that enables simultaneous isolation and on-chip characterization of cell-free DNA (cfDNA), extracellular vesicle RNA (EV-RNA), and exosomal proteins using AC electrokinetics (ACE). By applying electrical signal to the microelectrode array, non-uniform electric field is induced that reversibly captured target of interests onto the array. Isolated cfDNA, EV-RNA and EV-associated proteins can be characterized directly on the chip using specific dyes. Moreover, isolated biomolecules can be further eluted off from the chip for downstream analyses including PCR, RT-PCT, and next-generation sequencing.

Taken together, a myriad of isolation methods is available each with its pros and cons. The most suitable tool would depend on the constraint posted and subsequent processing required.

CONCLUDING REMARKS

Exosomes are now becoming increasingly clear that they possess substantial cell-relevant and decease-relevant information via their protein and nucleic acid cargo. Hence, exosomes in the circulation should be a viable target for assessment of AD and other neurodegenerative diseases. Since, secretion of exosomes is a part of the cyto-physiological process, potential remains that exosomes can provide preclinical and/or prodromal assessment of neurodegenerative diseases.

To realize this goal, isolation of high-quality exosomes

remains a lofty goal, especially for high throughput clinical utilization. Nonetheless, substantial progress is being made to concretely utilize exosomes for benefit of patients in early diagnosis.

Conflict of Interests:

CCL and WFH are employed by Reliance Biosciences Inc., a start-up from the National Taiwan University (NTU) on the use of exosomes as target to realize their clinical utility. NTU approved AMW to serve as CEO of Reliance Biosciences, Inc.

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