Exosomes: Extracellular communicators of health and disease

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Abstract
Exosomes are small microvesicles implicated in cell-cell communication locally and at distant body sites. Evidence suggests that exosomes can modulate normal cell function and are able to elicit disease alterations. For example, exosomes from Alzheimer brains have been shown to carry tau tangles and Parkinson patient exosomes contain α-synuclein. These putative “signals of pathology” are derived from intraluminal vesicles formed as endosome products either destined for degradation at the lysosomal-endosomal interface or extruded into extracellular space to be captured by recipient cells. Exosome cargo, packaged from selective cytoplasmic entities, can be proteins, RNAs or other biomolecules like lipids. Exosome cargo from disease cell lines has revealed both translatable mRNA and non-coding RNAs (lncRNA and microRNAs) that potentially have a gene/cell regulatory role. Exosomes appear important for establishing micro-niches for cancer metastasis and are being investigated as participants in neurodegenerative diseases. Numerous exosome proteins and RNAs may be useful as biomarkers of disease. Finally, since exosomes are natural cell shuttles of a variety of cargo and pass the blood-brain barrier, investigators are evaluating whether synthetic cargoes can be loaded into exosomes and used as a therapeutic approach to alleviate disease.

Introduction
Interest in extracellular vesicles (EVs) has exploded in recent years leading to the exploration of exosome content and function. Yet, dissecting the role of EVs and their spectrum of functions remains a complex task. Cells release a variety of extracellular vesicles at their plasma membrane surface (ectosomes (100-1000 nm), apoptotic bodies (50-5000 nm) and exosomes (30-140 nm). Exosomes are the smallest of the extracellular vesicle types and are released from non-apoptotic cells [1]. Pan and Johnstone [2] first observed exosomes in reticulocytes and hypothesized that they were a way for that cell to extrude extra cell membrane and/or act as carriers of waste products expelled from the cells. Despite the explosion of research into the role of exosomes found in all organisms, we are still learning about their biogenesis, trafficking to the extracellular space and their physiological roles in health and disease. This short review summarizes the current focus in exosome research.

Biogenesis and trafficking
Exosomes are the smallest of the extracellular vesicle types. They form by invagination of endosome membrane into the endosome space capturing cytoplasmic content generating intraluminal vesicles (ILVs). Endosomes loaded with ILVs become a multivesicular body (MVB) (Figure 1). Exosomes [3] are membrane bound vesicles approximately 30-140 nm in size and within the vesicle contain a variety of cargo. Exosomes are extruded into the extracellular space and can enter recipient cells through (1) endocytosis at the recipient cell’s plasma membrane surface, (2) recipient cell receptor-mediated endocytosis and/or (3) direct fusion with the recipient cell’s plasma membrane [4] or (5) they can enter the organism’s circulation from the extracellular space. An alternate fate of the ILV containing MVB is fusion with the lysosome where the MVB contents are degraded. Assembly of exosomes occurs in at least three ways (1) by protein sorting of specific ubiquinated proteins and the ESCRT (Endosomal Sorting Complex Required for Transport) machinery [5], (2) by association with lipid rafts contained in the exosomes lipid bilayer membrane [6] and (3) by a ceramide dependent pathway that captures biomolecules [7]. Specific proteins are required to load the exosome (ESCRT complex), energy from ATP lysis (Rab GTPases) is needed for release at the plasma membrane and proteins embedded in the exosome lipid membrane are needed for recognition and capture at the recipient cell’s surface (transpanins, flotillins and others). The ESCRT complex and associated proteins are used to sort and cluster ubiquinated proteins like receptors [8]. The tetraspanins along with CD9 and CD63 initiate exosome formation by clustering in microdomain regions of the endosome membrane. Segregation of proteins in the microdomains occurs and an additional recruitment of ESCRT complex proteins begins invagination of the endosomal membrane capturing cytosolic cargo in the ILVs (reviewed by Kalra [8]). Also, It is shown that there is an ESCRT complex independent pathway for formation of ILVs; this glycoprotein pathway involves the formation of lipid rafts [9] where ceramide may be a key component. Oligodendrocytes seem to form exosomes via sphingomyelinatease cleavage of sphingomyelin to ceramide [7]. Ceramide induces invagination and then formation of ILVs. Subsequent steps in biogenesis involve moving the MVB to the plasma membrane surface, fusion with the plasma membrane and release of the exosomes. YKY6 SNARE protein and Rab GTPases direct exosomes to the plasma membrane surface [4,10]. Interestingly,

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Received: August 10, 2016; Accepted: August 19, 2016; Published: August 23, 2016
Exosomes are extracellular vesicles (EVs) generated by both phagocytic and non-phagocytic cells. These vesicles are generated when multivesicular body (MVB) membranes fuse with the cell’s plasma membrane, releasing their contents to the extracellular space. Exosomes contain both extracellular and intracellular proteins, lipids, nucleic acids (RNA and DNA), and metabolites. They can function as a regulatory messenger between cells and can be induced by a variety of stimuli, such as cytokines, growth factors, and other extracellular cues. Exosomes are involved in cell communication and can be used for the delivery of proteins and nucleic acids. They play a role in intercellular signaling and can contribute to the initiation and progression of various diseases. The selective sorting mechanism used to load the ILV destined to be an exosome is still a topic of debate. The content of exosomes is selected based on various factors, including the expression of specific proteins and the environment in which the cell resides. The role of exosomes in disease states, such as cancer and neurodegenerative diseases, is an active area of research.
Isolation and characterization methods

Exosomes are released by all cell types examined in the CNS either to the intracranial space or into the cerebral spinal fluid (CSF) or blood circulation [4,35]. Exosomes may act both positively and negatively on recipient cells. As described, exosomes can remove cellular waste, communicate signaling information between local and distant sites and may be vehicles to transfer pathogens like infectious viral particles [36] or prions [37]. Thus, exosomes are cellular communicators of disease and they can be captured from CSF and medium from cultured neural cells. They have been studied in several neurodegenerative diseases including Alzheimer’s disease [38,39], Creutzfeldt-Jakob Syndrome [40,41], Parkinson’s [42,43] and Amyotrophic Lateral Sclerosis (ALS) [44]. Extracellular vesicles from one neural cell type may interact with other neurons, astrocytes, microglia, and oligodendrocytes transporting both healthy and diseased proteins, lipids or RNA species [4]. In Alzheimer’s disease the accumulation of amyloid plaques, insoluble protein complexes, have been isolated as content in exosomes [45]. Transfer of the amyloid complexes via exosomes to other neural cells may “seed” protein misaggregation in the recipient cells and, thus, spread disease [46]. This mode of exosome transfer of aggregated protein is also reported for Parkinson’s disease where α-synuclein is found in exosomes and ALS where mutant SOD1 is located in glia-derived exosomes [4]. What remains to be investigated is if the exosomes are simply a transport vehicle for disease spread or if their composition is also contributing to disease. Recent investigative reports do suggest a role for mutant proteins encased by exosomes playing a role in transmitting neurological disease. For example, a paper investigating the progressive nature of ALS, found mutations in the FUS (Fused in Sarcoma) gene in both sporadic and familial ALS [47]. Protein pull-down analyses identified the proteins associated with the normal and mutant FUS gene product. The authors showed these proteins are active in many cellular pathways including nuclear organization, transcription, RNA transport and stress response, thus, demonstrating a broad role for FUS in cellular activity. FUS protein and many of it’s interacting proteins were found localized to exosomes suggesting that release of these exosomes to adjacent recipient cells is one route of disease spread in these ALS patients.
Frontotemporal lobar degeneration (FTLD) primarily affects patients younger than 65 years where loss of function is in the frontal and anterior temporal brain lobes [48]. Upwards of 50% of patients have a family history and genetic factors are considered the primary cause of FTLD. Granulin mutations (GRN) are associated with FTLD that cause haploinsufficiency or loss of progranulin protein in these patients. Benussi and colleagues [48] cultured fibroblasts from 16 FTLD patients with GRN mutations and appropriate cell controls. They found progranulin associated with exosomes from culture medium in its N-glycosylated form and the quantity of GRN was reduced in exosomes from the FTLD fibroblast cultures. They also show that exosome numbers from FTLD patients, where GRN is deficient, are reduced and contain LAMP1, a lysosomal protein. These findings were not consistent with cell apoptosis or impaired cell viability. Progranulin is secreted as a soluble neurotrophic factor that promotes neurite outgrowth and neuron viability but is also found in exosomes. Thus, loss of progranulin in FTLD may impact development of neurodegeneration by a reduction of exosome extracellular communication.

Exosome release in some neurons is coupled to glutamate receptor activation as Ca$^{2+}$ enters the neuron via N-methyl-D-aspartate (NMDA) receptors [49]. Exosomes released from cultured N2a cells were captured by both neurons and glial cells. In contrast, GFP-TTC (green fluorescent protein coupled to non-toxic tetanus toxin C-fragment) labeled exosomes released by synaptic activation were selectively transferred only to other neurons at their synapses [35]. This work demonstrates a specific signal for release of exosomes from neurons and that these exosomes are selectively accured by other neural cells. This phenomenon apparently occurs by receptor recognition at the recipient cell interface or recipient cell endocytosis via the plasma membrane [39]. Exosome cargo may affect neural cell function in positive ways mediating oxidative stress [49] and altering gene expression patterns in recipient neurons [50].

Exosome shuttle RNAs (esRNA) are found in exosomes and contain small RNAs and miRNA that can enter the extracellular space and affect cellular function in recipient cells [46]. Many observations [45] support the conclusion that exosomes from diseased cells transfer both coding RNA and non-coding miRNA that may alter cell function producing neurotoxicity. Neurons communicate with their support cells (astrocytes and neuroglia) responding to peripheral stimuli. It is not hard to envision extracellular transport of exosomes as a signaling method both for healthy and disease outcomes. Whatever the source of RNAs or protein found in exosomes and the relative ease of capture from urine, CSF and blood, there is increasing interest in using exosome preparations as potential biomarkers of disease [4].

Exosomes may act as mediators of neurological cancers. Glioblastomas have been characterized to release a variety of extracellular vesicles (exosomes and the larger oncosomes) whose composition diverges from cytoplasmic content [51-53]. These “tumor vesicles” are reported to increase a variety of oncogenic processes that promote tumor growth and development, like immune suppression, establishment of tumor micro-niches and angiogenesis [52,54]. Li et al. [55] showed that glioblastoma extracellular vesicles carry both non-coding RNAs and RNAs to recipient cells that alter transcriptional profiles. Extensive analysis of neural tumor cell’s exosome cargo is needed to establish what is being transferred via this route of extracellular communication and how it is abnormally altering the function of the receiving cell. The role of exosomes from tumor cells found in all parts of the body is reviewed in [3,56-58].

Potential therapeutic roles for exosomes

Diagnostic or prognostic biomarkers

Exosomes are suggested as promising carriers of biomarkers where protein cargo profiling may be especially informative [59] and where exosomes could easily be captured from the patient’s blood. Exosomes are accessible from the blood, urine, CSF and other body secretions making collection methods low risk and minimally invasive. They might be particularly useful in capturing information from different tumor types, for prognostic staging of tumor progression and, potentially, for identifying a patient specific drug regimen [60]. The role of exosomes as biomarkers in cancer and its metastasis is reviewed in [61]. Another application using blood exosomes from neonates to assess neurotoxicity is profiled in Gillet et al. [62]. Profiling exosome miRNA to identify biomarker candidates for early Alzheimer’s disease is discussed in Kumar and Reddy [63]. Cheow et al. [64] showed the utility of using exosomes as biomarkers for therapy of myocardial ischemic injury. In a neurodegenerative disease example, mutations in LRRK2 (leucine rich repeat kinase 2) are one of the most frequent causes of familial Parkinson’s disease and suspected in idiopathic Parkinson’s disease. The mutation G2019S in LRRK2 allows phosphorylation of the serine in the now mutant protein and this could act as a potential biomarker of Parkinson’s disease. Fraser et al. [65,66] captured urine exosomes from Parkinson’s patients that contain LRRK2 and showed that the mutant LRRK2 serine was indeed phosphorylated. The level of phosphorylated serine in the exosome was significant when compared to control patients. The phosphorylated serine level also was found to correlate to the degree of cognitive dysfunction seen in idiopathic Parkinson’s patients [66]. The potential of exosomes to be used as biomarker appears limitless but takes extensive validation to arrive at a potent biomarker [67]. As these analyses broaden and become more sophisticated and reliable, exosome biomarkers will enter the biomedical mainstream and likely be approachable as liquid biopsies [68].

Exosome carriers of ex vivo therapy

Exosomes have four characteristics that make them a putative therapy approach for treating both metabolic disease and various forms of cancer. First, they are a naturally encapsulated package that is able to avoid cellular degradation [4]. Exosomes are received at all cellular membranes, carried in the blood or CSF and able to cross the blood-brain barrier making them a good extracellular candidate as a carrier of therapeutics. Exosomes might also be better tolerated by the immune system since they are natural nanocarriers of cellular cargo [69,70]. Lastly, exosome cargo can be substituted for synthetic proteins or drugs that could induce therapeutic reactions. As an example, bone regeneration has focused on stimulating repair but has not investigated the putative cell-cell communication need for stimulation and continuity of repair. Exosomes exhibit both stimulation of repair in osteogenic cells and can carry “messages” between cells [71]. Hence, it appears that exosomes can directly regulate bone repair by stimulating osteoblast function. Studies of the exosomes role in alleviating pathophysiology require much more investigation. It is proposed that exosomes, loaded with specialized cargoes, could also be engineered by adding receptor ligands to target specific cell populations. This approach offers exciting opportunities for treatment of cancer, neurodegenerative or metabolic disease. Reproducible exosome therapies await assessment of delivery to selected tissues and validation of reaching a therapeutic threshold of efficacy. These developments were recently reviewed in [67,72-75].
Conclusions

A better understanding of the molecular mechanisms that regulate formation of ILVs into MVBs, selection of ILVs cargo and trafficking of MVBs to either lysosomes for degradation or to the plasma membrane for release will advance our understanding of diseases where pathogenic proteins, lipids or infectious agents are present in the exosome [76]. Critical to understanding the role exosomes play in health and disease is defining the contents of exosomes and understanding how exosomes from diseased cells differ from healthy cells. Exosomes from diseased cells should be a rich source of identifiable biomarkers for a specific pathology. Due to the nature of the exosome, a bilayer lipid bound vesicle that protects its contents from proteolysis, the exosome itself, in pathogenesis is defining the contents of exosomes and understanding how exosomes for release will advance our understanding of diseases where pathogenic proteins, lipids or infectious agents are present in the exosome [76].

Many avenues of exosome research have progressed over the past 10 years and one anticipates new insights and broader knowledge of the exosomes role in altering a cell’s functionality and the potential use of exosomes as biomarkers for pathology and for treatment of disease.

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