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Review Article

Exosomes: new molecular targets of diseases

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Abstract

Extracellular vesicles (EVs) comprise apoptotic bodies, microvesicles and exosomes, and they perform as key regulators in cell-to-cell communication in normal as well as diseased states. EVs contain natural cargo molecules, such as miRNA, mRNA and proteins, and transfer these functional cargos to neighboring cells or more distant cells through circulation. These functionally active molecules then affect distinct signaling cascades. The message conveyed to the recipient cells is dependent upon the composition of the EV, which is determined by the parent cell and the EV biogenesis. Because of their properties such as increased stability in circulation, biocompatibility, low immunogenicity and toxicity, EVs have drawn attention as attractive delivery systems for therapeutics. This review focuses on the functional use of exosomes in therapy and the potential advantages and challenges in using exosomes for therapeutic purposes.

Keywords: exosomes; microvesicles; noncoding RNAs; biomarker; drug delivery; exosome mimics

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Introduction

Recently, extracellular vesicles (EVs) have earned a significant contribution in understanding and targeting different diseases. Over the last few years, many studies have been performed in the field of microvescicles to study their role in regulation of different pathophysiological conditions, such as cancer[1], immunological disorders[2], and other systematic disorders in different organs. Furthermore, recent research has been performed to understand the impact of stem cell-derived microvesicles in therapeutic intervention and tissue repair[3]. EVs are membrane-bound endocytic vesicles released in an evolutionally conserved manner by cells ranging from organisms such as prokaryotes to higher eukaryotes and plants that are capable of intercellular communication. Due to their capacity to transfer proteins, lipids and nucleic acids, they can influence various physiological and pathological functions of both recipient and parent cells[4].

Recently, several studies have demonstrated that these membrane-bound vesicles serve as biomarkers to identify different diseases and are essential for effectively combating disease. Moreover, due to their ability to carry biological materials to tissues, exosomes have vast application potential in therapy. Nonetheless, many concerns have arisen regarding the therapeutic use of stem cells, such as undesirable proliferation, heterogeneity and immunogenic effects. In this context, stem cell-derived cell-free exosomes are considered a major emerging tool for curing many diseases. Previously, we discussed the major contents and paracrine-mediated functions of exosomes. These exosomes have both beneficial physiological and detrimental pathological roles during their biological processes. Moreover, studies have shown that exosomes are carriers for different micro-RNAs (miRNAs) that are responsible for different cardiac diseases, including hypertension, cardiac hypertrophy, cerebral ischemia (CI) and stroke[5, 6]. This review gives a brief overview of studies that have been reported regarding how exosomes serve as a new molecular target for various diseases, and it discusses the use of exosomes in therapy.

Classification

EVs are small membrane vesicles heterogeneous in size (20 nm to 2 μm) bounded by a phospholipid bilayer and released by all cell types in various biological fluids and extracellular space[7]. They are released through exocytic budding of the plasma membrane in response to cellular activation or apoptosis[8]. These vesicles contain membrane surface protein and lipid components as well as nucleic acids originating from their original cells[4]. EVs can be classified into different subpopulations, including apoptotic bodies (ABs), microvesicles (MVs) and exosomes, each with specific characteristics, including their

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Table 1. Classification of different types of extracellular vesicles.

	Exosomes	Microvesicles	Apoptotic bodies
Size	20–100 nm[10]	50–1000 nm[9]	500–2000 nm[15]
Density (g/mL)	1.13–1.19[10]	1.04–1.07[9]	1.16–1.28[16]
Biogenesis	Endolysosomal pathway, inward luminal budding of membrane, and fusion of multivesicular bodies with cell membrane[22]	Cell surface, outward budding of cell membrane[9]	Cell surface, outward budding of apoptotic cell membrane[123]
Composition	Elevated level of aminophospholipids, and lipid ceramide in the membranes compared to the outer leaflet of the plasma membrane and lipid ceramide. Production of ceramide is an essential step in the sorting and generation of exosomes[33]	Lipid composition is similar to that of cell membrane, but lacks the asymmetric distribution of lipids. Aminophospholipids, phosphatidylserine and -ethanolamine, are not sequestered to the inner leaflet of the membrane, but distributed homogenously across the bilayer membrane[123]	Externalization of phosphatidylserine but distributed on the cell surface, presence of Annexin I and calreticulin, may also include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5[124]
Contents	mRNA, miRNA, other non-coding RNA, cytoplasmic and membrane protein, major histocompatibility complex (MHC)[125]	mRNA, miRNA, other non-coding RNA, cytoplasmic protein and membrane protein, receptor proteins[126]	Nuclear fractions, DNA, cell organelles[127]
Biomarkers	Tetraspanins family (such as TSPAN29 and TSPAN30,CD81, CD82, CD9, CD63), ESCRT proteins (alix, TSG101), actin, flotillin, Hsc70, Hsp 90, Hsp60 and Hsp20 clathrin, integrins (such as α3, α4, β1, β2)[35]	Integrins, selectins, flotillin-2, CD40 ligand, metalloproteinase[128]	Annexin V positivity, phosphatidyl serine[129]

biogenesis, size, cellular origin, protein composition, mRNA and miRNA content, and/or biological function (Table 1)[9].

Exosomes

The meaning of the term exosomes has changed over time in efforts to better understand their endocytic origin and distinguish them from other microvesicles. Exosomes are currently defined as heterogeneous molecules with a diameter of less than 100 nm and a density of 1.10–1.18 g/mL. They also reflect the phenotypic state of the parent cells from which they are generated[10]. Exosomes originate from repeated invagination of the lipid bilayer membrane of multivesicular bodies, and at any given point, they may contain all known molecular constituents of a cell, including proteins, RNA, and DNA[11]. The fusion of multivesicular bodies with the plasma membrane facilitates the release of exosomes into the extracellular space.

Microvesicles

Microvesicles, described by Wolf for the first time in 1967, have been studied for the past two decades[12]. Microparticles or microvesicles (MVs) originate directly from the membrane surface through outward budding of the plasma membrane. They are characterized by the presence of phosphatidylserine (PS) in the outer leaflet of the membrane and a size ranging between 100 and 1000 nm in diameter[9]. MVs are heterogeneous populations with irregular shape and varied size and densities between 1.04 and 1.07 g/mL[9, 13]. MVs can be separated from different biological samples by size along with a combination of typical cell-specific markers, which will also help to detect their cellular origin[9]. At present, less information has been found for markers to identify MVs compared with exosomes. However, the most common MV markers used thus far in studies are CD40 ligand, adenosine diphosphate ribosylation factor 6, and several integrins and selectins[14]. MV inner contents are similar to those of exosomes and are mirror those of their parent cells.

Apoptotic bodies

In 1974, Kerr first described apoptotic bodies as vesicle-like structures that formed as a result of cell fragmentation in the process of programmed cell death (apoptosis). Among EVs, apoptotic bodies are the largest vesicles, with a size ranging from approximately 500 to 4000 nm and a density between 1.16–1.28 g/mL[9, 15, 16]. Apoptotic bodies are characterized by the presence of DNA fragments and histones along with proteins as the vesicular cargo. Apoptosis initiates with condensation of the chromatin, followed by plasma membrane blebbing and encompassing of the cellular content into distinct membrane-enclosed vesicles known as apoptotic bodies or apoptosomes. Finally, these bodies are released into the extracellular space. The outer leaflets of the lipid bilayer of apoptotic bodies contain phosphatidylserine, similar to the membrane of exosomes and MVs. These translocated phosphatidylserines can bind to Annexin V, which is recognized by phagocytes to undergo phagocytosis[17]. Oxidation of surface molecules causes membrane alteration that triggers binding of thrombospondin[18] or the complement protein C3b, which is in turn recognized by phagocyte receptors[19-21]. Therefore, Annexin V, thrombospondin, and C3b, serve as accepted markers of apoptotic bodies[21]. Unlike the other two EV types, apoptotic

bodies are only released during programmed cell death.

Biogenesis and composition of exosomes

Biogenesis

Among the three main classes of EVs, this review focuses on exosomes. EVs are cell-derived vesicles, enclosed by a lipid bilayer, with varied diameter depending on their origin. In contrast to microvesicles, which are generated by budding from the plasma membrane, exosomes are derived from the endolysosomal pathway (Figure 1). Microvesicles are closer than exosomes to the parent cells in their membrane composition, but exosomes typically contain some additional defined components[3]. Exosomes are formed intracellularly via endocytic invagination within the endosomal network. Endosomes at their early stage fuse with endocytic vesicles and incorporate their content into vesicles destined for recycling, degradation, or exocytosis. Early endosomes then undergo a sequence of alterations to become late endosomes or multivesicular bodies (MVBs), which are characterized by the presence of multiple small interluminal vesicles (ILVs)[22]. When MVBs undergo maturation, the "cargo" within them is sorted, and they then fuse with lysosomes for lysosomal degradation or fuse with the plasma membrane to be released as exosomes in the extracellular space. During this process, transmembrane proteins are incorporated into the invaginating membrane, maintaining a topological orientation similar to that of the plasma membrane[1].

On the other hand, the biogenesis of microvesicles is distinct from exosome biogenesis. Microvesicle formation is initiated through direct outward budding followed by a fission that resembles the abscission step in cytokinesis. These vesicles, also called ectosomes[23], are usually larger than exosomes, and their sizes overlap. There is an asymmetric distribution of phospholipids within the plasma membrane that is tightly regulated by aminophospholipid translocases such as flippases and floppases[24-26]. Flippases help to transfer phospholipids from the outer leaflet to the inner leaflet. Membrane budding and vesicle formation is induced by translocation of phosphatidylserine to the outer-membrane leaflet. This process is accomplished by contraction of cytoskeletal protein actin–myosin interactions[27].

Composition of EVs

EVs contain signaling proteins, transcriptional regulators, nucleic acids and lipids[28]. The composition is based on the components of the parent cells[28]. For example, EVs derived from lung epithelial cells contain lung surfactant proteins[29]. The presence of these cell-specific proteins can act as markers to identify the origin of EVs. Moreover, the presence of certain biomarkers in body fluids also helps to identify particular disease types and the severity of the prognosis of the disease state. For example, melanoma patients have higher levels of CD63 and CAV1 in plasma exosomes compared with healthy individuals[30]. Furthermore, isolating exosomes from body fluid in order to engineer them using lectins or antibodies against exosome markers such as CD63, CD81, EpCAM or

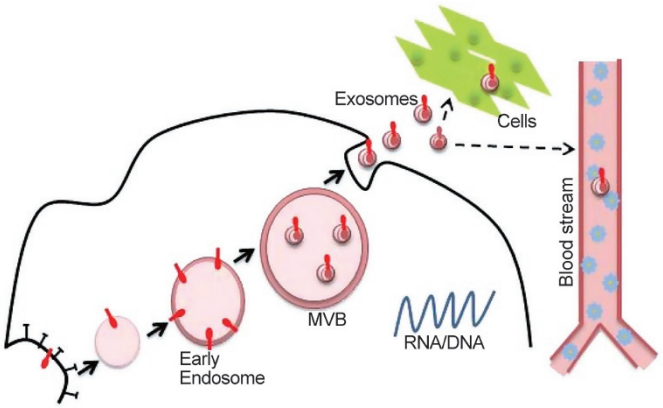

Figure 1. Biogenesis and release of extracellular vesicles: represented diagram depicts a typical extracellular vesicle biogenesis and release.

Rab5 is possible[31]. Aminopeptidase N and nebulin are two abundant membrane-associated proteins found in the MVs of nephrotic urine after ultracentrifugation[32].

Compositions of exosomes

To understand the cellular composition of exosomes, a wide variety of techniques has been used, including trypsin digestion and mass spectrometry, Western blotting, and fluorescence-activated cell sorting (FACS) analyses in various types of cells. These techniques have identified a subset of cellular proteins that specifically target exosomes. Strikingly, a study involving humans and mice found that 80% of proteins from exosomes derived from dendritic cells (DCs) are conserved in both[33]. The protein composition of the exosomes is still mostly unknown. Due to their endosomal origin, all exosomes contain membrane transport and fusion proteins (GTPases, Annexins, and flotillin), tetraspannins (CD9, CD63, CD81, and CD82), heat shock proteins (Hsc70, Hsp 90, Hsp60 and Hsp20)[34, 35], proteins involved in multivesicular body biogenesis (Alix, TSG101), lipid-related proteins and phospholipases (Figure 2). Exosomes released by APCs are rich in antigen presenting proteins such as MHC class I and class II[2]. Exosomes derived from DCs contain CD86, an important co-stimulatory molecule for T-cells. It has been shown that different α- and β-chains of integrins, ICAM1/CD54, A33 antigen and P-selectin, or cell-surface peptidases (CD26 and CD13) are also present in exosomes[33]. Other than the characteristic morphology, the protein and lipid composition of exosomes is unique, providing an additional tool for their identification. Furthermore, exosomes are unique in enrichment of cholesterol, ceramide or other sphingolipids, and phospoglycerides with long and saturated fatty-acyl chains[36]. A recent study has shown that exosomes from mast and dendritic cells have increased levels of phosphatidylethanolamines, which have a higher rate of flipping between the two leaflets of the exosome bilayer than in cellular membranes[37]. There are findings that show that

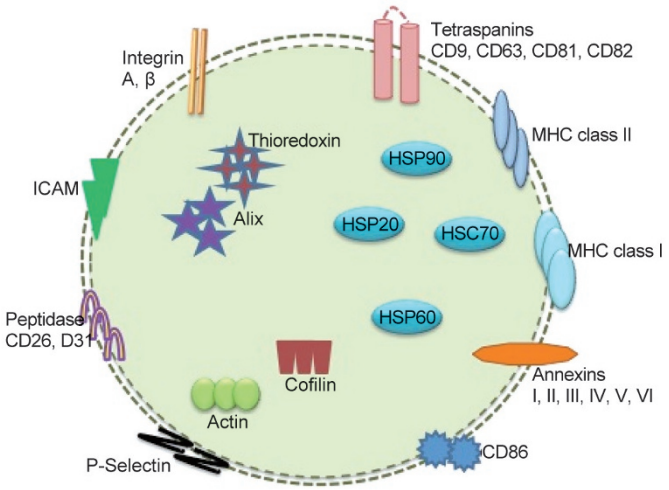

Figure 2. Proposed structure of a mid-size exosome (40–60 nm in diameter). A vesicle contains cytosol of the parent cell and expresses the extracellular domains of different transmembrane proteins reflecting the type of parent cell from which it is derived.

exosomes play a role in delivering prostaglandins to the target cells[37]. The outer surface of exosomes also contains saccharide groups, which are enriched in mannose, polylactosamine, α-2,6 sialic acid, and complex N-linked glycans[38].

Exosomes have been reported to contain significant amounts of miRNA, other non-coding RNAs, and mRNA, which can all be transferred between cells and modulate gene expression in recipient cells[39]. A few studies have indicated that the RNA content in exosomes differs significantly from that of its parental cell[40]. Similarly, circulating EVs are carrier vehicles for a large number of miRNAs involved in cardiovascular disorders. miRNA packaging in EVs is different from the parent cell and is largely influenced by external stimuli. EVs treated with miR-150 mimic increase endothelial cell migration. miR-126 is elevated in various types of EVs and promotes re-endothelialization *in vivo* and therefore serves as an important regulator of angiogenesis and vascular integrity[32]. Another study on atherosclerotic mice injected with miR-143/145-containing EVs showed a reduction in atherosclerotic lesion formation[41]. Furthermore, fibroblast-derived exosomes that carry miR-21 act as a paracrine signaling factor in cardiac hypertrophy[42].

Functions of exosomes

Biological function

There are many studies that have shown that exosomes derived from antigen presenting cells, such as DCs, can express major histocompatibility complex (MHC) class I and II molecules on the cell surface, which helps to induce specific immune responses by activating immune cells, such as $CD8^+$ and $CD4^+$ T-cells[43]. EVs regulate normal biological processes in a pleiotropic fashion, either directly activating cell-surface receptors of neighboring cells or merging into the plasma membrane of neighboring cells and delivering its cargos, including transcription factors, oncogenes, miRNAs, mRNAs and infectious particles[44-46]. The exosomes thus serve as important effector molecules that modulate normal physiological functions of the body, such as stem cell maintenance[45], tissue repair[47], immune surveillance[48] and blood coagulation[49].

EVs either activate or inhibit the function of regulatory T cells; suppress natural killer cells (NKs) and $CD8^+$ cell activity; or activate monocytes, B cells and NK cells[3]. EVs have an intrinsic adjuvant effect, which enables them to become efficient immune modulatory molecules that transfer antigens between APCs. EVs isolated from mast cells contain relatively high HSP60 and HSC70 content that promotes DC maturation in mice[2]. In addition, bacteria-infected macrophages release EVs carrying microbial antigens and pathogen-associated molecular patterns that promote an inflammatory response by macrophages in a TLR-dependent manner[50].

Pathological function

Role of exosomes in cardiac disease

Exosomes are known to be involved in many cardiovascular physiological and pathological disorders, such as cardiomyocyte hypertrophy, peripartum cardiomyopathy and sepsis-induced cardiomyopathy. In response to cardiac stresses, such as myocardial infarction, cardiac valve disease, and systemic hypertension, the heart undergoes extensive cardiac remodeling that results in cardiac fibrosis and pathological growth of cardiomyocytes or hypertrophy[51, 52]. In the hypertrophic heart, cardiac fibroblasts induce and modify cardiomyocyte hypertrophy by secreting different growth factors and extracellular matrix components[53]. Recently, a study by Bang *et al* showed that cardiac fibroblasts secrete exosomes enriched in miRNAs, which are often degraded intracellularly. Approximately 25.5% of fibroblast-derived exosomes contain these "star" miRNAs. miRNA profiling of exosomes revealed that exosomal miR-21* is a potent paracrine-like miRNA molecule that induces cardiomyocyte hypertrophy. This can be mediated by silencing SORBS2 (sarcoplasmic protein sorbin and SH3 domain-containing protein 2) and PDLIM5 (PDZ and LIM domain 5) proteins[52].

Peripartum/postpartum cardiomyopathy (PPCM) is a critical, potentially life-threatening pregnancy-associated cardiomyopathy characterized by sudden heart failure during the last month of pregnancy and/or in the first few months postpartum[51]. Cathepsin D is cleaved from a 16-kDa N-terminal prolactin fragment (16K PRL) in the full-length nursing hormone prolactin (PRL) and is believed to be a potential factor in initiating PPCM[54]. Although the underlying molecular mechanisms are not clear, Halkein *et al* reported that 16K PRL not only induced the expression of miR-146a in endothelial cells (ECs) but also enhanced the release of miR-146a-enriched exosomes from ECs. These EC-derived exosomes were absorbed by cardiomyocytes, resulting in elevation of miR-146a levels. Consequently, the expression of Erbb4, Notch1, and Irak1 was decreased in cardiomyocytes, which ultimately led to impaired metabolic activity and contractile function[51, 55]. Furthermore,

levels of exosomal miR-146a were found to be significantly higher in plasma from patients with acute PPCM than healthy postpartum controls and patients with dilated cardiomyopathy. Therefore, exosomal miR-146a may also serve as a highly specific blood biomarker that is useful for diagnosis of patients with PPCM.

Early during diabetes, high glucose levels in the bloodstream can lead to endothelial dysfunction. This promotes abnormal vascular growth that triggers the progression of atherosclerosis in patients with diabetes mellitus[56]. In a recent report, Wang *et al* showed that in response to hyperglycemia, exosomes derived from cardiomyocytes harbor a variety of miRNAs, which may be transferred to adjacent ECs and modulate their function[57]. Furthermore, their findings revealed that cardiomyocyte-derived exosomes from diabetic type 2 diabetic Goto-Kakizaki GK rats contain higher levels of miR-320 and lower levels of miR-126 and Hsp20 proteins than exosomes collected from healthy cardiomyocytes. Importantly, cardiomyocyte-derived exosomal miR-320 can be transferred to ECs and consequently down-regulates the expression of IGF-1, Hsp20, and Ets-2, leading to inhibition of EC proliferation, migration and tube formation. It has also been shown by Wang *et al* that diabetic cardiomyocytes release exosomes containing lower levels of Hsp20 than exosomes from normal cardiomyocytes, which is responsible for hyperglycemia-induced cell death. Overexpression of Hsp20 significantly reduced cardiac dysfunction, hypertrophy, apoptosis, fibrosis, and microvascular rarefaction in diabetic mice. Overall, this study uncovered a novel mechanism underlying the impairment of myocardial angiogenesis in diabetes, which may be caused by the secretion of anti-angiogenic exosomes from cardiomyocytes[58].

Myocardial dysfunction is one of the main predictors of poor outcome in septic patients, with mortality rates of approximately 70%[59]. Evidence suggests that platelet-derived exosomes might be involved in myocardial dysfunction in sepsis patients[60]. Numerous studies have indicated that the presence of exosomes in the plasma of sepsis patients may cause vascular and cardiac dysfunction in sepsis[51]. Studies have shown that lipopolysaccharide (LPS) triggers increased generation of nitric oxide (NO) and subsequently induces the release of exosomes from platelets during sepsis. These LPS-induced platelet-derived exosomes contain higher levels of NADPH oxidase, nitric oxide synthases (NOS) and protein disulfide isomerase (PDI) than healthy exosomes. Furthermore, it has been shown that NO-induced and human septic platelet-derived exosomes induce caspase-3 activation and apoptosis of target ECs through generation of active ROS/RNS by NADPH oxidase and NO synthase type II[61]. In addition, one report provided evidence that circulating platelet-derived exosomes from septic patients induced myocardial dysfunction in isolated heart and papillary muscle preparations. This effect was further enhanced by *in vivo* pre-exposure of exosomes to LPS[60].

Role of exosomes in Neurodegenerative disease

Neurodegenerative disorders are characterized by the deposition of misfolded, aggregated forms of specific proteins in defined neuroanatomical locations. Recent research has revealed that the majority of proteins involved in neurodegenerative diseases are transported in exosomes. Exosomes containing aggregation-prone proteins involved in Parkinson's disease (PD), Alzheimer's disease (AD), Creutzfeldt-Jakob disease (CJD), and amyotrophic lateral sclerosis (ALS) have all been found in the cerebral spinal fluid and blood of patients affected by these disorders. Moreover, exosomes provide a supportive environment that can induce conformational change of native proteins into aggregates that can be transmitted to aggregate-free cells in the brain[62]. In Parkinson's disease, mutated α-synuclein proteins form intracellular oligomers (known as Lewy's bodies) that can be secreted via exosomes to the extracellular milieu and taken up by nearby cells, thus spreading the disease from cell to cell within the brain[63]. Further investigation on this subject by Danzer *et al* has shown that autophagy acts as a protective mechanism in cells and constitutes a major degradation pathway for α-synuclein oligomers[64]. Any deregulation of neuronal autophagy might promote the aggregation of these proteins and their secretion by exosome release, thereby spreading and causing neurodegeneration[63].

In Alzheimer's disease, *tau* protein aggregates form filamentous intracellular inclusions that can spread from affected nerve and glial cells to healthy cells, thus spreading the disease. Studies have shown that extracellular α-amyloid aggregates can induce *tau* pathology in transgenic mice and finally promote neurodegeneration[65, 66]. Furthermore, exosome-associated *tau* and α-amyloid have been proposed to be present on the exosomal surface, which can act as a platform for β-amyloid aggregation after protein conformational modifications[63, 67]. Prions are abnormally folded proteins with the ability to propagate in the central nervous system, causing fatal neurodegenerative disorders such as Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Scheinker syndrome. Infectious prion proteins (PrPSc) have been identified in exosomes derived from the conditioned media of mammalian neurons[68, 69]. These exosomes were internalized by normal cells and transformed naturally occurring cellular prion proteins (PrPc) into misfolded infectious prion proteins (PrPSc) and thus spread the disease[63]. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of motor neurons. Mutations in superoxide dismutase 1 (SOD1) and TDP-43 are involved in inherited forms of ALS, and both these proteins have exhibited template-directed induction of pathological misfolding. Furthermore, these proteins have been found to be associated with exosomes, suggesting a potential role of extracellular vesicles in intercellular transfer of misfolded SOD1 and TDP43[62, 70].

Role of exosomes in cancer

Direct interaction between tumor cells and their environment is absolutely necessary for cancer progression. Growing evidence indicates that exosomes from cancer cells transfer oncogenic proteins and nucleic acids that modulate the activity of recipient cells and play decisive roles in tumorigenesis, cell proliferation, progression, metastasis, and drug resistance.

Prostate cancer cell-derived exosomes containing oncogenic proteins (Ras superfamily GTPases), mRNA (H-ras and K-ras), and miRNAs (miR-125b, miR-130b, and miR-155) can induce neoplastic transformation of adipose-derived stem cells (ASCs)[71, 72]. Tumor-derived exosomes can also activate endothelial cells to support tumor angiogenesis and thrombosis. Exosomes derived from hypoxic glioblastoma cells are potent inducers of angiogenesis[71]. K562 leukemia cell-derived exosomal miR-92a targets integrin α5 to enhance endothelial cell migration and tube formation[73]. Hypoxic K562 cells released miR-210-enriched exosomes that promote the angiogenic activity of endothelial cells[74]. Exosomes also contribute to creating an immunosuppressive microenvironment by inducing apoptosis and impairing the function of effector T cells and natural killer cells (NKs), inhibiting dendritic cell (DC) differentiation, expanding myeloid-derived suppressor cells (MDSCs), and promoting regulatory T cell (Treg) activity. Exosomes derived from tumors can also convert fibroblasts and mesenchymal stromal cells into myofibroblasts to facilitate tumor angiogenesis and metastasis. Tumor-derived exosomes can mobilize neutrophils and skew M2 polarization of macrophages to promote tumor progression. Moreover, tumor-derived exosomes can help tumor cells develop drug resistance by transferring multidrug-resistant proteins and miRNAs, exporting anti-cancer drugs, and neutralizing antibody-based drugs. In turn, exosomes from activated T cells, macrophages, and stromal cells can promote tumor metastasis and drug resistance[71].

Role of exosomes in liver inflammation

Studies have shown that exosomes are also involved in liver inflammation. It has been revealed that repeated injection of exosomes isolated from peripheral blood of mice fed a high-fat diet to mice fed a regular diet resulted in activated immature $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ myeloid cell accumulation in the liver that caused chronic inflammation and promoted obesity-related disorders, such as fatty liver disease[75, 76]. These studies clearly indicate the pathological role of exosomes in metabolic liver disease. Electron microscope images showed that exosome number is increased in the cells after treatment with LPS compared with the normal unstimulated cells (Figure 3). This suggests that exosomes may make a significant contribution during inflammation-based modulation of cells.

Different strategies to attenuate adverse functions of exosomes during disease

After understanding extracellular vesicle-mediated disease pathogenesis, it is important to understand the ways that they could be inhibited. Several approaches could be used to attenuate extracellular vesicle function and inhibit the development of diseases. These include their formation, release, cell uptake or targeting extracellular vesicle components that are responsible for disease pathogenesis.

Targeting formation

Different cellular components are recognized to be critical for extracellular vesicle formation. The best approach to inhibit their formation is inhibiting cellular components that aid in formation of exosomes by small molecules or RNAi. Therefore, using inhibitors of sphingomyelinase or amiloride, a blood-pressure-lowering drug, can inhibit ceramide formation and thereby vesicle formation[3]. The use of amiloride was also found to be effective in reducing mouse and human tumor cell growth by blocking EVs harboring membrane-associated heat shock protein 72 (HSP72)[77]. Inhibiting the interaction of syndecan proteoglycans and their cytoplasmic adaptor syntenin with the exosomal protein ALIX could attenuate exosome release. Blocking specific tetraspanins, *eg*, TSPAN30/CD63, which are important for extracellular vesicle formation, could be another alternative approach to inhibit EVs by reducing the formation tumors[3].

Targeting release

Many proteins have been found to be important for the release of EVs, including exosomes, but the detailed mechanism of vesicle release remains unclear and is likely to vary among cell types. One approach is to inhibit their release into the extracellular space so that their effect on other cells is prevented. For example, EV formation and release into the extracellular space relies on the endosomal sorting complex required for transport (ESCRT) and/or ceramide and sphingosine 1-phosphate. Targeting these complexes with small molecules or RNAi inhibits EV release into the extracellular space. The GTPase RAB27A73, another molecule involved in exosome release in some tumor cells, could also act as a therapeutic target for reducing tumor growth[78]. Furthermore, using GW4869 and specific small interfering RNA blocks the biosynthesis of ceramide and thereby inhibits exosome release from the affected cells to the neighboring non-transformed cells. This could be another approach to control intracellular communications mediated by exosomes. This approach has been shown to be effective for reducing sepsis-induced inflammatory responses and improving cardiac dysfunction, as well as lowering the presence of circulating miRNAs in blood cancer patients[79, 80].

Targeting uptake

Inhibiting extracellular vesicle uptake would be another approach to inhibit disease-causing EVs. There are many mechanisms for uptake of extracellular vesicles, but the detailed mechanisms in extracellular vesicle trafficking and targeting are not as well understood. Phosphatidylserine (PS) is an important surface molecule, and therefore, blocking PS with diannexin can reduce adhesion of extracellular vesicles released from tumor cells to non-tumor cells[81, 82]. Although this strategy had some effect on reducing tumors in a mouse model, there are also some disadvantages[3]. EV uptake is also mediated by clathrin-dependent endocytosis and caveolin-mediated (clathrin-independent) uptake, macropinocytosis, phagocytosis, and lipid raft-mediated internalization. It is also important to further look into these cellular processes to inhibit the uptake of EVs and thereby minimize disease progression[83].

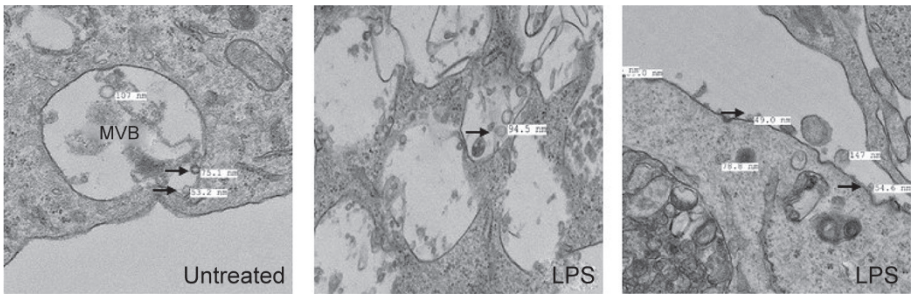

Figure 3. Electron micrographs of mouse bone marrow derived primary macrophage cells treated with or without lipopolysaccharide (LPS). (A) Exosomes in the untreated cells; (B) exosomes after treatment with LPS; (C) exosomes secreted outside the cells; MVB, multivesicular body; Bars: 100 nm.

Targeting trafficking

Blocking specific signaling molecules responsible for the trafficking of EVs might be another approach to inhibit their activity. For example, in a melanoma model, it has been shown that monoclonal antibodies for FASL could target FASL1 present on the cell surface of EVs and thus reduce tumor development. However, one of the great limitations of this process is the specificity, which affects the immune system globally[3, 84]. Recent studies have shown that there are specific cofactors for nuclear exosomes that aid in exosome RNA synthesis and stability. For example, nucleoplasm-specific Nuclear EXosome Targeting (NEXT) complex constitutes a non-nucleolar hMTR4-containing complex that targets PROMoter uPstream Transcripts (PROMPTs) for rapid exosomal turnover in humans[85]. Therefore, targeting these complexes could be an approach to inhibit overall exosome turnover.

Potential role of exosomes and microvesicles in therapy

The natural cargo carrying property of EVs makes them a promising therapeutic agent for different disease states, including cancer and degenerative diseases.

Stem cell-derived exosomes and microvesicles have a protective effect

For a long time, mesenchymal stem cells (MSCs) have been used for preclinical and clinical studies, including acute myocardial infarction, stroke, acute kidney failure, and many others (http://clinicaltrials.gov). Recent studies have found that lung-trapped MSCs secrete an anti-inflammatory protein, TSG-6, which plays a beneficial role in myocardial infarction[86]. Another study that explored the use of MSC-derived exosomes after myocardial ischemia in mice observed a reduction in infarct size[87]. Taken together, these studies suggest that exosomes play an important role in regenerative therapies[88]. In a report on middle cerebral artery occlusions in a stroke model, mice were treated with MSC-derived exosomes, which were known to contribute to increased neurite branch numbers as well as total neurite length after stroke[89]. It has been shown that human umbilical cord MSC-derived exosomes promote proliferation and inhibit apoptosis of skin cells in rat burn models. Moreover, the wounds treated with MSC-derived exosomes exhibited accelerated re-epithelialization with increased expression of CK19, PCNA, and collagen I *in vivo*[90]. MSC-derived exosomes can potentially play regenerative roles in tendinopathy by developing exosomes as a non-surgical and non-cellular treatment option for tendon repair (Tetta C 2012). Recent studies have shown that cardiac cell-derived exosomes mediate protective cardiac functions in a murine model of myocardial infarction[91, 92]. Exosomes derived from MSCs are currently being used for fracture healing where they might exert an important regulatory function in osteogenic differentiation of bone marrow stem cells. These effects are mediated by upregulation of miRNAs, such as miR-21, miR-125b and miR-4454[93]. Another study has shown that mean pulmonary artery pressure (mPAP) and mean right ventricle pressure (mRVP) were reduced significantly in pulmonary arterial hypertensive rats who received intravenous injection of MSC-derived MVs or MSCs[94]. More recently, it has been shown that in diabetes the circulating MV and endothelial progenitor cell-derived MV levels are lower due to a decreased level of miR-126, and therefore, this could be used as a potential target to treat patients with diabetic vascular complications[95].

Pharmacological delivery systems for molecules of interest

Exosomes can serve as an efficient carrier system to deliver different pharmacological molecules to target cells or tissues. These molecules can be further modified and reinserted into exosomes for different therapeutic applications (Figure 4).

Advantages of exosomes as carrier vehicles

Liposomes and polymeric nanoparticles are two exciting drug delivery platforms that hold great promise. However, the major concern with liposome-mediated delivery is the stability, toxicity and ability to evade the host immune system[96]. Conversely, polymeric nanoparticles have better stability, but their toxicity and rapid clearance by the immune system is a concern[97, 98]. In this respect, exosomes or exosome mimetics are more advantageous for an ideal drug delivery system with more of the desirable features. Exosomes exhibit an increased circulating half-life that enables them to travel long distances within the body under both physiological and pathological conditions[99]. Additionally, exosomes have more biocompatibility, which makes them suitable to host soluble drugs at

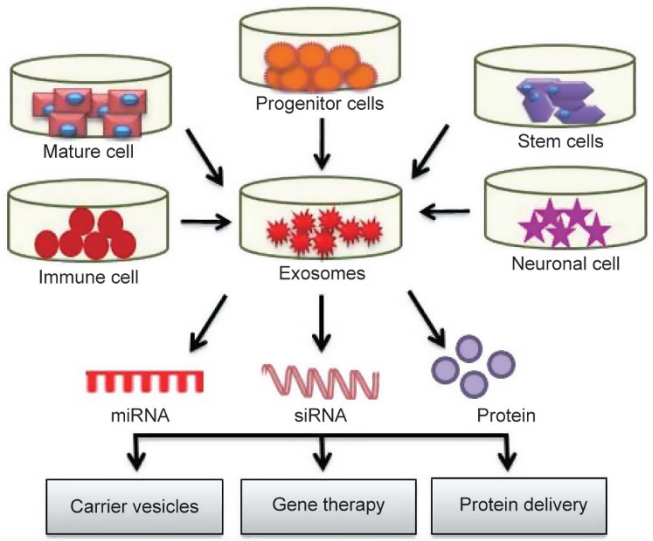

Figure 4. Exosomes play roles in drug delivery: exosomes isolated from different cell types are rich in miRNA, RNA and protein. These molecules can further modified and reinserted into the exosomes for different therapeutic applications.

room temperature (RT). Drugs such as the low molecular weight antioxidant curcumin[100], anticancer agents such as Doxorubicin (Dox)[101] and Paclitaxel (PTX)[102], and Rhodamine-123[102] can be incorporated within exosomes. However, the fact that exosomes are already packed with intrinsic natural molecules presents a problem, and thus, it is often hard to load additional molecules into them[103]. Because they are such small molecules and they carry cell surface molecules, exosomes have a high affinity for tissues[104-106], and they possess a natural targeting capacity and thus have less off-target effects[99], which makes them a better option for drug-delivery systems. Moreover, exosomes have been shown to cross the blood-brain barrier and selectively be taken up by brain microglial cells, and therefore, they can be used in the treatment of diseases of the central nervous system[107]. Furthermore, the immunogenicity of exosomes is very low compared to liposomes and virus-based drug delivery systems[106].

Manipulation of exosomes is relatively simple and can be performed by isolating them from cell cultures or a patient's body fluids, serum, and plasma, and after modification, they can be subsequently transferred back to the same patient[105]. In addition, exosome mimetics can be created synthetically by liposomes harboring only essential components of natural exosomes[99]. Phase I clinical trials have already been performed with dendritic cell-derived exosomes as cell-free cancer vaccines[108]. In other clinical trial for patients with stage III/IV metastatic melanoma, dendritic cells (DC) were collected and pulsed with MAGE 3 peptides to be used by MHC for antigen presentation. Next, autologous EVs were reintroduced into patients to evoke an immune response against melanoma. Mostly, patients tolerated EV administration for up to 21 months with mild inflammatory responses[109]. Another trial for patients with non-small cell lung cancer was performed to assess the safety, feasibility, and efficacy of DC-derived exosomes incubated with the MAGE tumor antigens weekly for 4 weeks, which manifested low-level inflammatory responses[110]. These results encouraged testing of DC-derived EVs for non-small cell lung cancer treatment in a Phase II clinical trial (http://clinicaltrials.gov/show/NCT01159288). EVs are also being tested as a curcumin delivery agent for colon cancer patients (http://clinicaltrials.gov/show/NCT01294072).

Extracellular vesicles as gene therapy devices

Accumulating evidence has revealed that small interfering RNAs can be utilized as therapeutic agents, particularly for cancer and viral infections[111]. However, due to the lack of appropriate delivery systems, their clinical application is still circumscribed. Certain intrinsic properties of exosomes, such as their small size and natural ability to carry miRNA, their cellular membranes with multiple adhesive proteins on the surface and their ability to cross the blood–brain barrier, make exosomes an excellent gene delivery system for gene therapy[104, 112, 113].

Ohno *et al* modified human embryonic kidney cell line 293 (HEK293) cells with forced expression of GE11 or EGF using a pDisplay expression vector. They then isolated exosomes from culture supernatants that carried GE11 or EGF on their surface. Finally, they injected these modified HEK293 cell-derived exosomes to EGFR-expressing cancer tissues[114]. Akao *et al* successfully entrapped chemically modified miR-143BP in microvesciles and transfected THP-1 macrophages with the same RNA molecules, followed by collection of exosomes by ultracentrifugation. The exosomes from these modified THP-1 macrophages were shown to effectively target tumors and the kidneys and treat cancer and other diseases[115].

Exogenous siRNA could also be loaded into exosomes by electroporation. In a model of Alzheimer's disease, Erviti *et al* loaded GAPDH siRNA into DC-derived exosomes to specifically deliver them to neurons, microglia, and oligodendrocytes in the brain. They also showed that exosome-mediated delivery of siRNA against the BACE1 gene resulted in strong mRNA (60%) and protein (62%) knockdown of BACE1 in wild-type mice[104]. This group thus demonstrated that exosome-mediated siRNA delivery is a promising therapeutic agent against neurodegenerative diseases.

Protein cargo

The size and structural proximity of exosomes with cellular components make them easy to use as a specific drug delivery medium in which the drugs are usually protein molecules. A study has shown that exosomes can cross the blood-brain barrier and improve PD status when loaded with the antioxidant protein catalase *ex vivo*, which makes the use of exosomes a promising option for PD therapy[116]. CD44 expression is high in tumorigenic and metastatic hepatocellular cancer stem cells (CSCs). Therefore, anti-CD44 antibody-coated liposomes that can deliver doxorubicin directly to these cells would have

great potential for targeted therapy in cancer[117]. Recently, work by Meyer *et al* has shown that vesicular stomatitis virus glycoprotein (VSVG) can both load protein cargo onto exosomes and increase their intracellular therapeutic protein delivery ability via exosome-based vehicles and thus shows promise for exosome-based delivery[118].

Challenges and future direction

Using exosomes as drug delivery vehicles has several advantages over nano-based or liposome-based therapies. Particularly, exosomes can avoid phagocytosis or degradation by macrophages and can also circulate for prolonged times; however, there are a number of limitations and challenges for translation into clinical therapies. A key problem prohibiting exploring exosomes in clinical applications is the availability of pure exosomes from the body[119]. Because of the low number of exosomes, purifying them is also difficult[120]. Each of the available methods of exosome isolation from cell culture supernatants or different biological fluids, including milk, urine, plasma, amniotic fluid, saliva, and cerebrospinal fluid[121] have different advantages and disadvantages. It is important to determine the best source from which more pure, well-characterized exosomes with high quality can be obtained[122]. Characterization of exosomes using different available techniques such as electron microscopy, FACS, and Western blot analyses have limitations and therefore cannot be used as independent methods to characterize the biophysical and biochemical properties of exosomes. There should be definite and reproducible techniques to characterize MVs clinically and use them as attractive biomarkers for different pathological conditions[44].

There are controversies regarding how to define EV dosage, number of vesicle particles, the amount of vesicle protein, or expressing dosage as a vesicle number to protein ratio. This optimization is important to define EV dosage for clinical trials[28]. Next, enhancing efficient loading of various cargos and targeting proficiencies of exosomes without altering the structure and content of exosomal membranes is important for increasing the use of exosomes while maintaining their functional efficacy. It has also been found that combining exosomes with different therapeutic cargoes often makes them immunogenic based on the nature of parental donor cells, and therefore, further studies are needed to delineate immunogenic reactions after administration. Ongoing research seeks to find ways of developing and standardizing appropriate methods to modify exosome contents in a loading process. Further exploration of the structure and function of exosomes will contribute to the clinical application of exosomes.

Conclusion

Exosomes have emerged as an important drug delivery vehicle because they can be loaded with a variety of cargo molecules that can transfer bioactive proteins, lipids, and nucleic acids. Due to their size and inherent similarity to the parent cell, they can be exploited for therapy in different clinical perspectives. The paracrine effect of exosomes and prolonged retention in the circulation make them increasingly important. Therefore, exosomes act as naturally occurring nanoparticles, and efforts will be made to better understand their properties and function.

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Abbreviations

EV, extracellular vesicles; AB, apoptotic bodies; MV, microvesicles; PS, phosphatidylserine; ILV, interluminal vesicles; MT1, membrane type 1; VAMP3, vesicle-associated membrane protein 3; MHC, major histocompatibility complex; NK, natural killer cells; PPCM, peripartum/postpartum cardiomyopathy; PRL, prolactin; EC, endothelial cell; PD, Parkinson's disease; AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; ALS, amyotrophic lateral sclerosis; PrPc, prion proteins; SOD1, superoxide dismutase 1; ASC, adipose-derived stem cell; HSP72, heat shock protein 72; ESCRT, endosomal sorting complex required for transport; ICAM1, intercellular adhesion molecule 1; CSC, cancer stem cell; VSVG, vesicular stomatitis virus glycoprotein.

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