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Therapeutic Uses of Exosomes

Review Article

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Abstract

Exosomes are membrane vesicles with a diameter of 40-100 nm that are secreted by many cell types into the extracellular milieu. Exosomes are found in cell culture supernatants and in different biological fluids and are known to be secreted by most cell types under normal and pathological conditions. Considerable research is focusing on the exploitation of exosomes in biological fluids for biomarkers in the diagnosis of disease. More recently, exosomes are being exploited for their therapeutic potential. Exosomes derived from dendritic cells, tumor cells, and malignant effusions demonstrate immunomodulatory functions and are able to present antigens to T-cells and stimulate antigen-specific T-cell responses. Exosomes have also been examined for their therapeutic potential in the treatment of infections such as toxoplasmosis, diphtheria, tuberculosis and atypical severe acute respiratory syndrome as well as autoimmune diseases. Attempts to find practical applications for exosomes continue to expand with the role of exosomes as a drug delivery system for the treatment of autoimmune/inflammatory diseases and cancers.

Keywords Exosomes, Therapy, Delivery

1. Introduction

Exosomes are membrane vesicles with a diameter of 40-100 nm, a sub-fraction of extracellular vesicles that are secreted by many cell types into the extracellular milieu [1, 2]. They are equivalent to cytoplasm enclosed in a lipid bilayer with the external domains of transmembrane proteins exposed to the extracellular environment. Exosomes form in a particular population of endosomes, called multivesicular bodies (MVBs), by inward budding into the lumen of the compartment. Upon fusion of MVBs with the plasma membrane, these internal vesicles are secreted. While the biological function of exosomes is still under investigation, they can mediate communication between cells, provide a protective effect against or induce intra-and extracellular stress and are involved in the exchange of functional genetic information [1-3].

Exosomes are found in cell culture supernatants and in different biological fluids and are known to be secreted by most cell types under normal and pathological conditions. So far, exosomes have been found to be

released by all cells examined today such as B-cells, dendritic cells (DCs), T-cells, mast cells, epithelial cells, and platelets and have been found to be present in physiological fluids, such as bronchoalveolar lavage (BAL) fluid, serum, urine, breast milk, cerebrospinal fluid, saliva, and malignant effusions [4-14]. The presence of exosomes in biological fluids could be exploited for biomarkers in the diagnosis of disease [12, 13, 15-20].

The protein composition of exosomes has been characterized using immunoblotting [21], peptide mass spectroscopy mapping [22], and affinity extraction into magnetic beads, followed by phenotyping by flow cytometry [23]. Exosomes derived from dendritic cells [22, 24], B lymphocytes [21], intestinal epithelial cells [25] and other cell types [26-33] revealed the presence of common as well as cell type specific proteins. Exosomes from different cellular origins sequester a common set of molecules that are essential for their biogenesis, structure and trafficking – as well as cell-type specific components which, presumably, reflect the biological function of the parent cell. Ubiquitous proteins in exosomes include cytoplasmic proteins, such as tubulin, actin and actin-binding proteins, the heat shock proteins Hsp70 and Hsp90, and trimeric G proteins, as well as membrane proteins, such as members of the tetraspanin family (CD9, CD63, CD81, CD82) [37], which have been suggested to be involved in cell adhesion, activation, proliferation and antigen presentation. In addition to the conserved set of proteins, exosome functionality seems to be determined by cell-type specific proteins that reflect the specialized function of the original cells. For example, exosomes originated from dendritic cells are enriched with major histocompatibility complex (MHC) class I and II and express co-stimulatory molecules like CD54, CD80 and CD86 (also known as ICAM-1, B7-1 and B7-2, respectively) suggesting a T-cell stimulatory capacity [15, 22, 24, 32-34]. Exosomes from synovial fluid contain citrullinated proteins (eg. fibrin α-chain fragment, fibrin β-chain, fibrinogen D fragment and Spα receptor) which might play an important role in converting nonimmunogenic proteins into autoimmunogenic proteins [35]. Exosomes collected in the urine contain aquaporin-2 which might be used as a biomarker for renal diseases [36].

Lipids found on exosomes are characteristic of the cell origin, with most of the lipid analytical work being performed on exosomes derived from cancer cells, reticulocytes, mast cells, B lymphocyte cell lines and human DCs [37-40]. The typical lipid composition of mast cell-derived exosomes includes lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, cholesterol and diglyceride [40]. Although most of these lipids are also present on exosomes isolated from other cell types, the ratios of these lipids vary. For example, the ratio of cholesterol/phospholipid is lower in exosomes derived from mast cells and reticulocytes when compared with B-cell-derived exosomes [39].

2. Isolation techniques

Exosomes represent only a small fraction of all components present in a culture medium, cytosol or body fluids. Recognizing the fact that the exosomes have a size that range between 40 to 100 nm, with density ranging between 1.13 and 1.21 g/mL, and contain cell type specific proteins, isolation procedures have focused on techniques based on size and density and biochemical properties. Usually, exosomes have been isolated by serial centrifugation of culture supernatant and body fluids to eliminate cells and debris which consists of multiple steps: first, a low speed spin (300 x g for 10 minutes) which eliminates dead cells and bulky apoptotic debris, followed by higher speed spins, which varies among different protocols, from 1000 x g to 20,000 x g and eliminates larger vesicles and debris. A final high speed spin at 60,000-100,000 x g for a period of more than 1 hour precipitates a pellet, which consists of extracellular vesicles including exosomes. Some protocols integrate the usage of filtration steps (like 0.8 μM and 0.22 μM filters) and spinning to eliminate cell debris and other contaminants. However, ultracentrifugation results in the formation of a pellet that could aggregate exosomes with other vesicles, particles, apoptotic bodies or other cell debris and interfere with purification. Moreover, applying excessive centrifugation force and time may lead to rupturing the exosomes. Also, taking advantage the density properties of exosomes, they can be purified from protein aggregates, apoptotic bodies and nucleosomal fragments by floatation into a sucrose density gradient. This procedure can eliminate impurity with composition different from that of exosomes. However, with these procedures, we can only obtain exosomes heterogeneous with microvesicles, because current methods could not distinguish a 50-100nm "exosome" from a 50-200nm "microvesicle". These processes result in variable recovery of the starting amount of exosomes [13, 41-44]. Although this branch of science is growing so fast, the quality and purity of these methods for exosomes preparation do not fulfill the common good manufacturing practice (GMP) criteria. Because exosomes prepared in this way are easily contaminated with media proteins and contain only 5-25% of starting concentration. Instead of differential centrifugation, a newer method for purifying clinical grade (cGMP) exosomes derived from antigen presenting cells employs ultrafiltration cartridges and pumps and is especially useful for purifying exosomes from large volumes (>1 liter) of conditioned medium. More specifically, the ultrafiltration process were incorporated through a 500-kDa NM WCO hollow fiber cartridge that allowed the passage of unaggregated media proteins through the pores of the membrane, while retaining aggregate proteins in the retentate without significant changes on the composition and performance of the media [44]. As it is believed, the protein aggregates are much more immunogenic than the soluble form because of preferential capture by antigen presenting cells [45]. Thus, the removal of co-purifying

proteins such as human haptoglobin and albumin aggregates prevents their undesirable immune responses to serum components [45-48]. Furthermore, during previous co-purifying with exosomes, these proteins can reach a higher concentration in the final product making it an essential aspect for the purification of cGMP exosomes.

Another isolation method, which is based on biochemical composition of exosomes utilizes magnetic beads coated with monoclonal antibody specific for a protein known to be present on the exosome membrane. For example, with the use of antibody-coated magnetic beads, using antibodies against tumor-specific proteins, it has been possible to collect HER2-expressing tumor exosomes from the culture supernatant of breast adenocarcinoma cell lines and ascites of an ovarian cancer patient [41, 43, 49].

3. Therapeutic use of Exosomes

In light of the fact that exosomes secreted by neoplastic cells are close copies of the originating cells in terms of their antigenicity, the use of exosomes in cancer immunotherapy holds promise. For example, melanoma-derived exosomes contain the highly immunogenic antigens MelanA/Mart-1 and gp100 and those released by colon carcinoma cells express CEA and HER2. This antigenic content is not only a feature of *in vitro*-released exosomes, but also can be found in microvesicles (or fragments of plasma membrane ranging from 50 nm to 1000 nm shed from almost all cell types) isolated from plasma of cancer patients as well, evidence that demonstrates the tumor origin of these organelles [14, 50]. Exosomes containing tumor antigens have been shown to stimulate $CD4^+$and $CD8^+$T cells and exosomes from *in vitro* cultured antigen presenting cells (APCs) administered *in vivo* can induce T-cell responses resulting in inhibition of tumor growth [51-53]. Also, dendritic cell-derived exosomes pulsed with tumor-derived antigens elicit potent antitumor T-cell responses and tumor regression in experimental animals [14]. However, while translating findings from mouse to human, we should be cautious about the difference between human and mouse immune systems. Despite many features conserved between human and mouse systems, there are substantial differences between them. Although extensive conservation exists when comparing activated immune T-cells, the pro-inflammatory response of mice is distinct from humans. Importantly, canonical Th17 differentiation signature (IL17A, F, IL23R, RORC, BATF, and CCL20) is different in human either because of an inherently higher responsiveness of the Th17 module in human or presence of fast reactive memory T-cells in human cells. In contrast, activation of CD24 or Lag3 seemed exclusive to mouse cells. Moreover, after pre-stimulation in similar conditions, mouse CD4 T cells activated slightly weaker than humans.

Phase I clinical trials in human cancer evaluated the effectiveness of patient-specific exosomes released by dendritic cells and loaded with tumor antigen-derived peptides (Dexosomes [Dex]) for melanoma and non-small cell lung cancer and showed that dexosome immunotherapy was feasible, safe and led to the induction of both innate and adaptive immune responses, disease stabilization and long-term survival for several patients [54, 55]. Also, ascites-derived exosomes derived from colorectal cancer patients were shown to be safe, nontoxic, and tolerable when used as a cancer vaccine, and in combination with GM-CSF can efficiently induce potent carcinoembryonic antigen (CEA)-specific antitumor immunity in advanced colorectal cancer patients [56]. It should be noted however that the potential antitumor effects of tumor-derived exosomes is still unclear as evidenced by the fact that in cancer patients with advanced disease, tumor-derived exosomes do not exert any effective immune-stimulatory or antitumor effects despite the abundant production of tumor-derived exosomes [53]. Tumor-derived exosomes have also been shown to be immunosuppressive with direct administration of tumor-derived exosomes actually resulting in promoted tumor growth [53, 54]. Tumor-derived exosomes were shown to directly suppress the activity of effector T cells or target myeloid cells to modulate their differentiation and function such as in the case where exosomes derived from human melanoma cell lines and colorectal carcinoma cell lines were demonstrated to skew monocyte differentiation into DCs toward the generation of myeloid-derived suppressor cells (MDSCs) and exert TGF-β1 mediated suppressive activity on T cells *in vitro* [53-55]. A better characterization of tumor-derived exosomes and understanding of their effects on cancer pathogenesis are warranted to further improve their use in cancer chemotherapy.

Exosomes are favorable as vaccine candidates in infections such as toxoplasmosis, diphtheria, tuberculosis and atypical severe acute respiratory syndrome. Toxoplasmosis is induced by the obligate intracellular parasite *Toxoplamsa gondii*. It has been reported that transfer of DCs pulsed with *T. gondii* antigens (TAg) to healthy mice induced protection against a virulent oral challenge of *T. gondii* but this approach is limited due to difficulty to obtain high quantity of DCs suitable for vaccination [57-59]. An alternative to DC-based vaccines being investigated is the ability of exosomes, especially those derived from DCs, to induce protective immune responses. Exosomes secreted by SRDC (CD8α+CD4− DC cell line) pulsed *in vitro* with *Toxoplasma gondii*-derived antigens (Exo-TAg) induced protective responses against infection with the parasite in both syngeneic and allogeneic mice. After oral infection, syngeneic CBA/J mice exhibited significantly fewer cysts in their brains and allogeneic C57BL/6 mice survived Immune protection is associated with the induction of humoral and cellular TAg-specific responses[60].

Exosomes have been examined for their therapeutic potential in the treatment of other infectious diseases as well. It has been shown that murine bone marrow-derived DCs (BMDCs) pulsed in vitro with intact diphtheria toxin

(DT)-released exosomes, which upon injection into mice induce immunoglobulin G (IgG)2b and IgG2a responses specific for DT [61]. Infection with M. tuberculosis primes macrophages for the increased release of exosomes and microvesicles bearing M. tuberculosis peptide-MHC-II complexes that may generate antimicrobial T-cell responses [62, 63]. Exosomes as a vaccine has also been explored in infection with the SARS-associated coronavirus (SARS-CoV) known to induce an atypical pulmonary disease with a high lethality rate. Studies by Kuate et al. demonstrated that exosomes containing spike S protein of SARS-CoV induced neutralizing antibody titres and this immune response was further enhanced by priming with the SARS-S exosomal vaccine and then boosting with the currently used adenoviral vector vaccine [64].

Exosomes may be potential candidates as vaccines for allergic diseases. Exosome-like vesicles isolated from the bronchoalveolar lavage fluid of tolerized mice by respiratory exposure to the olive pollen allergen Ole e 1 or found to induce tolerance and protection against allergic sensitization in mice [65]. Serum containing exosomes from OVA-fed experimental animals can induce tolerance to OVA when injected into naïve recipients [66, 67]. Exosomes found in breast milk [68] contain molecules such as MUC-1, MHC class I and II, CD86 and heat-shock proteins (Hsps) and have an immune regulatory role as they inhibit IL-2 and IFN-γ production and induce Treg cells ($FoxP3^+CD4^+CD25^+$cells); however, the biological role of exosomes in milk and their impact on allergy development remains under investigation [15].

Exosomes have also proved useful in treatment of autoimmune diseases in animal models. Kim et al. showed that administration of exosomes derived from DCs-expressing recombinant IL-4 was able to modulate the activity of APC and T cells *in vivo*, partly through a FasL/Fas-dependent mechanism, resulting in effective treatment against collagen-induced arthritis through suppression of the delayed-type hypersensitivity inflammatory response [69]. Also, vaccination of mice with exosomes from IL-10, FasL, and indoleamine 2,3-dioxygenase-modified DC reduced the clinical manifestation of mice with rheumatoid arthritis [70-75]. Exosomes from TGF-β1-modified DCs reduced disease activity and incidence of intestinal bleeding in a murine model of inflammatory bowel disease (IBD)[75, 76].

More recently, exosomes have been seen as an alternative to liposomes in the delivery of therapeutic agents [77-79]. Exosomes are comprised of natural non-synthetic components, and their small size and flexibility enables them to cross major biological membranes, while their bi-lipid structure protects the cargo from degradation, facilitating delivery to its target [80, 81]. In addition, these naturally-occurring secreted membrane vesicles are less toxic, and better tolerated in the body as evidenced by their ubiquitous presence in biological fluids [80]. For example, exosomes have been used to deliver anti-inflammatory agents, such as curcumin, to activated myeloid cells *in vivo*. Immune dysfunction is properly investigated during various tumor growth and progression. $CD8^+$cytotoxic T lympocytes play a substantial role in antigen-specific tumor destruction and $CD4^+$T cells assists CD8+T-cells in this scenario. Tumors frequently target and inhibit T-cell function to evade from immune response. Curcumin has been shown to inhibit the suppressive activity of T-cells via down-regulation of the production of TGF-β and IL-10 in T-cells as well as increasing the ability of effectors T-cell to destroy cancer cells. Curcumin delivered by exosomes is more stable and more highly concentrated in the blood [82]. Exosomes can be used therapeutically to target EGFR-expressing cancerous tissues with nucleic acid drugs [83]. In this situation, targeting can be achieved by engineering the donor cells to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide. Intravenously injected exosomes delivered let-7a miRNA to EGFR-expressing xenograft breast cancer tissue in RAG2(-/-) mice [83].

Exosomes are natural carriers of RNA making them a valuable tool for the delivery of RNA interference (siRNA) and microRNA (miRNA) regulatory molecules in addition to other single-stranded oligonucleotides [84]. It has been demonstrated that exosomes can be used as vehicles for delivering siRNA to suppress the growth of cancer cells [85]. In addition, tumor-suppressive miRNAs delivered via exosomes confer a gene silencing effect on recipient cells, inhibiting cancer proliferation [86]. Dendritic cell (DC)-derived exosomes have been exploited for targeted RNAi delivery to the brain after systemic injection [87]. Similarly, encapsulation of BACE (a therapeutic target in Alzheimer's disease) siRNA in exosomes derived from dendritic cells expressing Lamp2b, an exosomal membrane protein that reduces immunogenicity, fused to the neuron-specific RVG peptide resulted in delivery of BACE siRNA to the brain and decreased gene expression in neurons, microglia and oligodendrocytes in the brain [88].

Exosomes are being considered as a potential therapeutic tool in modulating neovascularization. Activation of neovascularization can lead to healing of wounds and reconstruction of hypoxic injury while hampering neovascularization delays tumor development [89]. Exosomes secreted from human CD34(+) cells have angiogenic activity in isolated endothelial cells and in murine models of vessel growth and have been postulated to represent a significant component of the paracrine effect of progenitor cell transplantation for therapeutic angiogenesis enhancing recovery from ischemic disease or injury [90]. Endothelial-derived exosomes carrying proteins such as Delta-like 4 (a transmembrane ligand for Notch receptors that is expressed in arterial blood vessels and sprouting endothelial cells) and matrix metalloproteinases lead to angiogenesis [66, 67, 89].

In conclusion, investigation in exosome biology has been a relatively new area of research and much work remains to be done to ensure the safe and effective use of exosomes for therapeutic applications. Exosomes appear to be non-cytotoxic and well tolerated. As our understanding of the biology of exosomes intensifies, so will the range of principles for the design of exosomes and exosomal conjugates used in the development of immunotherapeutics, vaccines, and angiogenesis modulators. The role of exosomes as a next generation drug delivery system appears to be advantageous over existing drug delivery systems because of their small size, lack of toxicity and target specificity although loading of exosomes without compromising their biological properties remains a challenge.

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