Arch Immunol Ther Exp, 2005, 53, 329–335
PL ISSN 0004-069X

WWW.AITE–ONLINE.ORG
Review

Received: 2005.01.31
Accepted: 2005.03.24
Published: 2005.08.15

Nanovesicular vaccines: exosomes

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Source of support: self financing

Summary

Exosomes are small membrane vesicles derived from late endosome. They are about 30–100 nm in diameter. The secretion of exosomes is a process in which multivesicular bodies fuse with the cell membrane, and all cells that contain multivesicular endocytic compartments could theoretically secrete exosomes. The surprising biological functions of exosomes are only slowly being unveiled, but it is already clear that they serve to remove obsolete membrane proteins and act as messages of inter-cellular communication. Exosomes derived from tumor or antigen-presenting cells have been extensively investigated. They are released into the extracellular environment and fuse with the membranes of neighboring cells, delivering membrane and cytoplasmic proteins from one cell to another. Exosomes carry immunorelevant structures which play important roles in immune response, such as MHC molecules, costimulatory molecules, heat shock proteins, and naive tumor antigens. Therefore they have been suggested as potential vaccines. Consequently, exosomes have shown considerable anti-tumor effect in several studies and are in phase I clinical trials.

Key words: **exosomes • immunotherapy • biogenesis • tumor • vaccine**

Abbreviations: **APC** – antigen-presenting cell, **CTL** – cytotoxic T lymphocyte, **DC** – dendritic cell, **HSP** – heat shock protein, **MHC** – major histocompatibility complex, **MVB** – multivesicular bodies, **TfR** – transferrin receptor.

Full-text PDF: http://www.aite-online/pdf/vol_53/no_4/7742.pdf

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INTRODUCTION

The secretion of exosomes was first reported in studies of reticulocyte maturation by Johnstone et al. in 1987[31]. They harvested vesicles that were released during the *in vitro* culture of sheep reticulocytes by centrifugation at 100,000×g for 90 min. They observed that these vesicles contained a number of structures of the reticulocyte plasma membrane, which diminished or disappeared upon reticulocyte maturation. They referred to these nanovesicles as exosomes. It has been suggested that exosome secretion is a mechanism to modulate membrane functions by releasing unnecessary membrane proteins during the maturation of reticulocytes. Later, a wide variety of cell types were reported to secrete these nanovesicles, including epithelial cell, mastocytes, fibroblasts, platelets, tumor cells, antigen-presenting cells (APCs), hepatocytes, and enterocytes[6, 12, 22, 46, 55, 70].

Exosome production and secretion is achieved by inward budding of the multivesicular bodies (MVBs), which then fuse with the cell membrane in an exocytic manner[51, 60]. Consequently, all cells that contain MVBs are considered to have the potential to secrete exosomes. Exosomes are commonly purified from cell culture supernatant and are identified by physical criteria and morphology. Exosomes float on sucrose gradients and their density ranges 1.13–1.19 g/ml. As observed by electron microscopy, exosomes present a cup-shaped morphology, with diameters ranging 30–100 nm.

The potential biological functions of exosomes might be much more important than the simple elimination of unnecessary cellular proteins[31, 32, 33]. In fact, evidence gathered from many experimental systems point to a decisive role of exosomes as complex information units which mediate intercellular communication by transferring material among cells[18, 45, 58]. This mode of signaling is thus clearly different from the classical intercellular communication by a mediator, as not only a single receptor-binding molecules is involved, but a complex nanovesicle with many biologically active molecules.

Exosomes secreted by APCs and tumor cells have been intensively investigated and show potent immunostimulatory activities both *in vitro* and *in vivo*. Exosomes secreted by APCs carry major histocompatibility complex (MHC) class I and II molecules, costimulatory molecules, and several adhesion proteins, and therefore have strong immunomodulatory capacities[1]. Exosomes secreted by living tumor cells contain and transfer tumor antigens to dendritic cells (DCs) and are considered a novel source of tumor-rejection antigens for T cell cross-presentation. Interestingly, tumor-derived exosomes need mature DCs to stimulate T cells efficiently[69, 70], although DC-derived exosomes may not[10, 27, 63, 71]. Due to their prominent anti-tumor effects *in vitro* and *in vivo*, human exosomes were successfully studied in phase I clinical trials.

BIOGENESIS OF EXOSOMES

Exosome formation and secretion includes budding at the limiting endosomal membrane, fusion of endocytic compartments with the cell surface, and final exosome secretion. The internal vesicles of MVBs are the precursors of exosomes[23, 68], and much evidence has been gathered over the last years in support of this notion. Fusion of MVBs with the cell surface has been reported in different cell types, including reticulocytes, DCs, T cells, platelets, and mast cells[25, 30, 36, 54, 59]. However, the protein composition of exosomes is limited. When analyzed by FACS or proteomics, all of the exosomal proteins identified so far are derived from the internal vesicles of MVBs, the cytosol, or the plasma membrane[17, 36, 54, 62]. However, the mechanisms of sorting proteins into MVBs and exosomes are still poorly understood[63]. Lemmon and Traub[41] showed that class E vacuolar protein-sorting proteins are required for protein sorting into MVBs in yeast, and lipids also play a central role in this involution process.

One important fact to keep in mind to avoid misunderstanding is that a kind of ribonuclease complex has also been named "exosome"[40, 49]. This "exosome" is a kind of protein complex which has been implicated in a variety of RNA processing and degradation processes. This is a completely different concept from the exosome we describe here.

PRODUCTION OF EXOSOMES

Since exosomes are secreted to the extra-cellular space, they are usually produced from the supernatants of cell-culture medium. The classical process for exosome production involves differential centrifugation to remove dead cells and large debris, with a final exosome pelleting step by centrifugation at around 100,000×g for at least 1 h[15]. The resultant exosomes can be further purified by ultracentrifugation through floatation on linear sucrose gradients, with the equilibrium ranging from 1.13 g/ml (for B cell-derived exosomes) to 1.19 g/ml (for intestinal cell-derived exosomes). Contaminating materials, such as protein aggregates or nucleosomal fragments released by apoptotic cells, can be separated from the exosomes by this further purification step.

The centrifugation process results in a relatively homogeneous population of vesicles. Under electron microscopy these vesicles have a characteristic cup-shaped morphology: a flattened sphere limited by a lipid bi-layer, with a size range of about 30–100 nm in diameter[19, 48]. Some molecules, such as MHC-I and MHC-II, are rich on the surfaces of these vesicles, as demonstrated by immunogold labeling and electron microscopy. Immunoblotting or mass spectrometry peptide mapping also prove the existence of several molecules enriched in exosomes. However, the classical process of exosome preparation is time consuming, complex, and might be contaminated with media and cellular components. Clayton et al.[14] developed a simple yet powerful method for the isolation and analysis of exosomes using antibody-coated magnetic beads and a flow cytometry technique. Magnetic beads are coated with monoclonal antibodies against molecules that are rich in exosomes. Subsequently, the coated magnetic beads are incubated with culture supernatants that are isolated after a series of differential centrifugation, but without the ultracentrifugation step for exosome purification[14]. This method is also suitable for an analysis of exosomal components, but it is not appropriate for large-scale exosome purification for *in vivo* or *in vitro* applications. Based on the unique size and density of exosomes, Lamparski et al.[72] established a rapid exosome purification process that is suitable for obtaining clinical grade exosomes. Ultrafiltration of the clarified supernatant through a 500-kDa membrane and ultracentrifugation into a 30% sucrose/deuterium oxide (98%) cushion (density: 1.210 g/cm^3) reduced the volume and protein concentration approximately 200- and 1000-fold, respectively. The percentage of exosome recovery ranged 40–50%, which is much higher than that of the classical method (5–25%)[54].

COMPOSITION OF EXOSOMES

One of the major components of exosomes is protein. The most common procedure to identify exosomal proteins involves trypsin digestion and MALDI--TOF/Q-TOF mass spectrometry to identify unknown or unexpected cellular proteins, but the presence of known cellular proteins in exosome preparations from various cellular sources can be analyzed by immunoblotting or FACS analysis of exosome-coated beads.

The available proteomic studies define a subset of cellular proteins from exosomes. These studies show that exosomes from different cells have a varied protein composition, but about 80% of proteins are conserved among exosomes derived from different cell types. Further, the protein types indicated that exosomes are only secreted by living cells and are significantly different from microvesicles derived from apoptotic cells.

The functions of most of the identified proteins are unknown, but the conserved proteins appear to be involved in exosome biogenesis and perhaps in some yet unknown common exosome functions. Thery et al.[63] summarized the identified proteins and divided them into several functional group. One of these groups functions in exosome biogenesis and mainly includes cytosolic proteins, such as tubulin, actin, and actin-binding proteins (i.e. cytoskeleton components), as well as annexins and rab proteins (involved in intracellular membrane fusion and transport). Cellular signal transduction molecules (protein kinases, 14-3-3, heterotrimeric G proteins) as well as several metabolic enzymes (peroxydases, pyruvate or lipid kinases, α-enolase) are found in exosomes. Interestingly, some immune response-associated molecules, such as heat shock proteins (HSPs), MHC class I and class II molecules, integrins, several immunoglobulin family members, and even antigen peptides[3, 71], have been found in exosomes. Several members of the tetraspanin family were also found in exosomes. Exosomes from APCs are most abundant in MHC class II molecules and also contain CD86, an important costimulatory molecule for T cells. The MHC ligand on T cells, the T cell receptor, is also specifically enriched on T cell-derived exosomes.

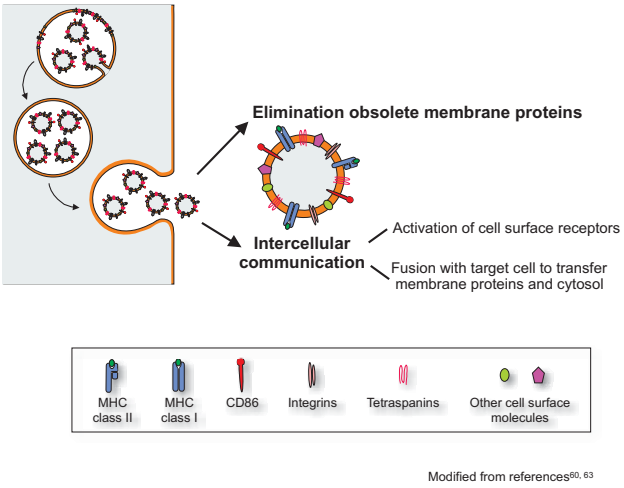
Another major component of exosomes is lipid, but very little data are available on this issue so far. Generally, the exosomal lipid composition is similar to that of the plasma membrane of the producing cell, and until now lyso-bis-phosphatidic acid, phosphatidylserine, and cholesterol have been reported to be present in exosomes[38].

FUNCTIONS OF EXOSOMES

The functions of exosomes depend on the cell types from which they are derived. Their putative functions have been divided into two major classes (Fig. 1). One is to eliminate obsolete proteins during cell maturation and the other is to mediate intercellular communication.

Elimination of obsolete proteins during cell maturation

Exosomes were initially observed during the differentiation of erythrocytes, and their postulated function was to clear redundant plasma membrane proteins, such as the transferrin receptor (TfR) and acetylcholine esterase[24, 33, 50, 51]. Reticulocytes are



Modified from references[60, 63]

Figure 1. Putative exosomes functions. The putative dual roles of exosomes: 1. elimination of obsolete proteins during cell maturation, and 2. regulation of intercellular communication.

immature erythrocytes containing ribosomes and remnants of subcellular compartments, such as mitochondria, the endoplasmic reticulum, the Golgi apparatus, and the endosomal system. Exosomes are known to originate from the inward budding of MVBs, and these internal vesicles are released into the extracellular environment during fusion of MVBs with the plasma membrane[16]. Exosome formation is a major route for the removal of plasma membrane proteins during reticulocyte maturation and differentiation. In the secreted exosomes, the TfR interacts with the heat shock cognated 70-kDa protein (Hsc70), which results in the complete loss of TfR from the erythrocyte's cell surface[21]. Johnstone et al.[33] showed that embryonic chicken reticulocytes lose their transferrin binding activity during maturation in the form of exosomes released into the extracellular medium *in vivo* and *in vitro*.

Mediation of intercellular communication by exchange of membrane proteins and cytosol between cells or cell types

As exosomes are derived from the late endocytic compartment fused to the cell membrane, they could bear complexes of both endosomes and cell membrane. These complexes may be ligands to different cell-surface receptors, which could mediate interaction between two cells without direct cell-to-cell contact. Whether exosomes fuse with target cells or not, they could bind to target cell membranes and transfer membrane material between different cells to mediate biological functions. For example, the presentation of MHC-peptide complexes to T cells is commonly known to take place at the cell surface. However, APCs contain a specialized endosome that harbors newly synthesized MHC-I and MHC-II molecules, and APC-derived exosomes are enriched in MHC class I and MHC class II molecules and play important roles in intercellular communication during the immune response. APC-derived exosomes may interact with T cells directly or indirectly. Exosomes derived from B lymphocytes and DCs bear MHC class I, II, and certain costimulatory molecules, including B7 and B2. B cell-derived exosomes bearing MHC-II molecules could stimulate $CD4^+$ T cells *in vitro*[54]. Exosomes produced by mouse DCs pulsed with tumor peptides stimulated T cell-mediated anti-tumor immune response *in vivo*[71]. Mast cell-derived exosomes incubated with autologous spleen cells induced their activation, which resulted in blast formation, cell proliferation, and interleukin 2 and interferon γ production[59].

Every cell that contains multivesicular endocytic compartments could potentially secrete exosomes. Wolfers et al.[70] analyzed murine mammary adenocarcinoma cell line TS/A and P815 mastocytoma cells. Their results showed that these cells contain numerous MVBs and exosomes could be isolated from the cell culture supernatants. Both human and mouse living tumor cells constitutively release exosomes which express certain membrane markers, such as MHC-I molecules, HSPs, and LAMP1. Interestingly, tumor-derived exosomes may require processing and cross-presentation by DCs to induce an immune response, whereas DC-derived exosomes may not. When added to human DCs, tumor-derived exosomes could transfer tumor antigens, which induces specific human cytotoxic T lymphocyte (CTL) activation *in vitro* and $CD8^+$ T cell-dependent cross-protection against mouse tumors *in vivo*. Exosomes from tumor cells contain native tumor antigens, and this might be how exosomes could establish a novel pathway of cross-presentation. The initiation of T cell-mediated anti-tumor immune responses requires the uptake and processing of tumor antigens by DCs and their presentation on MHC-I molecules. Exosomes have the capacity to transfer tumor antigens from tumor cells to APCs, which mediate $CD8^+$ T cell-dependent anti-tumor effects.

Tumor-derived exosomes are enriched with HSPs, which are known to evoke immune responses by binding tightly to immunogens and transferring them to immune cells[66, 67]. Because HSPs, such as gp96 and Hsc70, are known to bind to the surface of APCs and are internalized by receptor-mediated endocytosis[7], the presence of Hsp70 on the exosomal surface might facilitate uptake of the exosomes by APCs and thereby induce an elevated immune response.

EXOSOMES AS VACCINES

The aim of immunotherapy is to harness effective immune responses to eliminate harmful cells or to protect healthy tissues from immune destruction[26, 34, 35]. The establishment of immunity requires interaction between T cells and APCs, antigen-specific lymphocytes which undergo activation, expansion, and differentiation. Therefore, antigen presentation is a critical step in therapeutic strategies. Several artificial antigen-presenting systems have been developed, encompassing cell-based and acellular technologies[35].

The cell-based systems include mouse fibroblasts[37, 52, 56], human leukemia cell lines[47, 64], and genetically engineered insect cells[8, 28, 29, 61]. The acellular system includes magnetic beads[42, 43], artificial liposomes[53], and exosomes.

Exosomes have the potential to be used as cancer vaccines. As describe above, APC-derived and tumor-derived exosomes play an important role in immune response, especially in anti-tumor immune response[11]. Many studies have been done to investigate the application of exosomes as vaccines, including pre-clinical studies and phase I clinical studies[2]. Exosomes are attractive as safe and stable biomaterials. Their safety profile depends on the cell from which they were derived. DC- or tumor-derived exosomes are currently in clinical trials and no serious adverse reactions have been reported so far[4, 9].

DC vaccination is a new strategy in cancer immunotherapy. Several studies have already shown that isolated DCs loaded with tumor antigens *ex vivo* and administered as a cellular vaccine induce protective and therapeutic anti-tumor immunity in experimental animals[20, 57]. In pilot clinical trials, DC vaccination of patients with non-Hodgkin's lymphoma and melanoma induced anti-tumor immune responses and tumor regression[44, 65]. DC-derived exosomes can also trigger T cell responses without cell-cell contact. They harbor a discrete set of proteins, express high levels of functional MHC class-I-peptide complexes, thereby working as immunogenic vehicles of MHC class I/peptide complexes for the differentiation of T cells, and are stable reagents used safely in pioneering phase I studies[10]. All these preclinical studis prove that exosomes are functional for efficient T cell activation; however, their function requires mature or activated DCs for T cell activation *in vitro* and *in vivo*. They can also carry immunogenic antigens from immature DCs located on the periphery to other DCs, which become sensitized for T cell stimulation[12].

A phase I clinical study using DC-derived exosomes as vaccines has been completed in France. Different dosages of exosomes were given to metastatic tumor-bearing patients in an indirect or a direct loading process[12]. Exosomes showed no toxicity to patients and some patients achieved a regression of subcutaneous sites or exhibited objective response in skin and lymph nodes.

A study involving exosomes derived from different tumor cells showed that exosomes prohibited allogeneic tumor growth, indicating that tumor-derived exosomes may bear tumor antigens and induce antigen-specific immune responses[70]. Moreover, malignant effusions accumulate exosomes bearing tumor antigens and antigen-presenting molecules, and are thus able to generate antitumor T cell response[4]. Cho et al.[13] developed a novel method to generate an antitumor immune response by using exosomes engineered to express a specific tumor antigen. Human MUC1 (hMUC1) as a target tumor antigen was introduced in two mouse cell lines, CT26 and TA3HA. Exosomes derived from these two cell lines contained the target MUC1 antigen and other immune-relevant molecules, such as Hsc70 and MHC class I molecule. These exosomes stimulated the activation of immune cells and suppressed the growth of hMUC1-expressing tumors specifically. These data suggest that tumor-derived exosomes can deliver a target immunogen capable of inducing an effective immune response. Therefore, these studies support the idea that exosomes are a novel source of cell-free therapeutic cancer vaccines.

CONCLUSION

Over the last years, evidence is accumulating that exosomes are a potential source of novel vaccines. Exosomes are composed of ubiquitous proteins, such as HSPs, MHC class I molecules, tetraspanins, and cytoskeletal proteins, but some proteins, such as MHC class II, integrins, and cell-surface peptidases, are cell specific. Notably, APC- and tumor-derived exosomes have a pronounced capacity for immunomodulation, transferring tumor antigens to APCs, and triggering CTL responses. They have been used in phase I clinical trials and initiate and amplify antigen-specific immune responses. Furthermore, these types of selective and specific immunotherapeutic strategies are nontoxic to humans.

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Added in proof:

72. Lamparski H. G., Metha-Damani A., Yao J. Y., Patel S., Hsu D. H., Ruegg C. and Le Pecq J. B. (2002): Production and characterisation of clinical grade exosomes derived from dendritic cells. J. Immunol. Methods, 270, 211–226.