Role of exosomes in immune regulation

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Abstract

Exosomes are small vesicles originating from late endosomes, 30–100 nm in diameter with typical cup-shape morphology. They are reported to bear high levels of a narrow spectrum of molecules involved in immune response and signal transduction. Apart from removing obsolete membrane proteins, some surprising biological functions of exosomes were unveiled recently and their applications in immunotherapy of tumors are currently intensively investigated. Dendritic cell- (DC) and tumor-derived exosomes have considerable anti-tumor effects in experimental studies and several clinical trials. Despite their potential applications in eliciting a "positive" immune response, exosomes might induce some "unwanted" immune responses, such as immune tolerance and immune evasion. Therefore further investigations about the physiological functions of exosomes and the optimal way of exosome application in tumor immunotherapy are necessary. This review presents recent developments in the field of exosome research and focuses on its applications to tumor immunotherapy.

Keywords: exosomes • tumor • immunotherapy

Introduction

The term "exosome" was first used in 1987 to describe vesicles released during the *in vitro* culture of sheep reticulocytes in studies of reticulocyte maturation by Johnstone *et al.* [1]. They suggested that exosome secretion was a mechanism to modulate membrane functions by releasing unnecessary membrane proteins during the maturation of reticulocytes. Now exosomes are defined as small mem-

brane vesicles formed by inward budding of endosomal membranes, which generates multivesicular bodies (MVBs) and are released as exosomes following fusion of MVBs with the plasma membrane.

Exosomes are commonly purified from cell culture supernatants or human malignant effusions [2, 3] and identified by typical physical and morphological characteristics. Exosomes float on sucrose gradients and their density ranges from 1.13–1.19 g/ml. A variety of cells have been shown to release exosomes, including epithelial cells [4, 5], mast cells [6], platelets [7], antigen-presenting cells (APCs) [8] or tumor cells [9].

Western blot and flow cytometric analysis have identified many cellular proteins in exosomes derived from various cellular sources [10]. Further proteins are characterized from exosomes from dendritic cells (DCs) [11], tumors [2, 12], epithelial cells [5, 13] and B cells [14] by proteomic analysis. The results clearly show that exosomes are shed not only plasma membrane domains but also nano-organelles with a specific composition. Three groups of ubiquitous and cell-specific proteins are enriched in DC- and tumor-derived exosomes, namely antigen-presenting molecules, signal transduction proteins and cytoskeletal proteins. In addition to these three groups of proteins, other antigen presentation related molecules are detected as well [9, 11, 15]. Exosomes also have unique lipid compositions. Data from mast cell- and dendritic cell-derived exosomes show that the exosome membrane displays a tight lipid packing and is characterized by a non-asymmetrical distribution of phosphatidylethanolamine (PE) and a rapid flip-flop between the two membrane leaflets [14, 16, 17]. Exosomes contain abundant cholesterol which prevails in their precursor vesicles, and also other lipid compositions, such as sphingomyelin, and GM3 [14, 17]. Given the distinguished composition of proteins and lipids, exosomes are distinct from other types of cellular vesicles.

The biological functions of exosomes largely depend on the cell types from which they are derived, and are much more complex than the once accepted opinion of simple elimination of unnecessary cellular proteins [1, 18, 19]. Their putative functions can be divided into two major categories, one is indeed to eliminate obsolete proteins during cell maturation and the other is to mediate intercellular communication by transferring material among cells [20–22]. In fact, evidences gathered from many research groups have shown that exosomes play a role in modulating immune responses, including immune stimulation and immune suppression. B-lymphocyte-derived exosomes display abundant MHC Class I and II molecules, co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86), adhesion molecule ICAM-1 (CD54), also B cell marker CD20, and have the ability to activate CD4+ T cells in an antigen/MHC class II restricted way [10, 23]. Mast cells may contribute to the regulation of specific immune responses through secreted exosomes that have mitogenic activity on B and T lymphocytes both *in vitro* and *in vivo* [24]. Exosomes secreted by DCs and tumor cells have been most intensively investigated because of their potent immunostimulatory activities. Exosomes derived from CDs expressing MHC class I and CD86 were utilized to generate CD8+ T cell-mediated tumor lysis *in vitro*. DC-derived exosomes also show potent capacity to generate anti-tumor immune responses *in vivo* [8]. Tumor-derived exosomes, which are enriched in tumor antigens, are a novel source of shared tumor rejection antigens for CTL cross-priming [9]. However, tumor-derived exosomes require accessory cells to efficiently generate T cell responses *in vitro*. These accessory cells, like DCs, are supposed to generate appropriate microenvironment for exosome mediated immunomodulation [25, 26]. When pulsed to mature dendritic cells, the tumor-derived exosomes could elicit efficient antitumor T-cell response [27]. Based on their *in vitro* and *in vivo* anti-tumor effects, several phase I clinical trials have been successfully finished [28–30].

Despite the potential applications of exosomes in anti-tumor therapy, recent investigations also show that exosomes could exhibit immune suppressive effects. This review will introduce the biogenesis of exosomes, recent advances in exosomes preparation, developments in proteomic analysis of exosomes and general applications of exosomes to tumor immunology.

Biogenesis and preparation of exosomes

Exosomes are believed to originate from MVBs by inward budding from the limiting endosomal mem-

brane in a process that sequesters particular proteins and lipids. Endosomes are a set of heterogeneous, membrane-enclosed tubes extending from the periphery of the cell to the perinuclear region. Late endosomes are characterized by the presence of internal vesicles and are often referred to as MVBs, where exosomes accumulate and can be released in the extracellular milieu following fusion of the limiting membranes of MVBs with the plasma membrane. The process of exosome release is a reverse budding event, where exosomes contain cytosol inside, exposing the extracellular domain of receptors. The fusion of MVBs with the plasma membrane has been observed by electron microscopy of exosome-producing cells [8, 23, 31, 32]. Moreover, the endosomal origin of exosomes has been proven by the enrichment of certain proteins, which are known to be enriched in the endosomal pathway, in exosomes. Secretion of exosomes has been reported in different cell types [6, 33–35], however, unique proteins have been identified from these different types of exosomes. One recent proteome analysis of melanoma-derived exosomes shows that exosomal proteins are those proteins that are significantly reduced or excluded from whole-cell lysates, or mitochondrial, or lysosomal proteins. These findings further confirm the proposed endosomal origin of exosomes [36].

The classical process of purification of exosomes from cell culture supernatants involves a series of differential centrifugations to remove dead cells and large debris, followed by a final high-speed ultracentrifugation at around 100,000×g for at least 1 h to pellet exosomes [37]. As lipidic vesicles, exosomes float on sucrose gradients with a density ranging from 1.13 to 1.21 g/ml [23].

As DC-derived exosomes can mediate antitumor effects [8], they can be considered as attractive substitute for whole DC cultures. Tumor-derived exosomes are also present in large numbers in malignant effusions [27], and they can be an important source of tumor antigen and MHC class I molecules for immunotherapy. Therefore, exosomes were used in several preclinical animal models and clinical trials. Despite the rapid development of exosome applications, the quality and purity of the classic exosome preparation procedure is unsatisfactory, fortunately good manufacturing practice (GMP)-procedures for both DCs-derived and tumor-derived exosome preparation have been established [3, 38].

Lampaski *et al.* [38] developed a rapid purification process for the preparation of DCs-derived exosomes for clinical studies. Ultrafiltration of the clarified supernatant through a 500-kDa hollow fiber membrane and then ultracentrifugation on a 30% sucrose/deuterium oxide cushion (density: 1.210 g/cm^3) was successfully applied to enrich exosomes approximately 200-fold. The recovery of exosome range 40-50%, which is much higher than that of the classical method (5–25%) [23]. Immunocapture assays of MHC class I and II molecules and flow cytometric assay of exosome membrane proteins demonstrated the high quality of the resulting DC-derived exosomes. Furthermore, preparations of ascites-derived, GMP-grade exosomes have been developed as well [3]. GMP-grade, tumor-derived exosomes were prepared from ascites fluid obtained from ovarian cancer patients. Under sterile conditions, 200–500 ml of malignant ascites were collected and cell debris as well as protein aggregates removed by preliminary centrifugation and filtration steps. Thereafter, the PBS-diluted ascites fluid was ultrafiltrated through a 500-kDa membrane and ultracentrifugated on a 30% sucrose D$_2$O cushion at 100,000×g for 2h. The collected sucrose layer was filtered through a 0.2 µm filter and ultracentrifugated finally. The collected pellet was resuspended in a small volume of PBS and aliquoted for testing. The results of capture plate assay show that approximately 1×10^{11} MHC Class I molecules can be recovered from 1 ml of ascites fluid by using above method (shown in Fig. 1).

Exosomal molecules potentially involved in cancer therapy

The protein composition of exosomes from different sources has been studied by Western blotting [8, 23], flow cytometry of exosome-coated beads [10], and mass spectrometry [2, 6, 12, 39]. The results show that exosomes from different cellular origins share common groups of proteins and are distinct biochemically and morphologically from apoptotic vesicles [39]. Three groups of ubiquitous and cell-specific proteins are enriched in both DC- and tumor-derived exosomes, which are (*i*) antigen- binding and presentation-related proteins such as heat shock proteins(HSPs), MHC class I and II molecules, (*ii*) signal transduction related

Fig. 1 Different methods to prepare exosomes: Exosomes were initially purified from cell culture supernatants, but the classical procedure is tedious and time consuming. Methods suitable for GMP-preparation of exosomes for clinical studies have been developed. Here we show three different procedures and outline their advantages and disadvantages.
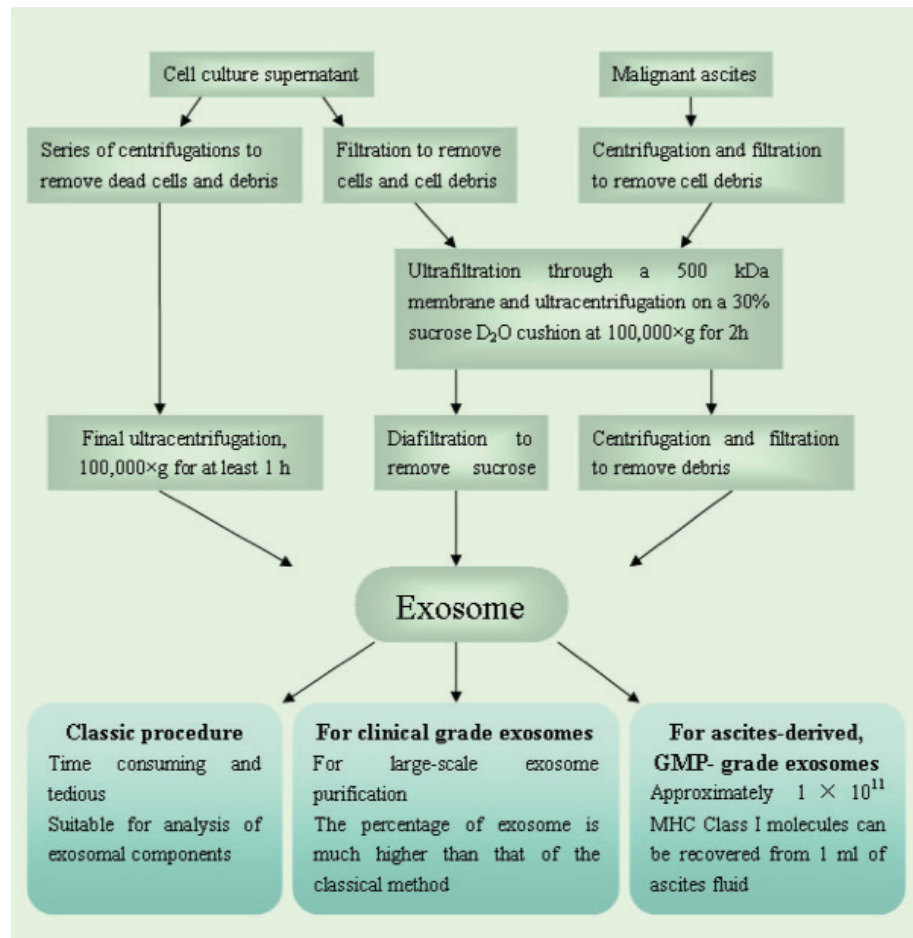
proteins such as annexins, tetraspanins and integrin proteins, (*iii*) cytoskeletal proteins such as actin, ezrin and tubulin. In addition to these three groups of proteins, other antigen presentation related proteins were detected [9, 15], especially in tumor-derived exosomes.

MHC class I and II molecules loaded with tumor peptides are found on exosomes originating from various cells, including dendritic and tumor cells, and these MHC/tumor peptides complexes can be involved in the presentation of polypeptide fragments of antigens to T cells and induction a specific immune response [8, 9]. HSPs are a group of common proteins that play a role in the cell responses to environmental stress, like oxygen deprivation, high temperature, infection, cytokine stimulation or metabolic starvation. Inside cells, HSPs act as chaperones [40, 41]. HSPs are also believed to be involved in the presentation of peptide fragments to the cell surface. Extracellular HSPs are very important to generate immune response to defend against infection and other diseases. The precise mechanisms by which

HSPs are released by viable cells have not been elucidated yet; however, exosomes are proposed to be one presumed way to release HSPs from cells [12, 38]. In these studies of exosomes, HSP70 was most frequently reported [9, 11, 14, 34, 42]. Many preclinical studies have shown that immune cells stimulated with HSPs can eliminate different kinds of cancers [43–48]. Therefore, exosomes carrying high amounts of HSPs from tumors could be good candidates for cancer immunotherapy.

Annexins are a family of Ca^{2+}- dependent, phospholipid-binding proteins [49], which are considered to play important roles in membrane traffic, regulation of ion channel activity, or control of inflammatory responses [50]. Proteome analysis show that DC-derived exosomes bear annexins, which are generally involved in intracellular membrane fusion [51]. Annexins are also found in human mesothelioma cell- and B cell-derived exosomes [14]. Apart from binding to intracellular membranes, they may play a role in the inward vesiculation process [52].
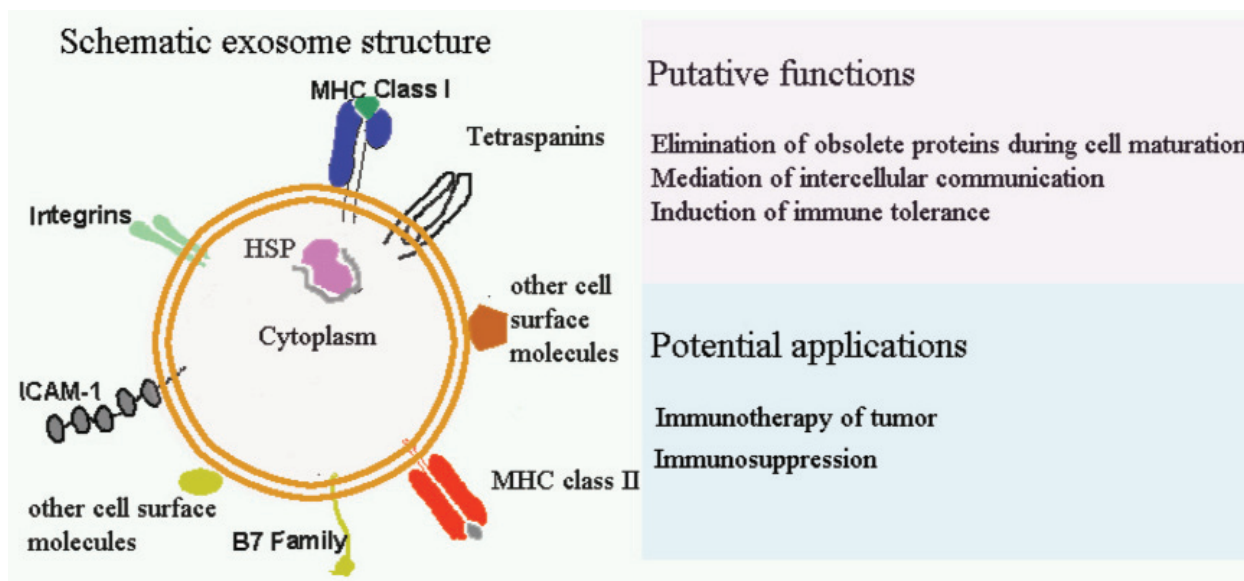
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Fig. 2 Graphical representation of composition of a typical exosome and their putative functions and potential applications.

Tetraspanins, also called tetraspans or transmembrane 4 superfamily (TM4SF), are cell-surface proteins that span the membrane four times. They are found not only in plasma membranes of many different cell types but also at the intracellular compartments, such as lysosomes and exosomes [32] to mediate cell adhesion, motility, activation, proliferation and antigen presentation. A major characteristic of tetraspanins is their ability to form cell-surface complexes with other cell adhesion molecules [53, 54]. It has been well demonstrated that exosomes contain tetraspanins on their surface. Tetraspanin formed protein complexes could be essential to keep immune proteins, such as MHC class II in an optimal and functional conformation [16]. The formation of these tetraspanin complexes between MHC class II and tetraspan molecules has been described [55], and they might be involved in cell adhesion [56]. Furthermore, due to the nature of exosome formation from endosomes, the concentration of tetraspanins on their surface could be the result of active sorting of proteins [32]. Tetraspanin complexes could play a key role in exosomal targeting to DC cells and the facilitation of cross presentation [57].

Cytoskeleton proteins play important roles in many physiological functions, like establishing cell shape, providing mechanical strength, locomotion, chromosome separation in and and intracellular transport of organelles. Given the putative biogenesis of exosome from the endocytic pathway, and considering the fact that actin and tubulin interact with endosomes and/or lysosomes [58], it is not surprising that actins, tubulins and several actin-binding proteins are found associated with exosomes [39]. Actin and tubulin are supposed to give structure to the exosome together with the associated proteins such as ezrin, moesin, actinin-4 and fascin [12].

Apart from common proteins, exosomes derived from different cell types contain a typical and unique set of proteins, which are important to their specific functions. For example, T cell receptors are abundantly present on exosomes secreted by T lymphocytes, showing a role of exosomes in intercellular communication [25, 59]. Tumor cell exosomes express substantial amounts of MHC class I molecules and tumor markers [27]. DC-derived exosomes are rich in MHC and co-stimulatory molecules which are involved in antigen presentation and stimulation of T cells [8].

Biological functions of exosomes

The functions of exosomes largely depend on their surface proteins and the cell types from which they are derived. Their supposed functions are shown in Fig.2.

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Removal unnecessary proteins during the differentiation of erythrocytes

Exosomes were initially observed during the differentiation of erythrocytes, and their supposed function was to partially or entirely remove specific proteins from the cell surface, for example the transferring receptor (TfR) and acetylcholine esterase [19, 60–62]. Exosome formation is a major route for the removal of plasma membrane proteins during reticulocyte maturation and differentiation. The TfR interacts with HSC70 bared on exosomes before or during the formation of exosome, which finally results in the complete loss of TfR from the erythrocyte's cell surface during the secretion of exosomes [63]. Johnstone *et al.* [19] showed that embryonic chicken reticulocytes lost their transferring binding activity during maturation in the form of exosomes released into the extracellular medium *in vivo* and *in vitro*.

Mediation of intercellular communication

Exosomes bear a selected subset of endocytic membrane proteins as a consequence of a specific sorting at the limiting membrane of endosomes during their formation. These complexes may be ligands to various cell-surface receptors, which can mediate interaction between two cells without direct cell-to-cell contact. Exosomes could bind to target cell membranes and transfer signals between different cells to mediate biological functions. For example, DC-derived exosomes are reported to induce cytotoxic T lymphocytes mediated responses, leading to the regression of established tumor in mice [8]. To induce such an immune responses, exosomes have to interact with target cells, and several major exosomal components, such as HSPs, tetraspan proteins may interact with membrane proteins including integrins and MHC class I and II molecules [64, 65], which could probably be involved in exosome-cell interaction.

Induction of immuno-tolerance

The capacity of DCs to induce tolerance is important in the maintenance of self tolerance in the immune steady state [66], and *in vivo* exosomes released by immature DC are supposed to be an important mediator of tolerance [67]. Data have been reported that donor bone marrow-derived DC exosomes, given 7 and 14 days prior to transplantation, can prolong or induce indefinitely rat heart allograft survival. Moreover, the *in vitro* test also showed significant decrease of CD4+ T cells from exosome treated recipients, suggesting an immuno-tolerance effect [68]. The putative mechanism could be that DCs exist in an immature form in the steady state can constantly process self antigens and secrete exosomes bearing MHC/self antigen complexes, but opposed to more mature or activated DC-derived exosomes, without CD86 [69]. These vehicles play a role in maintaining peripheral tolerance and controling autoreactive T cells [67].

Application of exosomes in cancer therapy

It was first reported in 1996 that B cell-derived exosomes induced antigen-specific MHC class II-restricted T cell responses [23]. Then in 1998, dendritic cell-derived exosomes were also shown to induce tumor eradication [8]. These observations showed for the first time that exosomes were bioactive vesicles with immuno-regulatory capacities and potent anti-tumor effects [70]. It has been demonstrated that exosomes are potential vehicle for immunotherapy and can trigger CD8+ T-cell dependent anti-tumor responses *in vitro* and *in vivo* [9, 56, 71]. These findings greatly stimulate research interests in this field [9, 32, 72]. Although the functions of exosomes remain largely unknown, they are potential immunotherapeutic agents and may have advantages over cells, being acellular, nonviable and thus rather stabile, and enriched in intact peptide–MHC complexes.

DC-derived exosomes

Exosomes derived from some DC populations stimulate T lymphocyte proliferation *in vitro* and have the capacity to generate anti-tumor immune responses *in vivo* [25, 31]. The anti-tumor effects induced by DC-derived exosomes are poor compared to that generated by peptide-pulsed DC *in*

vitro. However, DC-derived exosomes have showed relatively stronger immunostimulatory activity *in vivo* than that generated by peptide-pulsed DC. It has been shown that DC-derived exosomes, expressing MHC-I and CD86, have potent immunostimulatory potential *in vivo*, particularly in stimulating CD8^{+} cytotoxic T cell responses against established tumors [8, 73]. A single dose of exosomes derived from DC pulsed with tumor peptides completely stopped the growth of established tumors.

DC vaccination has already been applied to cancer immunotherapy. Several studies have already shown that isolated DCs pulsed with tumor antigens induce protective and therapeutic anti-tumor immunity *in vivo* in experimental animals and clinical trials [74, 75] . In clinical trials, DC vaccination of patients with non-Hodgkin's lymphoma and melanoma induced anti-tumor immune responses and tumor regression [76–78]. But pulsing of DCs with particular peptides has several disadvantages like short stability of MHC-peptide complexes and the need of direct interaction between cells. DC-derived exosomes are potential vaccines in tumor therapy. They harbor a discrete set of proteins, express high levels of functional MHC/peptide complexes, act as immunogenic vehicles for differentiation of T cells and trigger T cell responses. DC-derived exosomes are stable reagents used safe in phase I clinical trials. Autologous DC-derived exosomes loaded with tumor antigens were used in immunotherapy of patients with advanced non-small cell lung cancer, and their safety, feasibility as well as efficacy were also investigated. Prolonged disease stabilization was observed in some patients, and the results support further investigation of DC-derived exosome immunotherapy as a treatment approach for both advanced and early stage of non-small cells lung cancer and other tumors. [28]. Bernard Escudier *et al.* used autologous DC-derived exosomes as vaccines in metastatic melanoma patients. Different dosages of exosomes were given to metastatic tumor bearing patients in an indirect or a direct loading process. Such treatment showed no toxicity to patients and some patients even achieved a regression at subcutaneous sites or exhibited objective response in skin and lymph nodes and tumor regression. The possible mechanism maybe the result of NK cell activation *in vivo* [30].

The mechanism, particularly the *in vivo* mechanism by which DC-derived exosomes induce immunity, is still largely unknown. Direct antigen presentation may exist, and this hypothesis is supported by the analysis of DC-derived exosomes, which express MHC class I-peptide complexes, intracellular adhesion molecule (ICAM-1) and the costimulator B7. Derived from antigen-pulsed DCs, exosomes have the capacity to stimulate CD8+ T cells directly in the absence of antigen-presenting cells [56, 71]. Another possible mechanism of DC-exosome-generated immunity may be cross-presentation. The capacity of exosomes to stimulate T cells *in vivo* may be linked to capture and cross-presentation by DCs [79]. Exosomes contain high concentration of tetraspanins on their surface, and they form the tetraspanin complexes with MHC class II molecules, which are involved in targeting to DCs and facilitation of cross-presentation. DC-derived exosomes have stronger anti-tumor ability *in vivo* than *in vitro*, indicating the possible presence of accessory cell populations, such as mature DCs, which may generate appropriate microenvironment for exosome-mediated immune response. Exosomes may interact with APCs, directly or indirectly, to present MHC and costimulatory molecules. Probably, this is a major pathway in eliciting exosome immunity [57, 73].

Tumor-derived exosomes

Tumor cells harbor MVB, which could potentially fuse with the cell membrane and promptly release exosomes. Tumor-derived exosomes are similar to APCs-derived exosomes in their morphology, density and expression of certain membrane markers [9, 12]. Tumor-derived exosomes bear MHC-I molecules, tetraspanins, HSP70-80, LAMP1, as well as specific tumor antigens. The enrichment of HSPs on tumor-derived exosomes might facilitate the uptake of the exosomes by APCs and thereby induce an elevated immune response [80]. The migration and reactivity of NK cells could also be selectively stimulated by HSP70 surface-positive tumor-derived exosomes [81]. Therefore exosomes carrying high amounts of HSPs from patient tumors may be good therapeutic candidates for cancer immunotherapy. They are a new source of shared tumor rejection antigens for CTL cross-

priming and can trigger specific MHC class I restricted CTL activation [43–45]. Exosomes from heat-stressed carcinoembryonic antigen (CEA)-positive tumor cells pulsed autologous dendritic cells to induce CEA-specific CTL response and a substantial antitumor effect was observed in a murine model [82]. Vaccinated by a single dose (5 μg) of plasmacytoma-cells-derived exosomal protein, 80% of mice were protected against challenge with wild-type tumor cells. This protective effect could be linked to the immune system as vaccinated mice generated specific cytotoxic T lymphocytes and immunity was tumor-specific, while such effects were not seen in SCID mice [57]. However, the anti-tumor effects of tumor-derived exosomes are based on the activity of immune cells. Moreover, the tumor peptide repertoire presented on exosomes secreted by long-term tumor lines may not reflect the natural peptide repertoire processed by growing tumor cells *in vivo* [82, 83].

Malignant effusion-derived exosomes

Exosomes are present in larger amounts in malignant effusions from a number of tumors [2, 3]. Approximately 1×10^{11} MHC Class I molecules can be recovered from 1 ml of ascites fluid [27], showing that malignant effusions are abundant sources of exosomes and MHC class I/peptide complexes. Cancerous pleural fluid contains a combination of exosomes from various origins, mostly from lymphocytes and tumor cells [2]. Ascites exosomes are supposed to be as efficient as DC- or tumor- derived exosomes to sensitize dendritic cells and prime cytotoxic T lymphocytes. A preclinical study has shown that human malignant ascites-derived tumor exosomes loaded onto autologous dendritic cells activated cytotoxic T cells, which kill autologous tumor cells *in vitro* [27]. However, Martin P. Bard *et al.* pointed out that malignant effusion-derived exosome preparations might contain non-exosomal proteins, which could decrease the anti-tumor effect of the vaccine, as well as induce unwanted immune responses, like potentially dangerous autoimmune reactions. Given this potential, however unproven risk, purity of preparations must be taken into account before *in vivo* usage [2, 73]. Despite of this

hypothetical disadvantages, malignant effusion derived exosomes have strong immune stimulatory activity and are relatively easy to obtain under GMP-standards in sufficient quantity.

Potential drawbacks of exosome application in tumor therapy

Tumor-induced T-cell apoptosis is a mechanism of tumor immune evasion. T-cell killing molecules such as Fas ligand have been reported in a wide variety of cancers, as well as part of tumor-derived exosomes [84]. Dose-dependent apoptosis of T cells was induced by Fas ligand positive tumor-derived exosomes and anti-FasL antibody could block the apoptosis of T cells. Therefore the application of Fas ligand positive exosomes may induce the apoptosis of T cells, resulting in immunosuppression. This may be a new mechanism of tumor immune-evasion by secretion of circulating membrane-bound immune suppressive molecules [85, 86]. Tumor-derived exosomes could also suppress expression of T-cell activation signaling components, CD3-zeta and JAK 3 to induce T cells apoptosis [87]. Further, exosomes derived from immature DC produced in long term stromal cultures express LAMP-1, but lack expression of the costimulator CD86 and MHC-II. This kind of exosomes was found to be incapable of stimulating CD4+ T cells *in vitro* [73]. These tumor derived exosome-mediated immune suppression may be a critical factor in preventing cancer destruction by the host immune system. When they are used in tumor therapy, more attention should be paid to these exosomes.

Concluding remarks

The secretion of exosomes by various cells and their subsequent appearance in blood and malignant effusions of cancer patients has been investigated intensively over the last years. More and more surprising biological functions of exosomes are reported, and they are substantially involved in immunomodulation, antigen presentation, trigger-

ing of CTL responses but as well in immune evasion. DC- and tumor-derived exosomes have been shown to have anti-tumor effects *in vitro* and *in vivo* and have already to be tested in several phase I clinical trials. GMP standards are necessary for production of exosomes for clinical trials and purity of preparations and a clear definition of function are necessary. As immunomodulating agents, exosomes may induce the classical unwanted immune responses, like autoimmunity, tolerance and immune evasion. These potential reactions should be considered in the design of clinical trials.

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