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Review Article

Exosome-based cancer vaccines

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Abstract

Exosomes are 30-100-nm membrane vesicles originating from late endosomes and secreted by hematopoietic and epithelial cells in culture. The exosome protein and lipid composition is very unique and might shed some light on exosome biogenesis and function. Exosomes secreted from professional antigen-presenting cells (APCs), *i.e.,* B-lymphocytes and dendritic cells, are enriched in major histocompatibility complex (MHC) class I and II complexes, co-stimulatory molecules and heat shock protein Hsp70-90 chaperones, and have therefore been extensively studied for their immunomodulatory activity *in vitro* and *in vivo*. The first pilot phase I trials using exosome-based vaccines in cancer patients have just ended. This review will present the major biological features of exosomes, emphasizing their immunostimulatory functions in mice and humans, and will discuss their possible implementation in the immunotherapy of cancer.

Introduction

The biology of small vesicles (1-4) secreted from antigen-presenting cells (APCs) recently raised a great deal of interest with the demonstration of their potent immunostimulatory functions in tumor models (5, 6). The origin of vesicle secretion was first described by Pan and Johnston (7) in differentiating red blood cells, where multivesicular bodies (MVBs) fuse with the plasma membrane in an exocytic manner. This exocytic pathway was later shown to occur in a wide variety of cell types, such as B-lymphocytes, mastocytes, immature dendritic cells (DCs), platelets, cytotoxic T-lymphocytes (CTLs), fibroblasts, epithelial cells and tumor cells (5, 8-15). Vesicles exocytosed from MVBs into the extracellular medium are referred to as “exosomes”. Exosomes are unilamellar vesicles of 50-100 nm in diameter that sequester particular proteins and lipids (9).

Exosomes secreted from professional APCs, *i.e.*, B-lymphocytes and DCs, are enriched in major histocompatibility complex (MHC) class I and II complexes and co-stimulatory molecules, and have therefore been extensively studied for their immunomodulatory activity *in vitro* and *in vivo* (6, 14). This review will present the main biological features of DC-derived exosomes, with emphasis on their immunostimulating properties and implementation in cancer immunotherapy. Since DC-derived exosomes mediate antitumor effects (6), they may represent an attractive substitute for whole DC cultures for therapeutic cancer vaccine strategies.

Exosomes can be obtained by ultracentrifugation of culture supernatants of propagated cells (1, 6, 16). However, the quality and purity of such exosome preparations do not fulfill the common GMP criteria. Good manufacturing laboratory procedures for DC-derived exosome harvesting and purification have been set for clinical implementation (17). Exosomes derived from DC culture supernatants can be readily purified within 4-5 h starting from 2-3 l of culture supernatant based on their physical properties. A first-step ultrafiltration of the clarified culture supernatant through a 500-kDa hollow fiber membrane, followed by ultracentrifugation onto a 30% sucrose/deuterium oxide cushion (density: 1.210 g/cm^3), reduced the volume and protein concentration approximately 200- and 1,000-fold, respectively. Immunocapture assays assessing exosomal MHC class I and II molecules, as well as detection of membrane exosomal proteins by flow cytometry, have been validated for assessing the quality and calibration of therapeutic DC-derived

exosome preparations (17). From 2 x 10^{11}-10^{12} exosomal MHC class I molecules were reproducibly recovered in the supernatant pellets of 3 x 10^{7}-10^{8} immature human monocyte-derived DC cultures/24 h (from day 5 and 6 in AIM-V medium supplemented with IL-4 and GM-CSF). In metastatic melanoma patients, 10^{14}-10^{15} exosomal MHC class II molecules can be purified from monocyte-derived DC cultures (5 x 10^{8}-10^{9} cells) propagated from a single leukapheresis.

The immunostimulatory properties of DC-derived exosomes were extensively evaluated after a loading procedure of exosomal MHC class I molecules with molecularly defined peptides (18, 19). MHC class I molecules were more efficiently pulsed after acid elution and direct loading of the exosomal pellets with synthetic CTL epitopes (17, 20). However, exosomal MHC class II molecules can be indirectly loaded after pulsing of class II-restricted peptides onto monocyte-derived DC cultures (16). The combination of rapid and reproducible purification methods and quality control assays for exosomes allowed for their evaluation as new therapeutic tools in clinical trials.

Biological functions and preclinical data

Most of the studies on exosome clinical implementation have been performed using DC- or tumor cell-derived exosomes.

Dendritic cell-derived exosomes

Dendritic cells need to be triggered through Toll-like receptors (TLRs) or by foreign pathogens to mature into efficient APCs (21). A critical aspect of this maturation process is the back-fusion of the MVB internal vesicles with the limiting membrane and the subsequent transfer of peptide-loaded MHC class II by carrier vesicles from the endosomal limiting membrane to the plasma membrane (22). Consistent with the disappearance of MVBs in mature DCs, the production of DC-derived exosomes (dexosomes) is downregulated upon maturation, indicating that *in vivo*, exosomes are predominantly produced by immature DCs in peripheral tissues (23). Dendritic cells are, however, also pivotal for the induction of immunological tolerance (24). It is thus possible that exosomes secreted by immature DCs *in vivo* may have MHC-restricted tolerizing functions. A recent report showed that i.v. inoculation of allogeneic DC-derived exosomes could delay acute allograft rejection and induce a significant prolongation of allograft survival in rats with heart transplant (25). As discussed later in this review, DC-derived exosomes might exert immunostimulating properties under various conditions.

The biotechnological development of DC-derived exosomes emerged with the demonstration of their immunostimulating functions in tumor models. We have shown that DC-derived exosomes could eradicate established murine tumors in various models. Tumor rejection was tumor peptide-specific and required T-lymphocytes. These observations indicated that DC-derived exosomes stimulated antigen-dependent T-cell-mediated antitumor immune responses in a similar fashion as the gold standard mature DCs (6). To clearly show that exosomal MHC class I and II/peptide complexes could elicit peptide-specific T-cell priming in tumor-free mice, several experimental models were employed. Thery *et al*. found that DC-derived exosomes bearing an H-Y peptide activated specific naïve Marilyn $CD4^{+}$ transgenic T-cells *in vivo*. However, they discovered that exosomes could only be immunogenic when transferred to mature DCs which did not encounter antigens (16). Indeed, human DC-derived exosomes loaded with MHC class I-restricted peptides activate the peptide-specific $CD8^{+}$ T-cell clone only in the presence of DCs devoid of the appropriate MHC class I molecule (20, 26). In addition, it was demonstrated that DCs produce functional exosome-associated MHC class I complexes capable of CTL priming *in vivo,* but this priming required mature DCs (26, 27). Therefore, the design of an efficient exosome-based cancer vaccine ideally requires an adjuvant that optimally mimics or substitutes for mature DCs *in vivo*. Ligands for TLR3 or TLR9, such as CpG oligodeoxynucleotide (ODN) oligomeric sequences or double-stranded RNA, were efficient in inducing T-cell priming and tumor growth delay. Exosomes admixed with CpG ODNs exhibited comparable efficacy to mature DCs in initiating a peptide-specific $CD8^{+}$ T-cell response *in vivo*. We established –in transgenic HHD2 mice (28) using the melanoma B16F10 co-expressing human HLA-A2.1 and gp100 tumor antigen– that 10^{10} exosomal MHC class I/gp100 complexes admixed with CpG ODN mediated tumor rejection as efficiently as 3 x 10^{5} mature DCs-A2/gp100 and more efficiently than 50 g gp100 peptide admixed with CpG (27).

The role of $CD4^{+}$/$CD25^{+}$ T-regulatory cells in restricting T-cell-based immune responses has received renewed attention (29-31). T-regulatory cells prevent primary and secondary antitumor T-cell responses. Cyclophosphamide, at immunopotentiating concentrations, has been shown to inhibit $CD4^{+}$/$CD25^{+}$ T-regulatory cell activity (32). Therefore, we investigated the combined effects of cyclophosphamide and exosomes to elicit antitumor immunity leading to tumor rejection in poorly immunogenic melanoma. The DC-derived exosome-mediated antitumor effect was dramatically improved in cyclophosphamide-pretreated animals bearing established tumors. Cyclophosphamide pretreatment before DC-derived exosome vaccination was able to boost peptide-specific secondary responses, as assessed by immunomonitoring using specific fluorescent tetramers and inteferon gamma secretion by T-cells after *in vitro* stimulation (Unpublished data). Adoptive transfer of

T-regulatory cells blocked the synergistic effects of cyclophoshamide/ DC-derived exosomes, while natural killer (NK) cell depletion did not prevent the antitumor activity of the combination.

Altogether, DC-derived exosomes represent valuable cell-free peptide vaccines, especially when combined with TLR3/9 ligands or drugs inhibiting T-regulatory cells.

Tumor cell-derived exosomes

We first carried out biochemical and immunological analyses of tumor-derived exosomes released in the supernatants of tumor cell lines (5). Electron microscopy revealed that most murine tumor cell lines contain MVBs and constitutively release exosomes in the extracellular milieu (33-35). Western blotting demonstrated the presence of MHC class I molecules, tetraspanins, heat shock protein Hsp70-90 and lysosome-associated membrane protein 1 (LAMP1) in exosomes purified from murine tumor cell lines. As discussed below, tumor-derived exosomes are immunogenic *in vivo*. Riteau *et al*. have reported that tumor-derived exosomes from melanoma cells bear the nonclassical human leukocyte antigen G (HLA-G) class I molecule, known for its immunotolerant properties, suggesting a novel way for tumors to modulate the host immune response (35).

Immunotherapeutic strategies aimed at immunizing the host should ideally be able to elicit T-cell immune responses directed against a broad repertoire of tumor rejection antigens shared among various tumor models. While mature DCs appear to be the most potent natural adjuvants, suitable methods of tumor antigen loading that lead to efficient DC uptake, processing and cross-presentation into MHC class I molecules are still awaited. Several approaches involving the use of whole tumor RNA, tumor lysates, apoptotic or necrotic debris and fusion are currently under investigation. We extended the use of exosomes to this application by demonstrating that exosomes could be secreted by tumor cells. We reported that: 1) melanoma cell line-derived exosomes contain differentiation tumor antigens; 2) these tumor-derived exosomes loaded onto DCs transfer shared tumor antigens triggering MHC class I-restricted T clones *in vitro*; and 3) tumor-derived exosomes are a source of rejection tumor antigens since tumor exosomes promote T-cell-dependent cross-protection against syngeneic and allogeneic tumors in mice (5).

Tumor-derived exosomes are not only released *in vitro* by tumor cell lines in culture supernatants. We went on to examine malignant effusions from cancer patients for the presence of exosomes and analyzed their immunogenicity on autologous peripheral T-lymphocytes. Ultracentrifugation on sucrose and D2O gradients of 11 malignant effusions allowed isolation of abundant amounts of exosomal vesicles of 60-90 nm, *i.e.,* ascites-derived exosomes. These vesicles bear antigen-presenting molecules (MHC class I ± MHC class II, Hsps) and tetraspanins (CD81), and contain several tumor antigens (HER2/Neu, MART-1 [melanoma antigen recognized by T-cells], Trp1 [translocation protein 1], gp100). Up to 2×10^{14} exosomal MHC class I molecules are recovered from 2-3 l of malignant ascites. Exosomes from melanoma patients transport the MART-1 tumor antigen to monocyte-derived DCs for cross-presentation to MART-1-specific CTL clones. In 7 of 9 cancer patients, tumor-specific lymphocytes could be efficiently expanded from peripheral blood cells using autologous monocyte-derived DCs pulsed with autologous ascites-derived exosomes (1). Although the physiopathological conditions in which exosomes accumulate abundantly in cancer patients remain unclear, ascites-derived exosomes represent a natural and novel source of tumor rejection antigens, opening up novel immunization avenues for advanced ovarian cancer or mesothelioma.

Clinical studies with dendritic cell-derived exosomes

Their well-defined molecular composition and unique immunogenic properties, together with the availability of GMP processes, allowed for clinical trials using DC-derived exosomes in metastatic tumor-bearing patients. A first phase I feasibility and safety study was completed in France (Institut Gustave Roussy and Institut Curie, with the support of Anosys). Fifteen advanced stage III/IV melanoma patients bearing a tumor expressing the MAGE-3 antigen were enrolled (17, 19). Dendritic cell-derived exosomes were purified from the culture supernatant of day 7 autologous monocyte-derived DCs. MAGE-3 peptides (HLA-A1/B35) were loaded onto monocyte-derived DCs (in the first 6 patients) or directly onto DC-derived exosomes (in 9 patients). MAGE-3 class II peptides (DP04) or tetanus toxin class II epitopes were also loaded onto monocyte-derived DCs to confer a helper effect. Escalating doses of cryopreserved DC-derived exosomes (0.13 or 0.4×10^{13} MHC class II molecules) or peptides (10 *vs*. 100 μg/ml) were tested. Dendritic cell-derived exosomes were administered by weekly s.c./i.d. injections for 4 weeks, and then every 3 weeks, in patients who achieved stable disease or objective tumor response. Exosome production was achieved in all patients with a range of 6-120 vaccine doses. The therapy schedule was well tolerated, without any toxicity over grade 2 (NCI-CTC). Five of 15 patients experienced some clinical benefit (1 partial, 1 minor and 1 mixed response, 2 disease stabilizations) in skin and lymph node target lesions. It is noteworthy that 2 patients progressing on standard MAGE-3 vaccination exhibited a response with DC-derived exosomes (18, 19). T-cell immunomonitoring did not reveal any significant changes following exosome therapy, except for the CD122

molecule (IL-2Râ chain), which was significantly upregulated in $CD4^+$ T-cells after exosome therapy. Interestingly, while the lymphocyte pool remained stable throughout exosome therapy, the number of circulating CD3-/$CD56^+$ NK cells/mm^3 significantly increased after 3 vaccinations with exosomes. As for NK cell effector functions, more than 50% of evaluable patients exhibited enhanced NK cell cytotoxic capacity and interferon gamma secretion following an *ex vivo* boost with IL-2 or DCs (36).

This phase I clinical trial demonstrated the feasibility and safety of exosome inoculation in metastatic melanoma patients. Moreover, this clinical trial unraveled new biological attributes mediated by DC-derived exosomes *in vivo, i.e.,* NK cell activation, for which further investigation in preclinical studies will be required.

A second phase I study is ongoing in the U.S. to test the safety, feasibility and immunological efficacy of this approach in humans (37). The study is enrolling HLA-A2+ patients with MAGE-3-, -4- or -10-expressing stage III and IV non-small cell lung cancer who have stable, responsive or slowly progressive disease following chemotherapy. Patients undergo leukapheresis at the clinical site and the cell product is shipped to the centralized manufacturing site, where DC-derived exosomes are produced from *in vitro*-generated DCs. The HLA class I-restricted MAGE-3, -4 and -10 peptides and class II MAGE-3 peptides are either loaded onto the DCs prior to generation of the DC-derived exosomes (3 patients) or the peptides are loaded directly onto the DC-derived exosomes (3 patients). Patients receive 4 weekly immunizations of DC-derived exosomes (0.13×10^{14} class II molecules) s.c./i.d. Of the first 6 patients enrolled, 5 have completed immunization. Two patients have had prolonged disease stabilization (4 and 8 months), 1 of whom had been progressing prior to immunization. Three patients progressed and 2 died of their lung cancer. Pulmonary toxicities were attributable to disease progression, including 1 patient with grade 3 hemoptysis. Assessment of immunological responses consisted of delayed-type hypersensitivity (DTH) skin reactions and proliferation assays, ELISPOT, intracellular cytokine detection and tetramer analysis of peripheral blood lymphocytes. One patient with stable disease developed a 6-mm DTH reaction against MAGE-10. Two patients have been analyzed for immune response and no increases in antigen-specific immune responses has been detected in the peripheral blood at the low dose levels of this immunization approach. These preliminary results demonstrate the safety and feasibility of the DC-derived exosome vaccine. The final results of this study should be available within the next 6 months.

Dendritic cell-derived exosomes have completed phase I development as a treatment for advanced melanoma and lung cancer in Europe and the U.S. A phase II trial will start during 2005 in lung cancer patients in France. Dendritic cell-derived exosomes continue to undergo laboratory research programs and clinical development as a cancer vaccine.

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