Exosomes for cancer immunotherapy

F. Andre1,2, B. Escudier1, E. Angevin2, T. Tursz1 & L. Zitvogel2

1Department of Medicine and 2Immunology Unit, Institut Gustave Roussy, Villejuif, France

Introduction

Exosomes are small vesicles originating from late endosomes and are released in the extracellular medium by a broad array of cells. They were initially described by Johnstone et al. [1] in reticulocytes where multi-vesicular bodies (MVBs) fused with the plasma membrane in an exocytic manner. This exosome secretion was later shown to occur in a wide variety of cell types, including B lymphocytes, mastocytes, platelets, cytotoxic T lymphocytes and epithelial cells [2–6]. The most striking finding of initial reports focusing on exosome biology was that they concentrate molecules involved in the function of the cells. For example, exosomes derived from red blood cells concentrate transferrin receptor, while exosomes derived from B cells concentrate major histocompatibility (MHC) class II molecules. From these data the hypothesis emerged that dendritic cells (DC) could release immunogenic exosomes and that tumor cells could release exosomes enriched in tumor antigens.

In this review, we describe the definition and composition of exosomes and review the preclinical data that have allowed the evaluation of exosomes in cancer immunotherapy. This manuscript will also describe how basic knowledge has been translated into clinical trials evaluating cancer vaccines.

Definition and composition of exosomes

The definition of exosomes is based on morphological and biochemical criteria (Figure 1) and factors related to the process of purification. When analyzed by electron microscopy, exosomes present a characteristic ‘cup shaped’ morphology: a flattened sphere limited by a bilipidic layer. Their diameter ranges between 30 and 100 nm. These characteristics are consistent with the observed size and morphology of internal vesicles in multi-vesicular endosomes [7]. The hallmark of exosomes usually includes the presence of tetrastrians and heat shock proteins (Hsc73, Hsp80). The common procedure for purifying exosomes from cell culture supernatants involves a series of centrifugations designed to remove dead cells and large debris, followed by a final high-speed ultracentrifugation to pellet exosomes. The final step is purification on a sucrose gradient. Indeed, as for all lipid vesicles, exosomes float on sucrose gradients, and their density ranges from 1.13 to 1.21 g/ml. This physico-chemical property is used to purify exosomes.

Overall, as summarized in Table 1, the hallmark of exosomes is their electronic microscopic image, the presence of tetrastrians and a density between 1.13 and 1.21 g/ml.

The presence of known cellular proteins in exosome preparations from various cellular sources has been analyzed by western blot or by flow cytometric analysis of exosome-coated beads. Proteomic analysis to identify unknown or unexpected cellular proteins in exosomes, has also been performed on DC-, mast cell- and enterocyte-derived exosomes [8, 9]. These studies have shown that exosomes are clearly distinct from microvesicles produced by apoptotic cells and are only secreted by living cells. The proteomic analyses have shown that four groups of ubiquitous and cell-specific proteins are over-represented in DC-derived exosomes. Proteins involved in antigen presentation, e.g. MHC classes I and II molecules, are found on B-cell- and DC-derived exosomes. Hsc73 has been found in DC-derived exosomes [9], and might be involved in the direct transport of molecules to the exosomes. It has been suggested recently that heat shock proteins bind antigenic peptides and are involved in their loading in MHC class I molecules in the endocytic compartment. One of the most abundant ubiquitous protein families found on exosomes, and which constitute a hallmark of exosomes, are tetrastrians: CD9, CD63, CD81 and CD82. The function(s) of these proteins is still unclear. They form large protein complexes with the MHC classes I and II molecules, or with adhesion proteins of the integrin family. CD9 has been observed to be involved in the fusion of oocytes with spermatozoa and therefore could be involved in exosome targeting to antigen-presenting cells (APCs). DC-derived exosomes also contain a series of cell-specific trans-membrane proteins, including the integrin α and β chains (αMβ2 integrins) and Ig family members (ICAM-1/CD54). Within this group of proteins, MFG-E8/lactadherin, a milk fat globule protein, is probably the most important. This protein, is a ligand of αβ3 and αβ5 integrins, and has been recently involved in the uptake of apoptotic bodies by antigen presenting cells [10]. In addition to these four groups of proteins, other DC-derived exosome proteins are found to be overexpressed in proteomic analysis. These proteins are involved in exosome biogenesis, intracellular membrane fusion or transport (cytosolic tubulin, actin and actin-binding proteins, annexins and Rab proteins) and in intracellular signal transduction (protein kinase, 14-3-3, heterotrimeric G proteins). Various metabolic enzymes (peroxydase, pyruvate kinase) are also present in exosomes. Proteomic analysis suggests that DC-derived exosomes present all the biochemical material to present antigen to T lymphocytes.

Biochemical analyses of tumor-derived exosomes have shown that, as DC-derived exosomes, they also contain MHC class I molecules, heat shock proteins, lactadherin
Production and purification of DC exosomes for clinical use

Good manufacturing laboratory procedures for exosome harvesting and purification have been set up for clinical implementation [13]. In order to obtain accurate information, preclinical studies were performed mimicking the clinical process as far as possible. Exosomes derived from DC culture supernatants can be readily purified using ultracentrifugation followed by ultracentrifugation on a 30% sucrose/deuterium oxide cushion (density 1.13–1.21 g/cm³). From $2 \times 10^{11}$ to $2 \times 10^{12}$ exosomal MHC class I molecules were reproducibly recovered in the supernatant pellets of $3 \times 10^7$ to $3 \times 10^8$ immature human monocyte-derived DC (MD-DC) culture per 24h. In patients with metastatic melanoma [14], $10^{14}–10^{15}$ exosomal MHC class II molecules can be purified from MD-DC cultures ($5 \times 10^8$ to $5 \times 10^9$ cells) propagated from a single leukapheresis.

Background for the use of DC-derived exosomes (dexosomes) as cancer vaccines

Dexosomes induce tumor-specific CTL priming in mice

Preclinical data obtained in mice have shown that the injection of dexosomes purified from murine DC pulsed with tumor antigen lead to tumor rejection [15]. Further experiments have shown that tumor rejection is mediated through tumor-specific CTL expansion provided by the injection of dexosomes. More recently, we have shown that the injection of human dexosomes pulsed with Mart1 peptide, combined with molecular adjuvants, leads to the expansion of Mart1-specific CTL response in vivo in HLA-A2 transgenic mice [16]. In the same experiments, the injection of Mart1 tumor antigen combined with molecular adjuvants did not induce any CTL reaction in vivo. In addition, the magnitude of the CTL induction obtained with dexosomes and adjuvant was no different from that obtained with peptide-pulsed mature DCs. Indeed, we showed in the melanoma B16F10 tumor model co-expressing human HLA-A2 and the gp100 tumor antigen, that $10^{10}$ exosomal MHC class I–gp100 complexes mixed with unmethylated CpG sequences mediates tumor rejection as efficiently as $3 \times 10^6$ mature DC-A2 pulsed with gp100.

Overall, these data suggest that the injection of dexosomes could lead to tumor-specific CTL induction in vivo in animal models. Following these data, we have investigated the immunological properties of human dexosomes in vitro.

Human dexosomes induce tumor-specific CTL priming in vitro by transferring MHC class I–peptide complexes to APC

Based on the findings that immunological adjuvants increase the bioactivity of dexosomes, we hypothesized that dexosomes could activate tumor-specific CTL response through mature DCs. Experiments have shown that dexosomes transfer their MHC class I–peptide complexes to mature DC to induce a specific CTL response in vitro. Indeed, we have shown that
CTL induction in vivo in patients with stage III/IV lung cancer. Results showed that determine what effectors mediated clinical activity. stabilization. Immunomonitoring is ongoing and results will associated with some tumor regression and long-term study has shown that injection of dexosomes is safe and six patients exhibited objective regressions or long-term a regression of subcutaneous metastases. In the same group, direct loading but low exosome dosage, one patient achieved a regression of subcutaneous metastases. In the same group, a second patient exhibited a partial response at nodal sites. Using direct loading and high exosome dosage, three of patient achieved stabilization and received eight additional (NCI CTC). Using an indirect loading process, one stage III nogenic properties of dexosomes, as well as the availability of a clinically approved process for purification, allowed the performance of two phase I clinical trials in melanoma and lung cancer patients. The first clinical trial started at the end of 2000, 30 months after the original publication reporting that dexosomes induced tumor-specific CTL reaction [15].

Phase I clinical trials

The well-defined molecular composition and unique immunogenic properties of dexosomes, as well as the availability of a clinically approved process for purification, allowed clinical trials using dexosomes in stage III/IV melanoma patients and in stage III/IV lung cancer patients. A feasibility and safety phase I study was completed in patients with melanoma. Fifteen advanced-stage III/IV melanoma patients presenting tumors that expressed the MAGE-3 antigen were included [14]. Dexosomes were purified from the culture supernatant of autologous MD-DC after 7 days. MAGE-3 peptides (HLA-A1/B35 and -DP04 restricted) were loaded onto MD-DCs (in the first six patients) or directly onto dexosomes (in the other nine patients). Escalating doses of cryopreserved dexosomes were administered by four weekly subcutaneous or intradermal injections for 4 weeks, and then every 3 weeks in patients who achieved stable disease or tumor regression. Feasibility of exosome production was achieved in all patients with a range of six to 120 vaccine doses. The therapy schedule was well tolerated, without any grade 3 or 4 toxicity according to the National Cancer Institute Common Toxicity Criteria (NCI CTC). Using an indirect loading process, one stage III patient achieved stabilization and received eight additional vaccine injections with long-lasting disease control. Using direct loading but low exosome dosage, one patient achieved a regression of subcutaneous metastases. In the same group, a second patient exhibited a partial response at nodal sites. Using direct loading and high exosome dosage, three of six patients exhibited objective regressions or long-term stabilization (skin and lymph node lesions). Overall, this study has shown that injection of dexosomes is safe and associated with some tumor regression and long-term stabilization. Immunomonitoring is ongoing and results will determine what effectors mediated clinical activity.

A second phase I trial evaluated the tolerance of dexosomes in patients with stage III/IV lung cancer. Results showed that injection of dexosomes was safe and allowed long-term stabilization in four of the 12 patients included [20].

The two phase I trials have demonstrated that the injection of dexosomes is feasible, safe and associated with clinical activity. Phase II trials will be performed in patients with lung cancer.

Tumor-derived exosomes (texosomes): a new source of tumor antigens

The presentation of tumor antigens by DCs to naïve T cells requires an optimal antigen uptake and processing by DC. An optimal cancer vaccine should therefore contain an efficient antigen delivery system to induce tumor antigen presentation to naïve CTL. The ‘ideal’ antigen delivery system to be used in clinical trials should theoretically: (i) allow a polyepitopic presentation; (ii) be efficiently taken up and processed by DCs; and (iii) be quantified and produced for clinical use. Several approaches involving the use of whole tumor RNA, tumor lysates, apoptotic or necrotic debris and fusion are currently under investigation. Using the criteria previously reported, we determined that tumor cell lines release exosomes in culture supernatants [11]. We reported that texosomes loaded onto DCs transfer tumor antigens generating MHC class I-restricted T clones in vitro, and that they are a source of shared tumor rejection antigens since they promote T cell-dependent cross-protection against syngenic and allogeneic tumors in mice [11]. Therefore, these data show that exosomes are a source for an efficient uptake of tumor antigen and processing by immature DCs. Since a process for clinical use is available, these data suggest that texosomes could be used as a source of tumor antigens in clinical trials. To date, no clinical trial has specifically addressed the use of texosomes in cancer immunotherapy. Nevertheless, one randomized trial has evaluated whether the intradermal injections of polyvalent, shed antigen, melanoma vaccine could increase survival of patients with stage III melanoma [21]. This vaccine is mainly composed of the pellet of the ultracentrifugations of four tumor cell line supernatants. While this product is not well defined, the process is very close to that used in the purification of exosomes. This trial showed that the injection of polyvalent, shed antigen, melanoma vaccine improved median time to recurrence compared with placebo (P = 0.03).

Exosomes purified from cancer patients: a new entity [22]

Although studies have reported that a broad array of cells release exosomes, all the data were obtained using cells cultured in vitro. In order to show that exosomes as an entity are present in vivo, we looked for their presence in malignant effusions. The process used to purify exosomes from culture supernatants was successfully applied for the malignant effusions. Immunoelectron microscopy studies and biochemical analysis showed that malignant effusions contain large amounts of texosomes. In vitro studies further showed that
these texosomes purified from malignant effusions allow tumor-antigen uptake and presentation by DCs to CTL. Indeed, following in vitro stimulation of lymphocytes with DCs pulsed with ascites-derived exosomes, tumor-specific CTL priming was obtained in vitro in seven out of nine cancer patients. These data show that exosomes are released in vivo and allow tumor antigen cross-presentation by DCs in vitro. In the same study, it was interesting to note that the tumor-specific T cells expanded in vitro could not be found in vivo in the peritoneal cavity, suggesting that texosomes could not elicit CTL reaction in vivo.

Conclusions and prospects

Biochemical analysis and functional studies have allowed the clinical development of dexosomes as cancer vaccines. Further clinical studies (phase II and III) will determine the efficacy of dexosomes. In parallel with this standard clinical development, translational and basic research performed with material obtained from clinical trials will determine what components of exosomes mediate their bioactivity. The results of these studies will improve our knowledge on the use of exosomes in clinical trials, and what is their optimal formulation. Since exosomes are composed of a limited number of proteins, it should be possible in the future to develop a synthetic vaccine that will contain only the crucial components of exosomes. At that time, exosomes will not be considered as a product derived from cell therapy, but as a synthetic drug. The use of biology and biotechnology related to exosomes to develop a synthetic vaccine is a major challenge for teams involved in this field.

We have reported that exosomes have some immunostimulatory capacity. Exosomes also exhibit other properties that could be of interest to oncologists. Indeed, since they are released by tumor cells in vivo, it is possible that they could mediate many other functions. For example, it has been suggested that exosomes could mediate chemoresistance by allowing drug release from cells [23]. Other applications in the field of cancer research will probably be determined in the future.

References