Chapter 13

Cancer Exosomes as Mediators of Drug Resistance

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Abstract

In the last decades, several studies demonstrated that the tumor microenvironment is a critical determinant not only of tumor progression and metastasis, but also of resistance to therapy. Exosomes are small membrane vesicles of endocytic origin, which contain mRNAs, DNA fragments, and proteins, and are released by many different cell types, including cancer cells. Mounting evidence has shown that cancer-derived exosomes contribute to the recruitment and reprogramming of constituents associated with the tumor microenvironment. Understanding how exosomes and the tumor microenvironment impact drug resistance will allow novel and better strategies to overcome drug resistance and treat cancer.

Here, we describe a technique for exosome purification from cell culture, and fresh and frozen plasma, and further analysis by electron microscopy, NanoSight microscope, and Western blot.

Key words Tumor microenvironment, Pre-metastatic niche, Tumor-derived exosomes, Drug resistance, Cell culture, Plasma, NanoSight microscope, Electron microscopy

1 Introduction

1.1 The Tumor Microenvironment, the Pre-metastatic Niche, and the Role of Tumor Exosomes in Tumor Progression and Metastasis Development

Solid tumors are complex, organ-like structures, consisting of cancer cells along with a supportive stroma composed of multiple nonmalignant cell types, such as fibroblasts, endothelial cells, mesenchymal stem cells, and immune cells, sustained by an extracellular matrix and a vascular network [1]. The tumor-associated microenvironment differs from normal tissues by an increased number of fibroblasts and an altered extracellular matrix, and the most frequently found immune cells within the tumor microenvironment are tumor-associated macrophages (TAMs) [2]. TAMs have been shown to be involved in multiple steps of tumor development, namely angiogenesis, invasion, and intravasation [3]. In past years, an association between the presence of these cells and poor prognosis in several types of cancers, including breast, gastric, urogenital, and head and neck cancer, was demonstrated [4]. On the other hand, the presence of mature dendritic cells in the primary tumor is associated with a good outcome in lung and head and
neck cancers [5, 6]. Fibroblasts are present at increased numbers in the tumor microenvironment, and influence the ability of tumor cells to invade and metastasize through the synthesis of growth factors, chemokines, and adhesion molecules [7].

As important as the primary tumor microenvironment, the future metastatic organ structure and composition are crucial for metastasis to develop. The pre-metastatic niche model suggests that in order for tumor cells to engraft and to form metastatic lesions at secondary sites, a suitable microenvironment must evolve in these pre-metastatic organs [8]. This theory advocates that metastatic proliferation does not depend solely on the characteristics and genetic alterations of the cancer cell itself, but that the formation of this pre-metastatic niche is also essential for metastasis to occur. These niches form as a consequence of growth factors, e.g., VEGF or placental growth factor (PIGF), secreted by the primary tumor [9]. In response to these soluble factors, tumor-associated cells such as hematopoietic progenitor cells or macrophages are mobilized to the pre-metastatic niches. Besides immature myeloid cells, other cells are also involved actively in the formation of the pre-metastatic niche. Platelets, resident fibroblasts, and endothelial cells are also important in this process. At the pre-metastatic niche, the mobilized bone marrow-derived cells together with resident cells produce chemokines, growth factors, and matrix-degrading proteins (e.g., MMP9). These alter the surrounding microenvironment, making it more suitable for the engraftment of tumor cells and the formation of metastatic lesions [8, 10].

Besides the multiple cell types, tumor-derived chemokines, and growth factors mentioned above, exosomes are also important mediators of metastasis, being involved in a permanent cross talk between the primary tumor and local/distant host cells. Exosomes are small membranous extracellular vesicles, ranging in size between 40 and 100 nm in diameter, that contain microRNAs, messenger RNAs (mRNA), DNA fragments, and proteins [11]. These small vesicles consist of a lipid bilayer membrane surrounding a small cytosol, are devoid of cellular organelles, and are secreted by many kinds of cells, including tumor cells, reticulocytes, and hematopoietic cells [12]. Exosomes are formed by the inward budding of cells known as multivesicular endosomes. Fusion of these endosomes with the plasma membrane leads to the release of internal vesicles known as exosomes [13]. The major role of exosomes seems to be the transport of bioactive molecules between cells, with consequences in targeted cell phenotypes. Exosomes are involved in the normal physiology of the body, including immune regulation, tissue repair, and communication within the nervous system [14]. In cancer patients, the abundance of secreted exosomes suggests an important role of these mediators in cancer development. In fact, a positive correlation between increased
Exosome secretion and cancer stage and progression has been shown [15]. Exosomes travel to surrounding cells or distant tissues to execute important functions in tumor biology, such as angiogenesis, immune suppression, induction of proliferation, and transfer of genetic material [16]. The transport of oncogenic proteins and miRNAs by exosomes released by tumor cells and the uptake of these oncogenic elements by nonmalignant cells in the tumor microenvironment can result in the transfer of oncogenic activity [17]. Work by Peinado et al. demonstrated that tumor-derived exosomes promote metastatic niche formation by educating bone marrow-derived cells towards a more pro-vasculogenic and pro-metastatic phenotype, through the exosome-mediated transfer of the oncoprotein MET [18]. Further studies supported these results, and confirmed the importance of exosomes in tumor growth, angiogenesis, and metastatic development [19].

The acquisition of resistance to chemotherapeutic drugs continues to be a major obstacle in cancer treatment. Although it was believed for several years that drug resistance resulted primarily from selection of mutant tumor cells that were resistant to the cytotoxic effects of certain therapies, mounting evidence suggests that there is more to this story than once believed. Functional gene mutations that alter the expression of proteins involved in the uptake, metabolism, and export of drugs are main causes of drug resistance, as are nonmutational (epigenetic) changes that can be associated to transient drug resistance. However, as discussed above, the tumor cell is only part of a complex group of constituents, and this tumor microenvironment is a critical determinant not only of tumor progression and metastasis, but also of resistance to therapy.

In 1998 Brown and Giaccia proposed that the microenvironment could be a major mechanism of drug resistance through the reduction of drug distribution throughout the tumor, therefore protecting high proportions of cells from damage induced by the drug [20]. In fact, the tumor stromal components contribute to an increase in interstitial fluid pressure, and several studies have shown an association between high interstitial fluid pressure and poor drug penetration, with a suggested association to response to chemotherapy [21]. On the other hand, the increase in interstitial fluid pressure in association with an oncotic pressure gradient of almost zero can lead to the extravasation of macromolecules, which can decrease the effectiveness of the treatment if the administered drug is lost at the tumor periphery [22].

Response to chemotherapy is also influenced by the vasculature, not only because the delivery of cytotoxic drugs can be impaired as a consequence of vascular disorganization [23], but also because this disorganized blood flow results in an abnormal
and limited delivery of nutrients to the tumor, and the appearance of hypoxia \[24, 25\]. The first link between glucose deprivation and drug resistance was reported by Shen et al. in 1987. They showed that in Chinese hamster ovary cells stress conditions that induced the endoplasmic reticulum-resident stress proteins, such as hypoxia or glucose deprivation, were associated with significant resistance to doxorubicin. Moreover, it was shown that the removal of these conditions resulted in the disappearance of drug resistance \[26\]. Hypoxic conditions can lead to the activation of genes associated with angiogenesis and cell survival \[27\]. The expression of these genes may result in an expansion of biochemically altered cells, with a drug-resistant phenotype. As an example, transient hypoxia has been shown to be associated with an increased expression of genes encoding P-glycoprotein and dihydrofolate reductase, which induces drug resistance, and with selection for cells that are deficient in DNA mismatch repair which increases their resistance to platinum-based chemotherapy \[28\]. Furthermore, the limited supply of nutrients induces cell cycle arrest, with a consequent reduction of tumor cell proliferation rate \[29\]. As most chemotherapeutic drugs are more effective against proliferating cells, the slow-growing cells localized most distant to the tumor vasculature have a high likelihood of becoming resistant to therapy \[30\].

Another known mechanism by which tumor stroma can influence drug resistance is through the interactions between tumor cells and the extracellular matrix. Work performed by Garrido et al., 1997, demonstrated that confluent cells in culture are more resistant to anticancer drugs than non-confluent cells \[31\]. Moreover, tumor cell adhesion to extracellular matrix mediated by integrins has been shown to protect small-cell lung cancer cells from drug-induced apoptosis \[32\].

In recent years, mounting evidence has suggested that certain growth factors and immune suppressor cells within the tumor microenvironment can induce tumor growth and mediate resistance to therapy. Straussman et al. demonstrated that in BRAF-mutant melanoma, hepatocyte growth factor (HGF) secretion by stromal cells was associated with poor response to BRAF inhibition. Furthermore, it was demonstrated that HGF plasmatic levels were inversely related to the response to BRAF inhibition in BRAF-mutant melanoma \[33\]. Recent work by Sun et al. suggests that microenvironment-mediated therapy resistance in the clinical management of prostate cancer may also arise from an adaptive, reciprocal signaling dialogue between the microenvironment and tumor cells. Specifically, it was shown that WNT16B was increased within fibroblasts exposed to cytotoxic drugs both in vitro and in vivo, and that in human tumors, WNT16B expression was associated with higher rates of disease recurrence after chemotherapy. Furthermore, when high-expressing fibroblasts were co-cultured
with epithelial cells or xenograft tumors and then exposed to cytotoxic agents, there was a survival advantage as compared to cultures with low or absent WNT16B-expressing fibroblasts. This work demonstrated that WNT16B signals through a paracrine manner to tumor cells, increasing their proliferation and resistance to apoptosis [34].

More recently, the role of exosomes in drug resistance has begun to be explored. In a study published recently, MCF-7 (breast cancer) cells sensitive to docetaxel were exposed to exosomes extracted from the supernatant of a docetaxel-resistant MCF-7 variant. It was demonstrated that exosomes effectively transferred drug resistance characteristics from drug-resistant breast cancer cells to sensitive ones [35]. Another study using breast cancer-derived exosomes reinforced these results, showing that adriamycin and docetaxel-resistant breast cancer cells may spread resistance capacity to sensitive cells by releasing exosomes and that these effects are attributed to the intercellular transfer of specific miRNAs [36]. Moreover, it was demonstrated that docetaxel resistance in hormone refractory prostate cancer cells can be acquired by noninvasive cell lines also via exosomes [37]. The addition of cisplatin (DDP) to A549 tumor cells (lung cancer cell line) has been shown to increase exosome secretion and the interaction of these secreted exosomes with other cancer cells increased the resistance of these A549 cells to DDP [38]. This study also demonstrated that when A549 cells were exposed to DDP, the expression levels of several miRNAs and mRNAs reportedly associated with DDP sensitivity change significantly in exosomes, and that these changes probably mediate the DDP resistance of these tumor cells.

Exosomes may also contribute to chemotherapy resistance through drug expulsion. Exosomes released from tumor cells have been shown to contain cisplatin, potentially redirecting the drug away from the nucleus where it would normally act, causing DNA damage, cell cycle arrest, and apoptosis [39]. A recent study identified another method by which exosomes may contribute to chemotherapeutic resistance. It was observed that exosomes released from cancer cells might impede antibody and drug therapies by expressing cancer-derived cell surface proteins that sequester the compound away from the target cell [40].

Taken together, the current data suggests that accurate predictions of response to cancer treatment will be incomplete unless an integrative approach is undertaken. It seems proper to consider that more attention should be given to the role of the microenvironment in drug resistance, namely the role of exosomes in therapy resistance. Understanding how exosomes impact drug resistance will allow novel and better strategies to treat cancer and prevent the emergence of drug resistance.
2 Materials

Materials

1. Cell lines.
2. Fresh plasma.
3. Frozen plasma.
4. 1.2 μm nylon filters.
5. 0.22 μm filter.

Reagents

1. Culture media.
2. 40 % Tris/sucrose/D₂O solution (40 % sucrose cushion).
3. 40 g protease-free sucrose.
   (a) 2.4 g Tris base.
   (b) 50 ml D₂O.
   (c) Adjust pH to 7.4 with 10 N HCl drops.
   (d) Adjust volume to 100 ml with D₂O.
   (e) Sterilize by passing through a 0.22 μm filter.
   (f) Store for up to 2 months at 4 °C.
4. FBS, Hyclone.
5. PBS.
6. RIPA buffer.
7. Protease inhibitor tablet (Roche).
8. Antibodies against CD3, CD9, and MHC-I.
9. 2 % PFA.
10. 200 nm phosphate buffer (pH 7.4).
11. FormVar-carbon-coated grid.
12. 1 % glutaraldehyde.
14. 0.4 % w/v uranyl acetate.
15. 1.8 % w/v methyl cellulose.

Equipment

1. Sorvall Surespin 630 rotor.
2. Sorvall S100AT5 rotor.
3. Centrifuge.
5. Electron microscope.
6. SDS-PAGE equipment.
The exosomes are purified by ultracentrifugation: the first steps are designed to eliminate large dead cells and large cell debris by successive centrifugations at increasing speeds. At each of these steps, the pellet is thrown away, and the supernatant is used for the following step (Fig. 1). The final supernatant is then ultracentrifuged at $100,000 \times g$ to pellet the small vesicles that correspond to exosomes. The pellet is washed in a large volume of PBS, to eliminate contaminating proteins, and centrifuged one last time at the same high speed.

1. FBS (Fetal Bovine Serum, Hyclone) is depleted of bovine exosomes by ultracentrifugation at $100,000 \times g$ for 70 min (Sorvall Surespin 630 rotor).
2. Cells are cultured in media supplemented with 10 % exosome-depleted FBS.
3. Supernatant fractions are collected from 48 to 72-h cell cultures and pelleted by centrifugation at $500 \times g$ for 10 min.
4. The supernatant is centrifuged at $20,000 \times g$ for 20 min.
5. Exosomes are then harvested by centrifugation at $100,000 \times g$ for 70 min.
6. The exosome pellet is resuspended in 20 ml of PBS and collected by ultracentrifugation at $100,000 \times g$ for 70 min.
7. The exosome pellet is resuspended in PBS and then stored at 4 °C for short term (1–7 days) or −20 °C for long term.
3.2 Exosome Isolation from Fresh Mouse and Human Plasma

Circulating exosomes are isolated from mouse and human plasma in the same way as from cell culture with an extra purification step with a sucrose cushion and an additional filtration through 1.2 μm nylon filters (GE) before the last step of ultracentrifugation. The extra purification step with a sucrose cushion eliminates more contaminants, such as proteins nonspecifically associated with exosomes, or large protein aggregates, which are sedimented by centrifugation but do not float on a sucrose gradient. The filtration through 1.2 μm nylon filters will eliminate dead cells and large debris while keeping small membranes for further purification by ultracentrifugation.

1. Plasma is pelleted by centrifugation at 500 × g for 10 min.
2. The supernatant is centrifuged at 20,000 × g for 20 min.
3. The supernatant is diluted 1:10 in PBS.
4. Exosomes are then harvested by ultracentrifugation at 100,000 × g for 70 min on a 40% sucrose cushion solution.
5. The floating exosome fraction is collected again by ultracentrifugation as above.
6. The exosome pellet is resuspended in 20 ml of PBS and filtered through 1.2 μm nylon filters (GE).
7. The exosome pellet is collected by ultracentrifugation at 100,000 × g for 70 min.
8. The exosome pellet is resuspended in PBS and then stored at 4 °C for short term (1–7 days) or −20 °C for long term.

3.3 Exosome Isolation for Retrospective Studies Using Frozen Human Plasma

Plasma for retrospective studies is previously centrifuged at 3000 × g for 20 min before storing at −80 °C.

1. 2 ml of cell-free frozen plasma is centrifuged at 500 × g for 10 min.
2. Then the supernatant is centrifuged at 20,000 × g for 20 min.
3. Exosomes are then harvested by centrifugation at 100,000 × g for 70 min.
4. The exosome pellet is resuspended in PBS and collected by ultracentrifugation at 100,000 × g for 70 min (Sorvall S100AT5 rotor).
5. The exosome pellet is resuspended in PBS and then stored at 4 °C for short term (1–7 days) or −20 °C for long term.

3.4 Electron Microscope Analysis of Exosomes

Exosomes purified as described above are fixed in 2% PFA (w/v) in 200 mM phosphate buffer (pH 7.4). Fixed exosomes are dropped onto a formvar-carbon-coated grid and left to dry at room temperature for 20 min. After washing in PBS, the exosomes are fixed in 1% glutaraldehyde for 5 min, washed in water, and stained with saturated aqueous uranyl oxalate for 5 min. Samples are then
embedded in 0.4 % w/v uranyl acetate and 1.8 % w/v methylcellulose and incubated on ice for 10 min. The excess liquid is removed. The grid is dried at room temperature for 10 min and viewed at 20,000 and 50,000 magnification using an electron microscope (model 910, Carl Zeiss) (Fig. 2).

Exosomes are lysed with RIPA buffer containing a complete protease inhibitor tablet (Roche). Lysates are cleared by centrifugation at 14,000 ×g for 20 min. Supernatant fractions are used for Western blot. Protein extracts are resolved by SDS-PAGE and probed with the indicated antibodies. For Western Blot analysis the following antibodies are used to identify specific exosome markers: anti-CD3, anti-CD9, and anti-MHC-I.

The LM10 nanoparticle characterization system (NanoSight) equipped with a blue laser (405 nm) is used for real-time characterization of the vesicles.

3.5 Identification of Exosome-Specific Markers by Western Blot Analysis

3.6 Quantification of Exosome Size, Distribution, and Number by LM10 Nanoparticle Characterization System (NanoSight)

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References


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