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Chapter

Diversity of Extracellular Vesicles (EV) in Plasma of Cancer Patients

Theresa L. Whiteside and Soldano Ferrone

Abstract

Extracellular vesicles (EVs) are produced by all cells and are found in all body fluids. They function as intercellular messengers that carry and deliver signals regulating cellular interactions in health and disease. EVs are emerging as potential biomarkers of diseases and responses to therapies, and much attention is being devoted to understanding their role in physiological as well as pathological events. EVs are heterogenous in their origin, size, molecular characteristics, genetic content and functions. Isolation of EV subsets from plasma and characterization of their distinct properties have been a limiting factor in ongoing efforts to understand their biological importance. Here, we discuss the immunoaffinity-based strategies that are available for isolating distinct subsets of EVs from plasma and provide a road-map to their successful immunocapture and molecular profiling, with special attention to tumor-derived EVs or TEX.

Keywords: extracellular vesicles (EV), exosomes, tumor derived exosomes (TEX), immunoaffinity capture from plasma

1. Introduction

Recent progress in the understanding of the role tumor microenvironment (TME) plays in cancer development has identified intercellular communication within and outside the TME as one of the major mechanisms driving tumor progression. A detailed characterization of the crosstalk of the tumor with various immune and tissue cells has become a major goal in cancer research. For years, many soluble factors, including cytokines and chemokines, have been postulated to play a major role in the regulation of cellular interactions in healthy and pathological tissues. The recognition of extracellular vesicles, EVs, as major players in the intercellular communication network occurred only a few years ago [1]. Since then, EVs produced by cancer cells and by immune as well as non-immune cells residing in the TME have become the topic of numerous studies evaluating their involvement in the regulation of tumor progression on the one hand and of the host anti-tumor immune responses on the other. This double role of EVs in cancer as well as other diseases emphasizes their potential as reporters or markers of changes that preface or accompany the emergence of disease or its outcome.

2. The origin and characteristics of extracellular vesicles (EVs)

Extracellular vesicles (EVs) are produced and released into the extracellular space by all cells. EVs are classified based on differences in their biogenesis, size and

functions [2]. The current EV nomenclature recognizes exosomes (30–150 nm), microvesicles (MVs; 150–1000 nm) and apoptotic bodies (>1000 nm). However, within these EV categories, there is considerable heterogeneity. Thus, although all exosomes, now referred to as small EVs (sEVs), originate in multivesicular bodies (MVBs) and thus share the endocytic origin [3], they are divided into tiny exomeres (<35 nm), small exosomes (Exo-S, 50–100 nm) and large exosomes (Exo-L, 100–150 nm) [2]. In our studies of EVs, we consider sEVs derived from MVBs and sized from 50 to 150 nm as exosomes [4] and intermittently refer to them as either exosomes or sEVs. Unlike exosomes, MVs bud off from the surface of parent cells, differ broadly in size and molecular content from exosomes and are called ectosomes or, if they carry oncogenes, oncosomes [2].

During exosome biogenesis, when MVBs filled with intraluminal vesicle fuse with the cellular plasma membrane, exosomes are released into the extracellular space. Due to their endosomal origin, exosomes carry endocytic markers, such as TSG101, ALIX, syntenin-1, flotillin and others but do not contain cytoplasmic proteins, such as calnexin or GRPp94. Importantly, the topography of exosome molecular surface as well as molecular and genetic contents of exosomes resemble
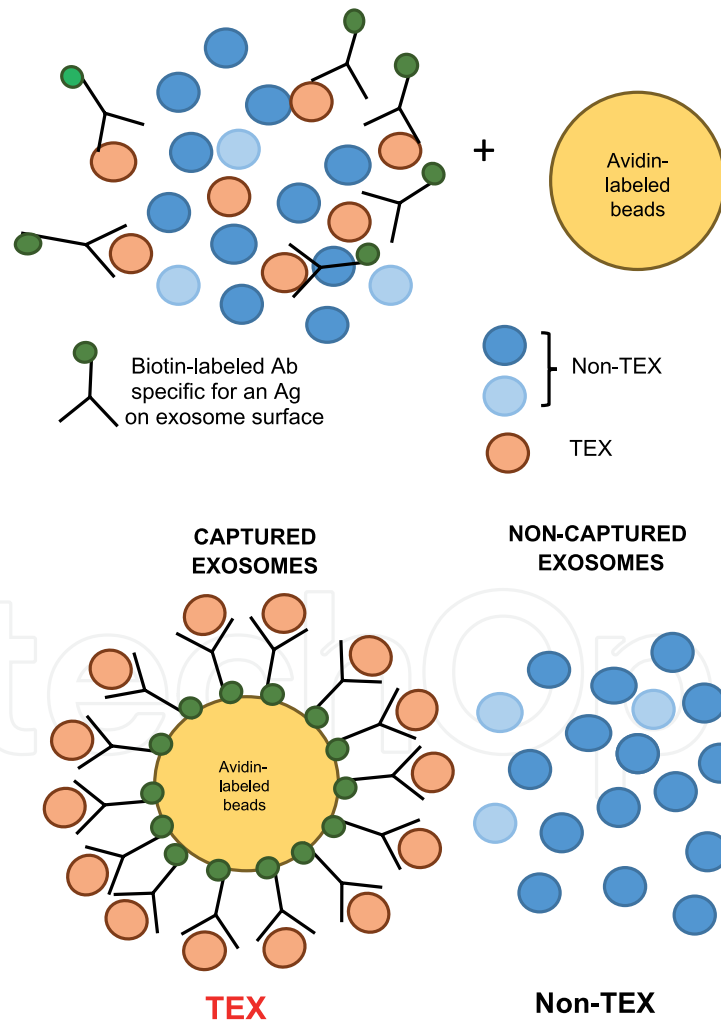

Figure 1.
Schematic view of an immune capture of tumor-derived exosomes (TEX) using an antibody (capture Ab) specific for an antigen epitope present on the surface of TEX. The capture Ab is labeled with biotin and is co-incubated with exosomes isolated by size exclusion chromatography (SEC) from plasma. These exosomes contain TEX and Non-TEX, which are produced by various non-malignant cells. TEX carry the target Ag and are captured by the Ab. Non-TEX do not carry the target Ag and are not recognized by the capture Ab. Streptavidin-labeled magnetic beads are added to the mix of exosomes and biotinylated Ab. TEX are captured on streptavidin beads and recovered using a magnet. Non-TEX are not captured and remain in solution. Captured TEX are then evaluated for their molecular content by on-bead flow cytometry.

those of their parent cells [5]. This similarity of molecular and genetic signatures of tumor-derived exosomes (TEX) to parent tumor cells is the main reason for considering TEX as a "liquid tumor biopsy" [6]. Tumor cells produce large numbers of exosomes, ranging, from 10^{10} to 10^{12}/mL plasma, and plasma of cancer patients is thus variably but significantly enriched in TEX [7]. However, not only tumor cells but also non-malignant cells in the TME, e.g., immune cells, endothelial cells or cancer-associated fibroblasts (CAFs), produce exosomes, which account for a considerable fraction of total EVs present in cancer patients' plasma [8, 9]. Thus, cancer plasma contains a diverse mix of small and large EVs of various cellular origins and presumably with different molecular content in the vesicle lumen and on the vesicle surface membrane. TEX represent a fraction of all circulating EVs that differs broadly among cancer patients; in patients with malignant melanoma, TEX constitute 20–80% of total plasma EVs (our data), and the ratio of TEX/non-TEX increases with melanoma progression **Figure 1** [7].

3. Up-take of EVs and reprogramming of recipient cells

Once exosomes are released from MVBs into the extracellular space, they are disseminated throughout all tissues, enter the circulation and freely cross the blood brain barrier (BBB) and all tissue barriers [10]. Exosomes are taken up and internalized by recipient cells in the circulation and tissues by a variety of mechanisms ranging from endocytosis, phagocytosis or membrane fusion to receptor-ligand mediated entry and signaling as reviewed elsewhere [11] and deliver their cargos to recipient cells, which may be either near or distantly located. Through this mechanism, "*exosome-releasing cells*" can profoundly alter the phenotype and function of "*exosome-receiving cells*". For example, immune cells found in patients with cancer (tumor-infiltrating as well as circulating cells) become polarized or reprogrammed through interactions with TEX, so that from effector cells, they turn into cells facilitating tumor growth [12]. Further, these reprogrammed immune cells now release exosomes that are equipped with immunosuppressive molecules and effectively suppress tumor antigen-specific immune responses. This process of the immune cell "corruption" or "subversion" by TEX is orchestrated by the tumor which utilizes TEX to initiate a "cascade" of secondary EVs, thereby changing the TME into one promoting tumor growth and suppressing anti-tumor functions of immune cells [13].

Body fluids are a diverse mixture of various EV subsets, and in cancer patients, TEX constitute a substantial and functionally important EV subset that is engaged in immune regulation. Mechanistically, TEX-mediated immune suppression involves activation in recipient immune cells of numerous inhibitory pathways, leading to a loss of anti-tumor functions [9, 13]. The result of TEX-driven reprogramming of immune cells is that not only TEX but also immune cell-derived exosomes in the plasma of cancer patients are enriched in immuno-suppressive proteins and upon co-incubation with primary normal immune cells or upon injection into experimental animals, these exosomes mediate immune suppression [14]. Although, TEX carry tumor-associated antigens (TAAs) and thus could be immunogenic, TEX interactions with reprogrammed antigen-presenting cells (APCs) in the TME do not support antigen processing/presentation which normally culminates in T cell responses [15]. Instead, T cells cross talking with TEX are suppressed or induced to acquire a suppressive phenotype (i.e., develop into Treg or myeloid-derived suppressor cells). Suppressive activities of TEX appear to be the major mechanism underlying negative regulation that prevails in the TME.

4. Isolation of exosomes from plasma

An existing barrier that has impeded the progress in EV research has been the lack of methods for their isolation from body fluids in a relatively "pure" form, i.e., without non-specifically attached plasma proteins, and in quantities sufficient for further studies. The current "gold standard" for the isolation of EVs has been the density gradient ultracentrifugation of pre-cleared plasma at 100,000x g for periods of time ranging from 12-24h [16]. Ultracentrifugation using iodixanol density gradients (24 spin time) is currently the preferred isolation method. However, for many reasons, including an inadequate recovery, vesicle aggregation and potential vesicle damage during prolonged ultracentrifugation as well as the isolation platform that does not lend itself to a high throughput required for clinical assessments, ultracentrifugation is being slowly replaced by other methods. The literature is replete in listing various technological advances for EV isolation from body fluids, including microfluidics and sophisticated ultrafiltration systems [17, 18]. Many factors need to be taken into account when selecting an EV isolation procedure, such as the volume of available fluid, desired recovery and purity of EVs and processing time. Among these various methods, size exclusion chromatography (SEC) emerges as the most efficient technique for the isolation of "purified" exosomes or sEVs from plasma [4, 19]. SEC is a readily applicable separation method based on differences in protein size, which removes unwanted soluble proteins from precleared plasma and allows for the recovery of partially "cleaned" exosomes in early fractions [4, 19]. Data from various studies indicate that upon sEV isolation by SEC, glycoprotein aggregates, albumin and other plasma proteins elute in the late fractions, while partly purified, tetraspanin-positive vesicles elute in the early fractions, allowing for a relatively simple, one-step separation of exosomes from most of "contaminating" plasma components. SEC outperforms various precipitation protocols which co-isolate contaminating plasma proteins [19]. It has been suggested that SEC has drawbacks, including relatively poor yield; however, as this results from removal of protein aggregates not a loss of vesicles, the lower yield is counterbalanced by increased sample purity. With relatively minor adjustments, SEC can be used for high throughput isolation of sEVs from serially collected body fluids, yielding partially purified sEVs in early fractions [4]. These exosomes retain their vesicular morphology and phenotypic as well as functional attributes, such as the ability to modify responses of recipient cells to exogenous signaling [4].

The use of SEC has facilitated the: (i) "cleaning" of exosomes from most, although not all, "contaminating' plasma proteins; (ii) separation of soluble Ags that might be weakly "associated" with exosomes from those embedded in or carried on the exosome membrane and (iii) recovery of morphologically intact, non-aggregated exosomes that retain their functional activity [4]. The isolation of non-aggregated vesicles is especially critical for the subsequent immunoaffinity capture of vesicles, because vesicle aggregation is likely to interfere with Ab-based capture. Equally important is the fact that the recovered vesicles retain their functional activity, e.g., are able to induce apoptosis following a brief co-incubation with activated T cells, after removal of soluble plasma proteins. It is for these reasons, that immune capture of EVs from body fluids should be preceded by SEC and not be used for direct EV isolation from plasma.

5. Rationale for sEV fractionation into TEX and non-TEX

In cancer patients, total exosomes isolated from plasma by SEC contain various proportions of TEX. In patients with melanoma, 20–70% of total plasma exosomes

are tumor cell-derived [7]. While total plasma exosomes with a high content of TEX might largely reflect the TEX characteristics, non-TEX present in the mix might influence the estimates of effects plasma exosomes to exert in recipient cells. Thus, the separation of TEX from a mix of other vesicles in plasma is a necessary step to evaluate their unique phenotypic, molecular and functional characteristics. This step may be especially important when TEX account for only a small fraction of total sEVs in plasma. Immunoaffinity capture of TEX from plasma has been introduced as an approach to the pulldown of TEX based on the use of Abs specific for the antigens selectively expressed or markedly overexpressed by cancer cells and carried by TEX [20]. Immunocapture-based exosome isolation from body fluids has been extensively used in diseases other than cancer, including neurological diseases, where Ab-based capture is broadly used for the isolation of neuron-derived L1CAM bearing EVs (NDEVs) [21]. In cancer, TEX separated from non-TEX are expected to serve as a liquid tumor biopsy that faithfully recapitulates molecular and genetic features of parental cancer cells.

6. Immunocapture of TEX from body fluids

In principle, EVs which resemble parent tumor cells and carry on their surface the antigens expressed by tumor cells, should be readily recognized and captured by Abs specific for these tumor-associated antigens (TAAs). There are two major components to this approach that are critical for immunocapture success: one concerns the general strategy used for capture and the other one is the selection of capture Abs.

6.1 Immunobead-based EV capture

The immunocapture-based methods generally use beads coated with selected Abs for EV pulldown. In the simplest approach, beads coated with Abs are added directly to plasma diluted in phosphate-buffered saline (PBS) with the expectation that all EVs bearing the target Ag on the surface will bind to the Ab coated beads. This strategy for capture may not be and usually is not very effective, because EVs in plasma carry a variety of soluble plasma proteins on the surface, such as albumin, immunoglobulins (Igs) and other "contaminating" plasma proteins. These plasma proteins form a protein "corona" associated with the sEV surface membrane which is likely to block the access of capture Abs to targeted Ags, leading to an incomplete pulldown or even lack of pulldown. Also, if the target Ag is present in soluble form in plasma, it might compete with the counterpart carried on the EV membrane, binding to the Ab coated beads either specifically as a soluble protein or as a "contaminating" EV surface-associated protein. The soluble target Ag present in plasma (especially when its abundance is high) could non-specifically associate with proteins/glycoproteins decorating EV surfaces. As a result, the target Ag could mediate the pulldown of EVs that do not constitutively express the Ag. The result will be a pulldown of EVs carrying a soluble Ag in addition to EVs genuinely endowed with the target Ag embedded in the EV membrane. Such capture will not distinguish between these two types of EVs, and thus the strategy is useless for selective capture of EVs carrying the targeted tumor-specific Ag. This capture strategy has been also used with EVs isolated from plasma by ExoQuick, which concentrates rather than "purifies" EVs, with the same unsatisfactory results [21].

Perhaps a good example of this strategy is immune capture from cancer patients' plasma of EVs carrying PD-L1 as recently reported [22]. This protein, commonly carried by TEX in most cancers, is also present in the plasma of cancer patients both

as a soluble protein derived from malignant and various non-malignant cells and as the integral membrane protein of non-TEX released by macrophages or other immune cells [23]. Therefore, beads coated with anti-PD-L1 Abs cannot be used for selective capture of PD-L1-positive TEX, because such beads will capture soluble PD-L1, non-TEX carrying PD-L1 as well as TEX carrying PD-L1, thus making it impossible to distinguish which EV subset delivers inhibitory signals to PD-1-positive recipient cells. The contribution of soluble PD-L1 to negative signaling by the captured vesicles may not be disregarded, because in addition to its specific binding to Ab-coated beads, soluble PD-L1 might non-specifically "associate" with all EVs in plasma, similar to albumin or other plasma proteins. Data in the literature [24] and the protein content of the ExoCarta data base [25] confirm that EVs isolated from plasma carry numerous non-specific plasma-derived proteins and suggest that the discrimination of "true" EV proteins from plasma "contaminants" is a major challenge in the field.

6.2 Selection of abs for immune capture of TEX

The selection of Abs for TEX immune capture depends on the convincingly demonstrated ability of such Abs to selectively bind to tumor cells expressing the target Ag on the cell surface, with the exclusion of any binding to non-malignant cells which do not express the Ag. This is a rigorous requirement and one that may be difficult to implement, because few tumor-specific antigens are known, except for mutated epitopes in cancer cells. To emphasize, the capture Abs selected for TEX capture must be specific for an antigen (or an epitope) present only in parent cells and in EVs these parent cells produce but not in any other cells or tissues. Even if such tumor Ag- specific Ab is available, it is necessary to ensure that its binding affinity for the target Ag is high and that the target antigen is not present in soluble forms in body fluids. Low-affinity Abs will not be effective in pulldowns, and the presence of a target Ag in soluble forms might not only interfere with Ab binding to TEX but upon its non-specific association with EVs, as described above, will lead to the capture of EVs derived from non-malignant cells, thus interfering with selective TEX capture. As most Ags expressed on the surface of cancer cells are enzymatically cleaved and are present in plasma, this requirement may not be readily addressed. Importantly, adding Ab coated beads directly to plasma without prior attention to these restrictions will jeopardize the selectivity and efficiency of TEX immune capture.

Clearly, the use of the best capture Abs is by far the most critical aspect of TEX immune capture from plasma. In the absence of such Abs, immune capture using a mix of Abs specific for Ags highly overexpressed on cancer cells relative to non-malignant cells and on the EVs these cells produce could be utilized for immune capture, and this approach has been successful [26]. It is possible to perform immune capture of TEX with a cocktail of Abs carefully selected for specificity to proteins overexpressed on tumor cells and weakly expressed on non-malignant cells. The complexity of immune capture increases with the use of Ab cocktails largely due to `Ab titration requirements and the need for extensive controls as well as the limitations imposed by the presence in plasma of the Ags recognized by the Abs used for immune capture, in soluble form.

7. A successful TEX capture from plasma of patients with melanoma

Compelling evidence indicates the immune capture method to isolate TEX from patients' plasma yields excellent results when essential requirements are met. For example, we have reported separation of TEX from non-TEX in plasma of

patients with melanoma [20] that have allowed for extensive characterization of the molecular cargo and functional repertoire of these sEV fractions [27]. The immune capture of TEX was performed using chondroitin sulfate peptidoglycan 4 (CSPG4)-specific mAbs developed by one of us [28]. These mAbs recognize CSPG4 which is selectively expressed on melanoma cells (and on the EVs these cells produce) but is not detectable on any other non-malignant cells in the body except for activated pericytes in the TME [29, 30]. Immunohistochemical staining with mAbs of more than 2000 melanoma lesions has showed that CSPG4 is expressed on about 80% of all investigated melanoma specimens [29, 30]. In melanoma tissues, CSPG4-specific mAbs decorate the surface of malignant cells; flow cytometric analysis of EVs stained with mAbs visualizes CSPG4 on their membrane [31]. Monoclonal Abs recognizing distinct CSPG4 epitopes are available to be selectively used for the capture of TEX, which is isolated and "purified" by SEC, and for subsequent antigen detection by flow cytometry confirmed that CSPG4 is expressed on TEX but is not detectable on non-TEX [7]. As with all immune capture experiments, titrations of the capture and detection Abs are critical for success as are the vesicle/Ab ratios, and these must be determined a priori and strictly adhered to during capture. As described in detail elsewhere [7, 20], all immunocaptured melanoma TEX are positive for CSPG4 and for melanoma-associated antigens (MAA), while non-TEX are negative. The exosome recovery ranged from 60 to 100 μg protein/mL plasma, and the ratio of TEX/total exosomes in plasma varied among melanoma patients from 0.2 to 0.6. The separation by immune capture of melanoma TEX from non-TEX yielded sufficient numbers of both exosome fractions for studies of their protein content by on-bead flow cytometry and by high-resolution mass spectrometry (HRMS) as well as of their function in co-incubation assays with immune cell subsets [7]. TEX were enriched in immunosuppressive and non-TEX in immunostimulatory proteins, and co-incubation of the fractionated exosomes with immune cells confirmed their distinct immunoregulatory functions [7]. Melanoma TEX carrying CD39, CD73, FasL, PD-L1, TGF-β and TRAIL, among other suppressive proteins consistently inhibited functions of immune cells, while non-TEX were stimulatory in co-incubation assays. Using LC–MS/MS-based proteomics, we identified a profile of 16 proteins highly overexpressed in TEX which discriminated TEX from non-TEX. These proteins were components of molecular pathways mediating cellular events such as vesicle transport, immune reactivity, signal transduction, and disease activity [27]. Further, by dividing the analyzed melanoma patients into two groups of 7 patients with no evident disease (NED) and 8 with progressive disease (PD) at the time of phlebotomy for exosome isolation from plasma, we were able to identify a signature of 12 proteins significantly and consistently overexpressed in TEX of patients with PD relative to TEX of patients with NED [27]. This ability of TEX bearing the signature of 5/12 most significantly ($p < 0.0003$) overexpressed proteins to discriminate melanoma patients with PD from those with NED within a very small patient cohort emphasizes the potential of TEX to serve as a biomarker of disease activity in melanoma [27]. In addition, this immunocapture-based study was the first to show that melanoma TEX, which are especially abundant in plasma of patients with advanced disease, are largely responsible for immune suppression that potentially promotes immune escape and tumor progression.

8. Immune capture of T cell-derived sEV from plasma

The T cell receptor (TCR) is expressed only on T lymphocytes, and an Ab specific for CD3, a protein component of the TCR complex, decorates the surface of sEV produced exclusively by T cells. A high-affinity Ab specific for CD3 proved

to be an excellent candidate for immunocapture and subsequent characterization of sEV in plasma of patients with cancer or HDs [32].T cell-derived sEV account for a considerable proportion of total plasma exosomes in cancer patients, and their phenotypic profiles examined by on-bead flow cytometry recapitulate those expressed by various T cell subsets as we reported [32]. CD3-based immune capture allowed for isolation from plasma of CD3(−) sEV fraction enriched in TEX and CD3(+) sEV fraction that was useful for evaluations of phenotypic and functional changes induced in the cancer-reprogrammed T cells [33]. Thus, this type of immune capture allowed for simultaneous analysis of molecular profiles in tumor-derived and immune cell-derived sEVs in the same plasma sample. The procedure for immune capture of CD3+ EVs from the plasma of cancer patients is described in Current Protocols in Immunology [34].

9. Conclusions

TEX are rapidly emerging as the major component of immunoinhibitory signaling that prevails in the TME. TEX present in body fluids a subset of circulating EVs. The large quantity and enormous diversity of circulating EVs in plasma of patients with cancer with respect to cellular origin, molecular characteristics, genetic content and functions imposes a need for the isolation of TEX and their separation from non-malignant vesicles. This approach allows for studies of impact TEX exert on cells in the TME and of TEX value as potential cancer biomarkers. To dissect the EV diversity in body fluids, strategies are necessary for their capture, isolation from body fluids and separation of various EV subsets without interference with EV molecular identities and functions. Among various isolation strategies, immune capture with Abs specific for proteins carried on the EV surface has been most frequently utilized with a variable level of success. While in principle, immune capture is the rational strategy for EV pulldown from plasma, its application to nanovesicles requires an understanding of EV characteristics and EV biology. As the latter is still largely lacking, all EV immune capture strategies may backfire, for reasons that may not be anticipated, such as high levels of a soluble target Ag in plasma or the presence on vesicles of a "corona" of contaminating plasma proteins. The set of stringent requirements for vesicle immune capture from plasma that we have discussed allows for overcoming some, but probably not all, of the barriers we might face in the future while attempting to study the diversity EVs in body fluids. As this diversity is of key importance in understanding the role EV subsets such as, e.g., TEX, play in health and disease, immunoaffinity EV capture is likely to remain the method of choice for selective TEX pulldown. However, only when performed correctly, immunoaffinity capture of TEX yields valuable insights into their potential as cancer biomarkers and as markers of immune competence.

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