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Biology and proteomics of extracellular vesicles: harnessing their clinical potential

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Abstract

Extracellular membrane vesicles have recently emerged as versatile mediators of intercellular communication, pathogenesis, drug and gene delivery and as potentially rich reservoirs of clinical biomarkers. Channeling their properties toward patient care is dependent on technological progress in approaches used for their analysis and molecular profiling.

Keywords

disease biomarkers; exosomes; microvesicles

Eukaryotic cells release membrane vesicles under physiological conditions, although aberrant shedding of vesicles into the extracellular environment can arise in disease states, as has been well documented in cancer [1,2]. These shed membrane vesicles vary with respect to morphological and functional characteristics. They may originate either at the plasma membrane (ectosomes) or are generated in intracellular multivesicular bodies and released upon fusion of the limiting membrane of multivesicular body with the cell surface (exosomes) [3]. Shed vesicles that originate at the cell surface are larger than exosomes and can range from 200 nm to a few microns in diameter. Vesicles that fit these latter criteria and derived from tumor cells have been referred to as tumor-derived micro-vesicles and oncosomes [4–6]. All of the aforementioned membrane-enclosed sacs, collectively referred to as extracellular vesicles (EVs), can impact a variety of cellular processes, including inflammatory responses and cell invasion [5,7,8]. They contain complex sets of cargo depending on the physiological conditions in which they are generated and released. EVs may indeed act as mediators of paracrine signaling because of their ability to transfer bioactive molecules, including lipids, proteins and functional nucleic acids to recipient cells

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in the extracellular microenvironment [1,2,5,7]. This transfer of functional molecules may result from direct membrane fusion or the internalization of the EVs into the target cell [2]. Moreover, their release into body fluids such as blood, saliva and urine, points to their potential as circulating biomarkers in the surveillance of disease progression [9]. EVs have been detected in the circulation of patients with several types of cancers such as glioblastoma, breast, lung, ovary, prostate, colorectal and gastric cancers [9]. Mass spectrometry (MS)-based proteomic profiling of EVs in the past several years have allowed their characterization from different types of cell lines, body fluids and various animal and human organ models of disease, generating a plethora of data stored in openly accessible databases such as ExoCarta [10] and Vesiclepedia [11].

Protein profiling of EVs released from human colon and prostate cancer cell lines shows that these vesicles contain signaling molecules that could potentially affect processes such as angiogenesis, cell metabolism, mRNA processing, cell invasion and growth, in addition to molecules that are likely required for vesicle biogenesis [12]. In early MS-based investigations, EV proteins were first separated by gel electrophoresis allowing identification of proteins enriched in EVs [13–15]. In more recent work, EVs purified by ultracentrifugation and more sophisticated fractionation methods, in addition to affinity binding have been used in high-throughput proteomic studies increasing the number of EV-associated proteins [16,17]. These and other studies [2] have suggested that inclusion of protein cargo into shed vesicles is not based on protein abundance or random blebbing but rather on selective sorting of cargo into shedding EV populations. Given the complexity of cargo, both proteins and nucleic acids, it is likely that more than one intracellular transport route engages in cargo delivery to sites of EV biogenesis. Co-sorting of cytoplasmic proteins via protein–protein interactions have also been proposed to play a role [17]. A recent study that analyzed 78 qualified high-throughput data sets on EV protein profiles, found that 797 common EV proteins were those originating from the cytoskeleton, cytosol, plasma membrane and intracellular vesicles [16]. Annotation of these proteins to biological processes revealed association with cell motility, metabolism, apoptosis regulation, small GTPase-linked signaling and vesicular transport. Furthermore, protein interaction network analyses reveal that these 797 proteins were extensively connected via physical interactions, whereas functional connections displayed modules for cytoskeleton remodeling and vesicular transport.

As stated earlier, given the heterogeneity of EV populations, it is likely that multiple shed vesicle populations were examined in the majority of investigations described to date. Particularly advantageous to the enrichment of EV antigens for use as diagnostic or prognostic indicators would be the ability to effectively concentrate and capture individual populations of EVs from body fluids. This approach, which is distinct from the analysis of whole tissue or unfractionated body fluid or even circulating tumor cells, would be especially significant if individual vesicle populations indeed concentrate molecular changes that occur in the parental tissue, as it would increase the sensitivity of detecting critical markers of disease progression. In this regard, molecular markers that would aid in the identification and/or capture of individual vesicle populations would be particularly beneficial. In a recent study utilizing immunobead-based capture, exosomes from colon cancer cells with protein marker A33 could be separated from those that contained epithelial

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cell adhesion molecule [18]. Proteomic profiling revealed significant differences in the two exosome subpopulations.

A largely unexplored hypothesis is that the proteins in individual EV populations might bear characteristic post-translational modifications. Unique glycosylation patterns of growth factors and signaling proteins, as they traffic through the endoplasmic reticulum and Golgi, might be one such modification. Similarly, investigations of the phospho-proteomic profile of individual shed vesicle populations could reveal unique modifications of cargo and will also shed light on current understanding of EV biogenesis. Lipid-based post-translational modifications in EVs are also yet unexplored. Palmitoylation of several signaling proteins, including Src family kinases and Ras, modulate protein localization in specific plasma membrane microdomains [19]. Such lipid anchors could promote stabilization of tetraspanin microdomains or even facilitate protein sorting to EVs, although this has yet to be explored. Importantly, post-translational modification could also potentially provide insight into physiological cellular changes.

EVs could also offer markers that are tissue specific. Panels of EV markers in combination with tissue-specific markers would potentially constitute unique and identifiable biosignatures for individual cancers or other types of disease. Furthermore, as the genome and proteome of tumors undergo changes with disease progression or in response to therapy, EVs might also be useful in disease staging as well as assessing the efficacy of response to therapy. This tenet is especially pertinent for tissues such as the ovary, prostate or pancreas, where repeat biopsies of these organs are difficult or unrealistic. Besides blood, sampling of other body fluids (e.g., saliva, urine, ascites or pleural effusions) could enable efficient disease detection. Complicating the utilization of EVs in cancer detection strategies is the presence of shed vesicles from other non-tumor cell types that likely might also be present in body fluids. Thus, it is equally important to identify markers that would allow selective capture of individual subpopulations of tumor-derived EVs, away from other shed vesicle populations in fluids. The methods currently used to discriminate between various shed vesicle populations found in peripheral fluids have been discussed [2,20], but a refinement of these procedures and additional approaches is needed.

Molecular profiling of EVs could form the basis of personalized therapeutics, especially as more reliable and rapid profiling technologies become available. For example, patients presenting increased expression of the breast cancer-associated oncogenic receptor, HER-2/neu, in subsets of gastric and ovarian cancers would benefit from existing breast cancer treatment strategies that target the HER-2 receptor [21]. Indeed, HER-2 has been detected in serum microvesicles derived from gastric tumors [22]. This type of information can be vital, as therapeutic treatments are coupled to ‘oncogenic’ mutations and cellular changes. The detection of such targets in EVs could pave the way for potential diagnostic and therapeutic strategies that preclude the need for major surgical intervention.

Recent work has explored the potential of EVs as novel therapeutic vehicles in cancer immunotherapy, as well as delivery of RNA-interference reagents and drugs [23,24]. EVs are poorly immunogenic and therefore protect its cargoes from rapid degradation *in vivo*, a distinct advantage over the packaging of similar cargo in liposomes [25]. Further

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investigation into the circulating levels and clearance dynamics *in vivo* is warranted to better define therapeutic effectiveness of EVs as delivery vehicles in clinical applications.

In conclusion, proteomic profiling studies represent a valuable, emerging strategy to gain better insight into the biology and clinical potential of circulating EVs in patients. Shotgun proteomic approaches, especially LC/LC-MS/MS-based techniques such as multidimensional protein identification technology that allow rapid and global protein identification and quantification [26], as well as multiplexed multiple reaction monitoring wherein peptides can be quantitated in a single analysis at high flow rates [27], coupled with sensitive immunodetection methods, would be especially helpful in this regard. Further, high-resolution MS, as has been recently reported to detect secreted proteins with picogram sensitivity [28], would be especially well suited for proteomic profiling of EVs in body fluids. However, resolving of EV heterogeneity and improvement in and standardization of isolation protocols are warranted to fully unleash the clinical potential of circulating EVs.

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