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Characterization and applications of extracellular vesicle proteome with post-translational modifications

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ABSTRACT

Extracellular vesicles (EVs) are a diverse population of complex membrane-encapsulated vesicles released by a variety of cell types and exist in most of body fluids. Continuously growing number of reports revealed that EVs participate in multiple biological processes, such as intercellular communication, immune regulation, and dissemination of cancer cells. Accordingly, recent attention has been given to the characterization of extracellular vesicles and their components. This review focuses on state-of-the-art proteomic technologies to analyze proteomes of EVs, especially their post-translational modifications (PTMs). With their strong biological relevance and the relatively noninvasive accessibility from body fluids, the promising potential and early applications of EV proteome and its PTMs as attracting biomarker sources are also evaluated.

1. Biological implications of EV proteome and its post-translational modifications

To date, the term extracellular vesicles (EVs) is typically given to include classes of vesicles released by cells into the extracellular environment, mainly including exosomes (30–100 nm in diameter), ectosomes or shedding microparticles/microvesicles (100–1000 nm), apoptotic blebs (50 nm-2μm), oncosomes (1–10 μm), and other EVs subsets, as reviewed by the International Society of Extracellular Vesicles (ISEV, www.isev.org). EVs are widely present in cell culture media and body fluids (urine, blood, saliva, etc.), and different EVs have fundamental differences in terms of origin based on their biogenesis or release pathway [1]. In general, microvesicles (MVs) are produced by direct shedding from the plasma membrane following stimulation so they demonstrate features of cell apoptosis, while exosomes are generated through endosomal processes in which multivesicular endosomes fuse with plasma membranes and release to extracellular systems. So far, the most conceivable way of MV and exosome generation involves the formation of intraluminal vesicles in multivesicular endosomes (MVEs) and exosome are released during the process of fusion between MVEs and lysosomes or plasma membrane. There is also another mechanism for the generation of exosome, especially that T cells and erythroleukemia cell lines may generate exosomes from plasma membrane immediately upon such stimulations as HIV Gag, Nef expression or special surface receptor cross-linkings. Exosomes formed by either of these two types of mechanisms have similar parameters in most aspects, including size, density and surficial protein markers (CD9, CD63 or CD81) [2]. Due to different formation procedures, MVs typically have the size of 100 nm–1000 nm, larger than exosomes (30 nm–100 nm), and this has been a basic criterion to distinguish MVs from exosomes [3]. In this review, we mainly focus on these two types of EV subsets, MVs and exosomes.

It has been known that EVs play important roles in circulating physiological and pathological behaviors of cells and in regulating a diverse range of biological processes. With cargoes like proteins, RNA, DNA, and lipids encapsulated in a lipid bilayer, EVs serve as messengers in intercellular communication networks. Communication between cells and EVs can be achieved through ligand-receptor interactions at the surface of cells and EVs or through releasing EV contents into recipient cells by fusion either directly with the plasma membrane or with the endosomal membrane after endocytosis [4]. Besides communicating information between cells, EVs have been reported to promote cellular migration and invasion through interaction with the extracellular matrix (ECM) [5]. EVs also play critical roles in the immune process, and tumor-derived

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EVs have been reported to stimulate or suppress tumor-specific and non-specific immune responses [6]. Importantly, in recent years, EVs have proved to have promising clinical potentials with their presence and stability in most bodily fluids and have raised considerable interest as a source for the discovery of biomarkers. Multiple RNAs or proteins from EVs are reported as potential biomarkers for prognosis or diagnosis of diseases. For example, an EV membrane-anchored proteoglycan molecule called glypican-1 (GPC1) was used to detect the early-stage pancreatic cancer, based on the EVs derived from blood stream [7]. EVs were also used as therapeutic methods of many diseases [8], such as being an inhibitor to suppress tumor growth [9] or being a vehicle to achieve drug delivery [10].

Owing to biological processes EVs are involved and their unique values in clinical applications, EVs have attracted a lot of attention in recent years. Particularly, recent applications of "-omics" techniques in the field of EVs provided greater information than before, and researchers become aware that EVs undoubtedly contain vital clues essential for better understanding of the function of vesicles [11]. Among many -omics techniques, proteomics of EVs is one of the most important fields because proteins represent actual functional molecules in the cell and most cellular functions are carried out by proteins. In a biological process where proteins participate, various PTMs finely tune the cellular functions of each protein, and EVs proteome may provide snapshots of the process. As PTMs are mainly the addition of a functional group covalently to a protein and thus influence numerous properties and functions of proteins, they engage in many cellular processes such as cellular differentiation, protein interactions, subcellular location, signaling and regulatory processes, regulation of gene expression, and protein-protein interactions. During the formation of EVs and in the biological process EVs participate, diverse PTMs of proteins are involved. Carbohydrate structures, like oligosaccharide, polysaccharide structures as well as glycoproteins, are important component of EVs surface. Studies indicated that glycosylation clearly participates in exporting and uptaking regulation during the formation of EVs [12]. Phosphorylation was reported to involve in preventing endosomal degradation of proteins, and influence EV biogenesis [13]. Ubiquitin and Ubiquitin-like modifiers (UBLs) are reported as major controllers of EVs protein loading. Although the detailed mechanisms are still not very clear, several studies showed that cargo proteins undergo ubiquitination and deubiquitination sequentially [14]. Conceivably, the importance of PTMs has highlighted the need for a better understanding of EV PTMs and particularly the need for deeper, comprehensive analyses of PTMs related to EV proteins.

Mass spectrometry (MS) coupled with liquid chromatography (LC) has been the most powerful tool for the large-scale identification of protein PTMs. Although large-scale analysis of several PTMs has been successfully achieved in the past decades using the LC-MS based proteomics strategy, high-throughput PTM discovery remains a challenge for a variety of reasons. First, although most proteins can undergo a wide range of PTMs, these modified proteins are often at low stoichiometry compared with unmodified proteins. After enzymatic digestion of proteins to generate peptides, peptides containing modifications can be further overwhelmed by unmodified peptides, making them difficult to be detected in the MS analysis. Second, some labile modifications, such as phosphorylation, may be unstable during in vitro sample preparation due to the phosphatase activity, resulting in the loss of their information. Third, some modifications increase the hydrophilicity of peptides or decrease the ability of protonation of peptides, making the modified peptides difficult to be detected due to the inefficient ionization. Moreover, proteins may have different abundance of PTMs at different times or under different conditions during development, further increasing the difficulty of comprehensive PTM analysis. To overcome these difficulties, it is increasingly apparent that expanding our ability to detect and identify these PTMs by MS become a prerequisite of understanding the role of PTMs.

Here we review recent advances in the characterization of EV proteome and its PTMs, particularly focusing on MS-based technical advances and challenges for EV analyses.

2. Proteomics strategy for studying EV proteomes and PTMs

2.1. General proteomic strategy for studying EV proteomes and their PTMs

A general proteomics strategy for studying EV proteomes and their PTMs involves the following several key steps (Fig. 1): 1) Separation EVs from cell culture media or body fluid; 2) Characterization the purity of EVs; 3) Extraction and digestion of EV proteins; 4) (Optional) label peptides with isotope tags for quantitation; 5) Enrich peptides with PTMs of interest; 6) LC-MS analysis for identification and/or quantification; 7) Database search to identify and quantify EV proteomes and their PTMs. Among these steps, Steps 3–7 are similar to procedures in a general proteomics strategy, and Steps 4 is optional according to the aim of a specific study. In this review, we will focus on discussing the separation and characterization EVs, enriching the modified peptides for identification and quantification, and their biological and clinical applications.

2.2. EV isolation and characterization

2.2.1. EV isolation

Isolation of EVs from cell culture media and biofluids with high purity and high yield is the most important prerequisite for the downstream EVs proteome analysis. First, contaminants like aggregated proteins and nucleic acids need to be minimized during the isolation to guarantee the purity of isolated EVs. Second, the isolation of EVs needs to be in high yield because the amount of proteins from EVs, especially the modified proteins, is typically low. Third, during the isolation process, EVs have to be intact to prevent internal cargoes from releasing to outside. Current approaches widely-used in EVs isolation includes differential ultracentrifugation (DUC), immunoaffinity isolation, size exclusion chromatography (SEC), polymer precipitation and etc. Each isolation method has its own advantages and disadvantages in terms of yield, purity, isolation time, cost, demand of instrument et al. (Table 1).

DUC is considered as a "golden standard" isolation method of EVs and probably the most commonly used method [15]. The overall protocols are similar, typically starting with sequential centrifugation steps with increasing centrifugal forces (g) to remove intact cells/cellular debris (300 × g) and apoptotic bodies (2000–3000 × g for 30 min). Then MVs are isolated with the speed of 10,000–20,000 × g. Finally, the supernatant is subjected to 100,000–200,000 × g for 60 min and longer to get exosome pellets at the bottom. Changes of centrifugation speed are necessary to fit for different samples, such as conditioned culture media and various biofluid types with different properties such as viscosity. According to different samples, it is also necessary to extend/shorten centrifugation time, adjust the temperature, etc. DUC has clear advantages, including tolerance of relatively low volumes of sample, low cost for a single experiment if high speed centrifugation instrument is available, no additional chemicals that would increase the complexity of samples, and so on. As little as 0.5 mL urine can be isolated using the DUC process and the maximum volume is limited by the total available positions in a rotor, which