

Functional Properties of Circulating Exosomes Mediated by Surface-Attached Plasma Proteins

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Abstract

Background: Exosomes and other types of extracellular vesicles present an important component of circulating plasma. Exosomes released by endothelial and blood cells account for majority of plasma exosomal population; exosomes secreted by other cells might cross tissue-plasma barrier and reach circulating plasma as well. Definitely, exosomes of different cellular origins are different by content and function. However, exosomal surface membrane interacts with plasma components. This interaction may alter composition of exosomal surface and hence, provide these vesicles with new functional properties. This study was aimed to estimate composition and possible functional role of proteins attached on the surface of plasma exosomes.

Methods: Here, extracellular vesicles from human plasma were isolated by ultracentrifugation and treated by trypsin. Trypsinized and native exosomes were analyzed by nanoparticle tracking analysis, Western blotting and quantitative high-resolution mass spectrometry.

Results: Surface-attached proteins were removed from exosomes isolated from plasma of healthy donors by incubation with serine protease (trypsin). Treatment did not impact exosomes integrity while

slightly reduced hydrodynamic radius. Mass spectrometry revealed 259 exosomal proteins; among them 79 proteins were completely removed and more than half of the proteins were partially removed by trypsinization. Gene ontology functional annotation revealed mostly extracellular locations of proteins cleaved from a surface of the plasma exosomes. Moreover, proteins cleaved from the exosome surface are supposed to be implicated into integrin-linked kinase (ILK), focal adhesion kinase (FAK) and other pathways connecting cell surface with intracellular signaling cascades.

Conclusion: Taken together, our results demonstrate that a surface of circulating exosomes is decorated by plasma proteins, and these proteins can mask tissue-specific characteristic of the exosomal surface membrane and provide exosomes with new and uniform properties.

Keywords: Exosomes; Plasma proteins; Mass spectrometry; FAK signaling; ILK signaling

Introduction

Exosomes are a sort of extracellular vesicles (EVs) derived from endosomal system and released out of cells upon fusion of multi-vesicular endosomes with a cell membrane. Exosomes differ from other types of EVs by relatively small size (50 - 130 nm) and expression of specific exosomal markers (CD9, CD63 and others). It was assumed earlier that exosomes involved in clearance of cellular waste, while recently exosomes are attracting considerable attention as mediators of intercellular communication [1]. Exosomes can be found in most of biological fluids. Concentration of the plasma exosomes isolated by differential centrifugation and measured by nanoparticle tracking analysis (NTA) is $9.5 \times 10^{10} \pm 1 \times 10^{10}$ vesicles/mL [2] that is equivalent to about 0.1-0.3% of the plasma proteins (166 \pm 50 μ g/mL). Assuming high level and variable aspects of functional activity, plasma exosomes might represent an important component of the blood in terms of its defensive, homeostatic and signal transduction properties.

The plasma exosomes have been investigated by different approaches including mass spectrometry [3]. Proteomics of plasma exosomes can reveal specific subsets of proteins reflecting various cellular functions. For instance, protein profile of plasma vesicles resembled protein profiles of vesicles from

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platelets, antigen-presenting cells, and natural killer T cells [4]. Moreover, cancerous disease or other systemic pathologies may affect composition of blood-cell derived vesicles in plasma or even change total content of plasma exosomes [4]. Currently, plasma circulating exosomes are considered as a promising source of markers for diagnostic and monitoring of various illnesses including cancer [5]. Despite great effort done in exosomes research, there are still considerable challenges associated with heterogeneity of plasma exosomes including cellular origin, molecular composition and functions [6]. Individual variability of donors [7] and methodological preferences of researchers [8] also introduce certain bias into exosome research.

One possible approach to identify different populations of plasma exosomes is subtraction of common characteristics. For instance, separation of exosomal samples from contaminating plasma lipoproteins allowed detailed analysis of exosomal proteome and discovery of new exosome-associated proteins [9]. Besides lipoproteins, other plasma components, like signal peptides or proteins, are interacting with circulating exosomes. Thus, plasma proteins can attach to the surface of vesicles and provide them with certain uniformity. This assumption is in line with results of recent study: serum exosome proteome is represented by extracellular proteins by 23% [10]. Extracellular components absorbed to the vesicular membrane during circulation can mask initial diversity of the exosomes and even provide them with universal functional properties. For example, fibronectin (FN) attached to exosomes is shown to be involved in various aspects of exosomal function [11, 12]. This study was aimed to estimate composition and possible functional role of proteins attached to the surface of plasma exosomes.

Material and Methods

Plasma collection, isolation and characterization of EVs

Design of the study and use of human material was approved by local research ethics committee of N.N. Petrov National Medical Research Center of Oncology (no. 4-14/13.02.2014). All participants have signed informed consent form before collection of the biological material (blood). In order to avoid possible impact of gender difference in content and quantity of plasma vesicles, only female donors were included in the study. Blood samples were collected from 10 healthy donors in the department of blood transfusion of N.N. Petrov National Medical Research Center of Oncology using EDTA-coated vacutainers. Exosomes were isolated by differential centrifugation, as previously described [13]. The size, homogeneity and concentration of isolated exosomes were evaluated by dynamic light scattering (DLS) and NTA according to the manufacturer's instructions (Malvern, UK). Techniques of atomic force microscopy (AFM) and cryo-electron microscopy (cryo-EM) were used for the direct visualization of exosomal particles, as described earlier [14, 15]. The presence of exosomal markers (CD63, CD9) was assayed by flow cytometry with Exo-FACS ready-to-use kit for human exosome analysis (HansaBioMed, Estonia).

Enzymatic modification and analysis of plasma exosomes

Exosomes isolated from plasma by ultracentrifugation were treated with 0.25% trypsin for 1 h, washed with phosphate-buffered saline (PBS) and purified again. Trypsinized and intact exosomes were analyzed by NTA, Western blotting (WB) and mass spectrometry (MS). For WB, protein concentration in extracts was normalized using Bradford method (Bio-Rad, USA). WB was performed as described [13] with primary antibodies against FN (ab2413; Abcam, USA) and CD63 (ab68418; Abcam, USA) followed by a peroxidase-labeled secondary antibody (A0545; Sigma, Germany). MS (shotgun analysis) of exosomal proteins was performed according to the filter-aided sample preparation (FASP) protocol [16, 17]. Briefly, the proteins were digested with trypsin (Promega Trypsin Gold), and MS/MS analysis of resulting peptides was performed in duplicate using an Orbitrap Fusion Lumos MS (Thermo Scientific, USA) [18, 19]. The data were searched by Mascot 2.4.1 (<http://www.matrixscience.com/>). As a protein sequence database, NeXtProt (October 2014) was used. To be included in the list of exosomal proteins, we considered only the proteins with high-quality identifications (≥ 2 peptides, 95 % confidence at least in one sample (treated or not treated by trypsin)). The exponentially modified form of protein abundance index (emPAI) defined as the number of identified peptides divided by the number of theoretically observable tryptic peptides for each protein was used to estimate protein abundance [20, 21].

Gene ontology (GO) functional annotation and pathway analysis

GO analysis of exosomal proteins was performed with functional annotation tool of The Database for Annotation, Visualization and Integrated Discovery (DAVID) [22, 23]. Human molecular pathways were extracted from the following databases: BioCarta (https://cgap.nci.nih.gov/Pathways/BioCarta_Pathways), KEGG [24], NCI [25], Reactome [26] and SABiosciences Pathway Central (<http://www.sabiosciences.com/pathwaycentral.php>). We calculated pathway activation score (PAS) for each pathway from the above-mentioned databases. PAS of the pathway P is the number of exosome surface genes, which are involved in the pathway P. P value calculation was performed using DAVID software (Pubmed PMID: 19131956). Briefly, P value was calculated with modified Fisher's exact test (column "P value", Supplementary Table 1). Adjustment for multiple testing was performed using the Benjamini-Hochberg procedure (column "False Discovery Rate corrected P-value", Supplementary Table 1).

Results and Discussion

Surface of circulating exosomes is decorated by various extracellular proteins

Isolated vesicles were characterized in accordance with Inter-

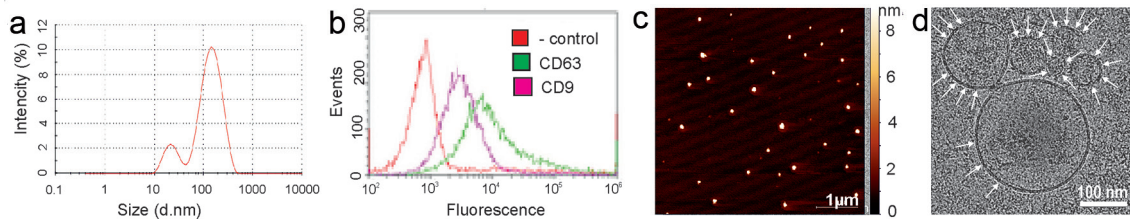


Figure 1. Exosomes characteristics. (a) Exosome size was estimated by dynamic light scattering (DLS). (b) Flow cytometry analysis of exosomes for the surface expression of exosomal markers CD9 and CD63 was carried out by ready to use kit for FACS analysis of purified exosomes (HansaBioMed). Negative control Exo(-) was performed without any vesicles. (c) The surface topography of plasma exosomes obtained with atomic force microscopy (AFM). The scale bar is 1 µm. On the right is the pseudocolor ruler indicating the particles' height (nm). (d) Cryo-electron microscopy (Cryo-EM) of the exosomes derived from plasma. The scale bar is 100 nm.

national Society for Extracellular Vesicles (ISEV) guidelines [27]. Size of vesicles was in a range of 70 - 200 nm, as it was measured by DLS (Fig. 1a). The morphology of single vesicles was assayed by AFM and cryo-EM (Fig. 1c, d). Presence of exosomal markers (CD9, CD63) on the surface of vesicles was confirmed by flow cytometry (Fig. 1b). Thus, nanovesicles isolated from human plasma had a typical spherical form, contained a bilayer membrane, were enriched with exosomal markers and were considered to be exosomes. Freshly iso-

lated and characterized exosomes from 10 individuals were pooled in equivalent quantities. Afterwards, half amount of pooled samples was treated with 0.25% trypsin solution and half amount was processed without trypsinization. Thus, treatment with trypsin led to reduction of vesicles size (Fig. 2a). Moreover, by NTA it is possible to estimate the intensity of irradiation reflected from the surface of the analyzed particles. As it can be seen by comparing the top and bottom panel (Fig. 2b), trypsinization leads to significant change in the structure of the exosome surface. It is logical to assume that such a treatment with trypsin leads to the complete or partial removal of proteins adhered to the surface of vesicles. For example, FN is a high abundance protein of plasma and can non-specifically bind to the surface of the circulating vesicles, regardless of their origin. To test this hypothesis, equivalent number of intact and treated with trypsin particles was used for the analysis of exosomal marker CD63 and FN by WB (Fig. 2c). As expected, incubation with trypsin leads to the destruction of exosomal FN, but did not significantly affect the content of transmembrane tetraspanin CD63. Next, we aimed to evaluate the whole spectrum of proteins attached to the surface of circulating exosomes by mass spectrometry. We reported here more than 250 high quality (≥ 2 peptides, 95% confidence) identifications of human exosomal proteins (Supplementary Table 2). Abundance (emPAI) of more than half of these proteins was being reproducibly reduced after processing with trypsin. Besides, 79 proteins were completely removed by trypsinization. (Supplementary Table 2). FN isoform 1 (FN1) is plasma abundant protein that was identified by MS in intact plasma exosomes and was almost completely cleaned from exosomes by trypsin. These results were confirmed by WB (Fig. 2c).

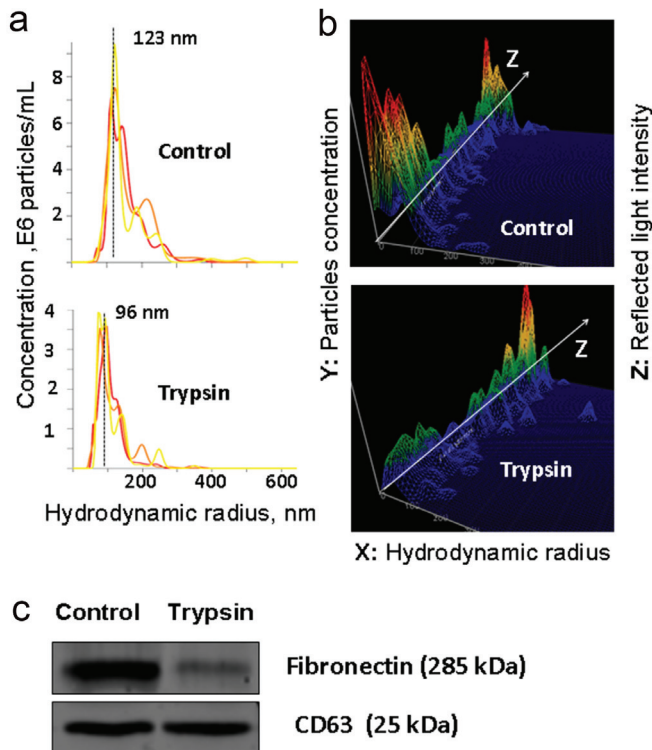


Figure 2. Analysis of trypsinized and intact plasma exosomes. (a and b) The results of nanoparticle tracking analysis (NTA) of exosomes isolated from plasma and treated with trypsin: the ratio of the size and concentration of exosomes (a); a three-dimensional graph, the Z axis shows changes in characteristics of light reflected from the surface of exosomes (b). (c) WB detection of fibronectin (FN) and exosomal marker CD63 in exosomes isolated from native plasma and exosomes treated with trypsin.

Plasma proteins attached to exosomes surface are implicated in ILK- and FAK-signaling pathways

GO analysis revealed several GO terms significantly over-represented in a set of the proteins cleared from the exosomal surface. Thus, clusters of secreted extracellular proteins and proteins modified by disulfide bond or glycosylation were significantly enriched among exosome-attached proteins, with enrichment score of 12.29 (Supplementary Table 1). Other clusters were enriched less significantly (enrichment score: 5.66) with GO terms relevant to various aspect of cellular

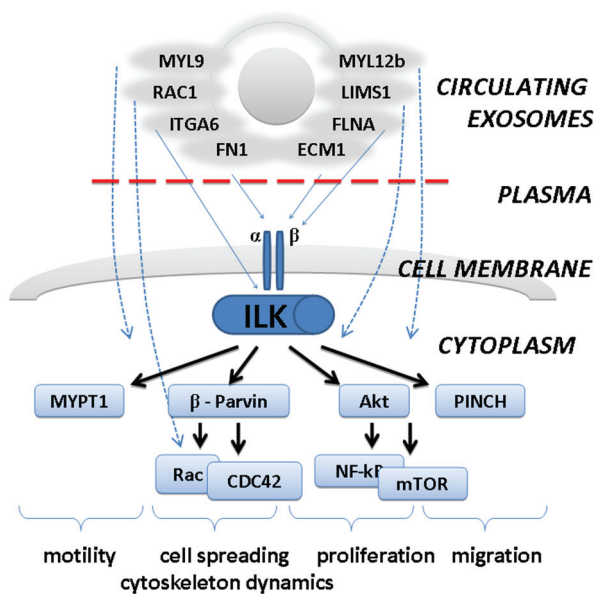


Figure 3. Predicted involvement of exosome-attached extracellular proteins in integrin-linked kinase (ILK) pathway. Plasma proteins attached to the surface of circulating exosomes may affect biology of the cells by interaction with surface receptors (integrins) and activation of downstream ILK-pathway. This would lead to stimulation of cellular migration, motility, proliferation, etc.

and humoral immunity as well as proteolytic plasma activity (Supplementary Table 1). Thus, exosomal surface is decorated by extracellular proteins that are abundantly present in plasma and are likely being attached to the exosomal membrane during circulation. It is not yet clear if interaction of plasma proteins with exosomes is selective in terms of exosome origin or chemical composition or all circulating vesicles are equally covered by plasma components. In any case, plasma proteins attached to an exosome surface may be involved in exosome-mediated cellular communication or can even provide exosomes with new regulatory functions. To predict intracellular pathways that may be affected by plasma proteins attached onto surface of circulating vesicles, pathway analysis was applied. List of intracellular regulatory cascades containing proteins cleared from a surface of the exosomes is presented in Supplementary Table 3. Signal transduction pathways mediated by integrin-linked kinase (ILK) and focal adhesion kinase 1 (FAK1) are predicted to be mostly affected by plasma exosomes. For examples, exosome-associated FN1, extracellular matrix protein 1 (ECM1) and filamin A (FLNA) can directly interact with heterodimeric transmembrane receptor integrin that is a vital cell adhesion receptor and an upstream activator of ILK-signaling cascade. Other five exosome-associated molecules can interfere with downstream events of ILK signaling (Fig. 3). Identified intracellular signaling pathways are involved in control of many various cellular functions including cell migration, adhesion [28], differentiation [29] (Fig. 3) and are essential for cell-to-cell communication and cell-to-extracellular matrix integration. Thus, our results highlight new aspect of regulatory function of plasma exosomes and need to be deeper investigated. It's important to note that plasma proteins attached to

the exosome surface may overcome new regulatory qualities as well as provide exosomes with new functions. Considering general character of observed phenomenon, it may have great impact into physiology and regulatory function of the blood.

Conflict of Interest

The authors declare no conflict of interest.

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