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Possible diagnostic role of cell membrane microparticles

Możliwe znaczenie diagnostyczne mikrocząstek błon komórkowych

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Summary

Extracellular vesicles (exosomes and ectosomes) are heterogenous group of spherical membrane structures released by most cells. They are secreted from the cell membranes during maturation and aging of cells. These membrane structures were detected, among others, in urine, blood, amniotic fluid and saliva. Cell activation, induced by various factors results in increased secretion of vesicles. These structures function, composition and their specific surface markers expression, depend on the cell type origin, from which they are formed. The article presents an overview of current knowledge on vesicle general characteristics, classification, research methods and role of membrane structures in various diseases. Microvesicles as biomarkers, mediators in cell interactions and proteins transporters can be applied in the diagnostics and prognosis of many diseases including cardiovascular, autoimmune, diabetes, infectious diseases and cancer metastasis. Determining the origin and the number of circulating microparticles facilitates explanation of their participation in the pathogenesis of various disorders. The elucidation of the biology and vesicles formation is important for understanding of their role in health and various disorders. Despite different applied methods (flow cytometry, microscopy, ELISA), the exact mechanism and components of the vesicle involved process formation remain only partially established and requires further studies.

Streszczenie

Mikropęcherzyki (egzosomy i ektosomy) są heterogenną grupą sferycznych błonowych struktur uwalnianych przez większość komórek. Wydzielane są z błon komórkowych podczas dojrzewania i starzenia komórek. Te struktury błonowe wykrywane są m.in. w moczu, we krwi, w płynie owodniowym i ślinie. Aktywacja komórek indukowana przez różne czynniki powoduje wzrost wydzielania pęcherzyków. Funkcja tych struktur, skład i specyficzna ekspresja markerów powierzchniowych zależą od typu komórki, z której powstają. Artykuł przedstawia najnowszy stan wiedzy dotyczącej ogólnej charakterystyki, klasyfikacji, metod badawczych i roli struktur błonowych w różnych chorobach. Mikropęcherzyki jako biomarkery, czynniki pośredniczące w oddziaływaniach międzykomórkowych oraz nośniki białek mogą mieć zastosowanie w diagnostyce i prognozowaniu wielu chorób, np.: choroby sercowo-naczyniowe, autoimmunologiczne, cukrzyca, zakażenia i progresja nowotworowa. Określenie pochodzenia i liczby krążących mikropęcherzyków ułatwia wyjaśnienie ich udziału w patogenezie różnych schorzeń. Wyjaśnienie biologii oraz powstawania pęcherzyków jest istotne w zrozumieniu ich roli w warunkach prawidłowych oraz w rozmaitych chorobach. Pomimo stosowanych kilku metod (cytometria przepływowa, mikroskopia, ELISA), dokładny mechanizm, jak również czynniki biorące udział w tworzeniu pęcherzyków są tylko częściowo wyjaśnione i wymagają dalszych badań.

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GENERAL CHARACTERISTICS OF VESICLES

Extracellular vesicles (EV) are spherical membrane structures released by different types of normal and also cancer cells (1). The best known example are the vesicles derived from blood platelets (2). These membrane structures were detected, among others, in urine, blood, amniotic fluid, saliva, or semen. Under physiological conditions they are released from the cell membranes during maturation and aging of cells. Cell activation, induced by various factors (complement proteins, cytokines, stress factors), results in increased secretion of these structures. Recently involvement of vesicles in many diseases, including cardiovascular, autoimmune, diabetes, infectious diseases and cancer

metastasis was studied (3). Determining the origin and the number of circulating microparticles facilitates explanation of their participation in the pathogenesis of various disorders. A few publications have revealed an increased release of these structures during long-term storage of blood for transfusions (4-8).

HISTORY OF VESICLES STUDIES

First report concerning the vesicles was published in 1967 and has been described by Wolf. The author termed the structures "platelet dust", the presence of which he found in human blood plasma. Structures secreted by activated blood platelets showed prothrombotic properties, owing to the presence of tissue factor – thrombospondin (9). The vesicles were isolated by ultracentrifugation, and observed by the electron microscopy. Discovery of the "platelet dust" marked the beginning of a new era regarding microvesicles formation and their role in the organism. Afterwards first publications on microvesicles released from erythrocytes (10), monocytes (11) and endothelial cells were published (12).

MICROVESICLES CLASSIFICATION

The membrane vesicles are a heterogeneous group of different sizes, origin, biological and physical properties, mechanism and source of formation. Due to the size and origin, the vesicles were divided into: smaller exosomes, with a diameter of 30-100 nm, and bigger, ectosomes, measuring from 0.1 to 1 μm (13). Previous nomenclature of these structures was based on the origin of the cells, from which they are formed. In recent years terminology was based on the mechanism of their formation. Consensus on terminology and classification of vesicles was obtained in 2013 (14). Among the vesicles, the exosomes generated within the multivesicular bodies (MVBs) were separated. During the fusion of MVBs with the intraluminal membrane, vesicles are secreted from cells. After that, their size ranges from 40 to 150 nm. Major markers are tetraspanins (CD9, CD63, CD81, CD82), and markers of lipid rafts: flotillins 1 and 2. Other markers, such as heat shock proteins (HSP), MHC molecules, various components of endosomal sorting complex required for transport (ESCRT) and member of the Ras superfamily of monomeric G proteins (Rab) are used to identify these vesicles (15, 16).

The exosomes are formed and maturate in the intracellular (endosomal) vesicles. Afterwards they are secreted out of the cell by the fusion of vesicle membrane with the cell membrane, what involves soluble N-ethylmaleimide – sensitive factor attachment receptor transmembrane proteins (SNARE). In the process of exosome formation, Ca^{2+} dependent, vesicle associated membrane proteins (VAMP) are included. It was shown that in leukemia cells, the VAMP-7 protein plays an important role in the formation of exosomes during their fusion with the cell membrane. VAMP-1,-2,-3 proteins were identified in the exosomes secreted from tumor cells (13). Recent studies have shown that there are 2 types of exosomes: immunologically active exosomes that are involved in antigen presentation and costimulation and those containing RNA and mediating the genetic communication between cells (17).

Ectosomes are formed extracellularly, directly from the cell membrane by blebbing. The vesicle detachment occurs due to the contraction of cell, and then peeling it in specific sites. On the cell surface, the buds are formed, which mechanism involves active participation of cytoskeleton proteins e.g. actin. In the cell membrane proteins and lipids many changes take place. Similarly to the apoptosis, disruption of phospholipid asymmetry appears. In the cell membrane movement of phospholipids: phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin takes place with the participation of flippase, floppase and scramblase. Ca^{2+} ions play an important role during the formation of microvesicles. As a result, the increased concentration of Ca^{2+} activates enzymes, such as gelsolin, aminophospholipid translocase, floppase, scramblase and calpain, resulting in the change of cell membrane asymmetry (18). Exposure of phosphatidylserine on the outside layer of the plasma membrane seems to be one of the main microvesicle properties, although there are also microvesicles without exposed phosphatidylserine. RhoA protein, belonging to the family of small guanosine-5'-triphosphates (GTPases), Rho-activated protein kinases (ROCK) and actin-binding (LIM) kinases are regulators of ectosome secretion. Other regulator, is calpain – Ca^{2+} dependent enzyme, which participates in the regulation of cytoskeleton proteins, where it plays a significant role in the ectosome formation. Furthermore it was also concluded that the ADP-ribosylation factor 6 (ARF6) protein is essential both in these structure formation and secretion (13).

SURFACE MARKERS

Vesicles express different characteristic surface molecules depending on their cellular origin. Specific markers to identify structures are listed in table 1 (19, 20).

RESEARCH METHODS

A variety of research methods are used to study microvesicles: ELISA, flow cytometry, electron microscopy, atomic force microscopy (AFM), spectroscopy and nanoparticles tracking analysis (NTA). While examining the structures, during the isolation and preparation, special care should be taken in the sampling procedure, and suitable type of the anticoagulant has to be used. Sodium citrate is the best anticoagulant for vesicles preparation because it in the smallest extent activates the cells in comparison with other reagents. It is also known that the number of extracellular vesicles increases under the influence of stress factors. Therefore, during the preparation a special attention should be paid to the temperature changes and gentle mixing of samples. The time between sample collection and isolation should be as short as possible (21). Flow cytom-

Table 1. Specific markers of vesicles releasing from different types of cells (by 19, 20, modified)

MP origin	Common name	CD	Role
Erythrocytes	Glycophorin A	CD235a	Blood group antigen
Leucocytes	LCA (T200, B220)	CD45	Tyrosine phosphatase
Monocytes	LPS-R	CD14	LPS – binding protein
Granulocytes	CGM6	CD66b CD66e	Cell adhesion, migration, pathogen binding, tumor progression
Th lymphocytes	T4	CD4	Th costimulation
Ts lymphocytes	T8 (Leu-2)	CD8	Tc costimulation
B lymphocytes	B1 (Bp35)	CD20	Regulation of B lymphocytes activation and proliferation
Platelets	GPIbα (β_3 integrin) GPIIb (αIIβ integrin) GPIX GPIIIa (β_3 integrin) P-selectin	CD42b CD41 CD42a CD61 CD62P	Adhesion and platelets aggregation Platelets aggregation, bind with CD61 molecule Adhesion and platelets aggregation Create αIIbβ_3 and $\alpha_v\beta_3$ integrins Leucocytes adhesion to endothelium
Endothelial cells	PECAM-1 GP 105-110 E-selectin α_v integrin S-Endo/Muc 18 VE-cadherin Endoglin	CD31 CD34 CD62E CD51 CD146 CD144 CD105	Leucocytes diapedesis Adhesion Leucocytes adhesion to endothelium Vitronectin receptor Transition into blood vessels Cell adhesion, endothelium marker Receptor for TGF-β1 and TGF-β3

etry is preferably used for the evaluation of number and determination of vesicle sizes (22, 23). This technique was used for the first time to study these structures in 1991 (24). Flow cytometry studies allow evaluation of circulating vesicles with using the antibodies directed against the surface markers. Cytometric method allows to obtain information on the morphology (size and granularity) of these structures as evidenced by the parameters: FSC (Forward Scatter Channel) and SSC (Side Scatter Channel) (fig. 1). In order to separate and determine structures in a proper way, calibration beads of appropriate diameter are used (25, 26).

During the formation of extracellular vesicles exposure of phosphatidylserine (PS) takes place, therefore annexin V, which binds to PS, is applied for staining. Additionally, antibodies against specific various cell markers should be used, since the vesicles are released from various cells. Proper separation, identification and evaluation of these structures is complicated and has many problems. Methods used for the isolation, qualitative and quantitative assessment are not standardized and create many technical issues. Key limitation in these studies is the lack of an internal validation process (27).

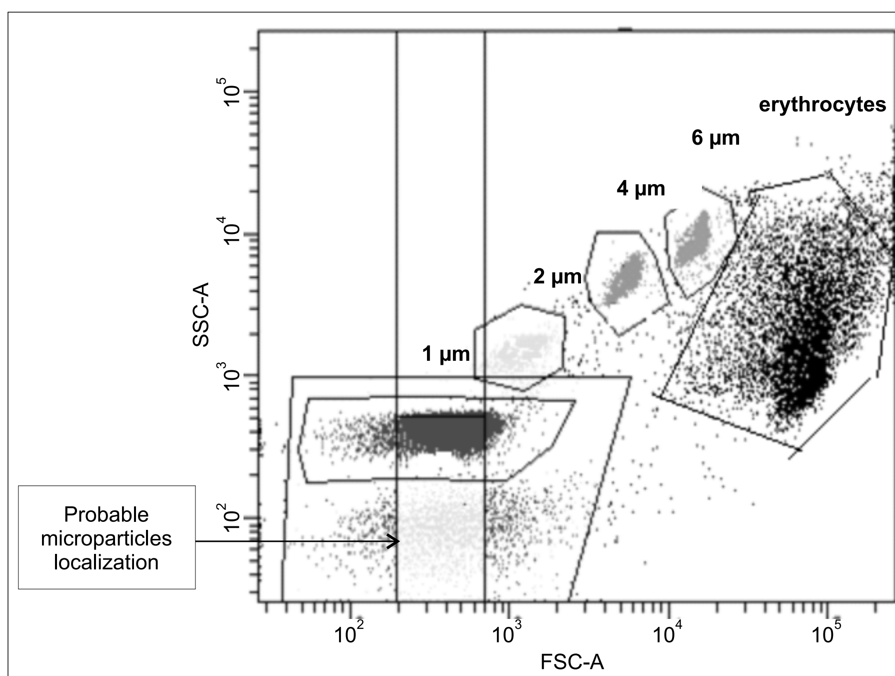

Fig. 1. Representative "dot-plot" obtained by flow cytometry. Determination of vesicles size. Flow cytometry analysis: calibration beads (size: 1, 2, 4, 6 μm), erythrocytes (7 μm) were used as an internal standard

Another method of vesicle detection is Nanoparticle tracking analysis (NTA) consists of measuring and visualizating particles in liquid. On the basis of the velocity measurement of the Brownian motion, which moves particles, the size of flowing molecules can be determined (28-30). This method allows determining the size distribution profile of the structures size, measuring 10-1000 nm in suspension. Some authors consider that defining the vesicles size by the cytometric method using calibration beads is inappropriate. The beads have various optical parameters: optical density, diffraction coefficient and light absorption. On the other hand NTA allows to define vesicle sizes without influence of molecule density or refractive index. The speed of particle movement is only influenced by temperature and viscosity of the fluid.

ROLE OF VESICLES IN VARIOUS DISEASES

Increasing interest in potential usage of vesicles, as biomarkers of disorders, is related to their role in the diagnostics of many diseases. Increase of the circulating vesicles number, their composition and shape changes may reflect the pathophysiological conditions of many different disorders. Their role has been recognized in, among others, in cardiovascular, autoimmune diseases, disorders of the blood coagulation, diabetes and during cancer metastasis.

Different patterns of cellular vesicles in type 1 and 2 diabetic patients were observed. Increase in the extracellular vesicles being released from platelets, monocytes, lymphocytes, granulocytes and endothelial cells was detected. These structures can cause diabetic complications: vascular thrombosis, vascular inflammation, and angiogenesis. Clinical studies indicated that level of endothelial and platelet-derived vesicles is significantly increased in T1 diabetes (31). Omoto et al. (32) observed increase in the number of vesicles released from monocytes and platelets in T2DM patients. Moreover the elevated endothelial and erythrocytes-derived vesicle number was estimated in T2 diabetes (33, 34).

In cardiovascular diseases changes of endothelial and circulating cells-derived vesicles were also observed. Correlation between endothelial vesicles level and the severity of coronary artery disease, heart attack and brain stroke was detected.

Role of these structures during the tumor progression was analyzed by the scientists at the Canadian University. Studies were conducted on the glioblastoma multiforme cells, and vesicles were released from these cells termed oncosomes. In the oncosomes, the molecules of the epidermal growth factor receptor III (EGFR III) and epidermal growth factor (EGF) were identified that stimulate uncontrolled cell divisions. Vesicles float through the blood, migrate and attach to a normal cells or these in the early stage of cancer. Structures fuse with the normal cells, providing the receptor. Additional molecules of EGFRvIII stimulate excessive cell proliferation leading to the cancer progression (35). Furthermore, the oncosomes can also transfer mRNA, microRNA what involves the phenotype change of the normal cell.

Vesicles derived from blood cells may play role in the transfusion medicine. Several observations have shown a link between elevated number of vesicles released from blood cells during storage of blood concentrates and post-transfusion complications (36-38). During early blood banking, RBC vesicles formation and membrane remodeling was observed (6, 39). The exposure of phosphatidylserine on the outside layer of cell membrane takes place (39, 40). Level of RBC-derived vesicles gradually increases with storage time, as already observed in transfused patients with paroxysmal nocturnal haemoglobinuria (41). However, level of vesicle formation in erythrocyte concentrates depends not only on storage period but also on preparation and the storage solution (41-44). Structure of vesicles in erythrocytes concentrates is similar to those released *in vivo*. However differences in the levels of some proteins, e.g. stomatin (elevated level in membrane of RBCs vesicles in concentrates), integral membrane or cytoskeleton proteins were detected. Vesicles found in concentrates are devoid of most of these proteins (3, 6, 10). Vesicles in erythrocyte concentrates are more heterogeneous, with increase of size and content of components of proteasome.

Despite many methods, e.g. flow cytometry, proteomics, microscopy, ELISA, nanoparticle tracking analysis, spectroscopy, the exact and complete mechanism and components of the vesicle involved process formation remain only partially established. Understanding the microvesicle biology, quantitative and qualitative analysis could be one of the links useful to improve the diagnosis and prognosis of various diseases.

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