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REVIEW

Extracellular vesicles in physiological and pathological conditions

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ABSTRACT

Body fluids contain surprising numbers of cell-derived vesicles which are now thought to contribute to both physiology and pathology. Tools to improve the detection of vesicles are being developed and clinical applications using vesicles for diagnosis, prognosis, and therapy are under investigation. The increased understanding why cells release vesicles, how vesicles play a role in intercellular communication, and how vesicles may concurrently contribute to cellular homeostasis and host defense, reveals a very complex and sophisticated contribution of vesicles to health and disease.

1. Introduction

The release of vesicles by cells is a common and evolutionary conserved process, because both prokaryotes[1,2] and eukaryotic cells[3,4] release such vesicles into their environment. The underlying molecular mechanisms of formation, cargo sorting, and release of vesicles are still largely unexplored.[5–7] It is appealing to consider why cells release vesicles. In complex multicellular organisms or within (mixed) populations of bacteria, vesicles offer an elegant solution to exchange biomolecules such as proteins, second messengers, and genetic information[3,4] or to get rid of redundant and/or dangerous intracellular or membrane-associated compounds.[8,9] Once the biomolecules have been packaged within vesicles they will be less susceptible to degradation. Packaging also offers the opportunity to store cargo in a highly efficient manner, and vesicles can be equipped with cell type-specific adhesion receptors so that the cargo will be delivered only at dedicated target cells. In the case of clearance of vesicles, concentrating harmful or redundant components into vesicles, such as chemotherapeutic drugs or (parts of) microorganisms, reduces the risk of "environmental contamination"[10,11] and at the same time facilitates cellular survival and may protect the host, e.g. by supporting defense processes such as coagulation and inflammation.[3,4,12]

Phospholipid bilayer-enclosed vesicles from eukaryotic cells will be collectively called extracellular vesicles (EVs) in this review when appropriate. Recent review reports that at least four different types of EVs have been defined based on phenotype and physical characteristics.[3] These types of vesicles are microvesicles (MVs), exosomes, membrane particles and apoptotic vesicles, but it is unclear whether each of these types indeed represents distinct types of vesicles.[3] Despite the lack of consensus on classification of EVs, three common types, MVs, exosomes, and apoptotic vesicles, are distinguished unanimously. MVs and exosomes have attracted much attention in the past years because the evidence is increasing, although mainly from in vitro studies, that both types of vesicles can contribute not only to intercellular communication, but also to processes such as coagulation, angiogenesis, cell survival, waste management, modulation of the immune response, and inflammation.[3,4]

EVs are widely distributed, and they have been found in all human body fluids that have been investigated thus far in both physiological and pathological conditions, including blood, urine, saliva, mother milk, and cerebrospinal and synovial fluid.[3,4] The numbers, cellular origin, composition and functional properties of EVs are associated with the type of body fluid, diseases and disease states such as cancer,[13–15] cardiovascular disease,[16,17] and inflammation.[18,19]

Despite extensive research on EVs, there are several major challenges to be faced, including the proper detection of EVs. Most information on diameter and size distribution of EVs comes from measurements by transmission electron microscopy (TEM).[20–22] As based on TEM measurements, most EVs have a diameter less than 100 nm, which is too small to be detected by standard cell-based methodologies. To which extent the diameter of single vesicles and the size distribution of a population of vesicles as determined by TEM reflects the true size and size distribution of vesicles in solution, however, are unknown, because TEM measurements require sample fixation and dehydration, i.e. processes likely to affect the size and morphology of vesicles. New methodologies such as atomic force microscopy (AFM), nanoparticle tracking analysis (NTA) or resistive pulse sensing (RPS) are capable of detecting

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single vesicles directly in solution and no fixation or dehydration is required. Thus, these methodologies are more likely to provide information on the real diameter of vesicles. Importantly, development of commonly accepted and acceptable reference materials will be essential, not only to define the original diameter and size distribution of EVs, but also to be able to compare results between laboratories.

In this review, we will present an overview on the presence and biological relevance of EVs in human body fluids in normal and pathological conditions, and we will provide an overview on their potential clinical applications, including their use as biomarkers and novel therapeutic agents.

2. Terminology of EVs

As mentioned before, there is no consensus regarding the classification and terminology of different types of EVs.[3] Recent evidence suggests that different types of EVs have more similarities than thought previously.[4,21] For example, the membranes of EVs are relatively enriched in detergent-resistant membrane domains, also known as lipid rafts, compared to plasma membranes[23–26] and there is much overlap in the density and diameter of EVs.[3,4] In fact, even for a single type of vesicle conflicting size ranges have been reported, and there is no consensus on this matter as illustrated in Table 1. The size of exosomes is below 100 nm in most references, but the size of the MVs (also called microparticles) varies widely between investigators. Furthermore, supposedly different types of EVs may share common membrane proteins. For example, P-Selectin (CD62p), which is exposed on activated platelets and platelet derived-MVs (PMVs), is also exposed on platelet-derived exosomes.[20] In addition, it cannot be excluded that many unique characteristics that have been ascribed to an isolated and purified population of vesicles, such as the presence of a particular mRNA or miRNA in exosomes, are due to contamination by larger vesicles, vice versa. Thus, extreme care is necessary when terms for specific subsets of vesicles are being used.

3. Formation and shedding of EVs

Cells release EVs upon activation and during apoptosis in vitro, i.e. under conditions of cell stress.[10,11,25,27–30] Under cell stress MVs and exosomes are being formed (Fig. 1). The formation of MVs seems to be initiated by an increase in the cytosolic concentrations of calcium ions. The increase of calcium ions activates scramblase and calpain, which leads to a loss of membrane phospholipid asymmetry (scramblase action) and calcium dependent degradation of various proteins (calpain action), which in some way allow the outward budding of MVs from the plasma membrane.[5,31] As a consequence, cells and MVs may expose phosphatidylserine (PS). This is illustrated in a rare bleeding disorder, Scott syndrome, in which a defective scramblase activity results in a reduced transport of PS to the platelet surface as well as the release of a reduced number of PS-exposing MVs.[8,32] Although many studies have shown that MVs may expose PS, also here there are still many questions to be answered. Exposure of PS by MVs seems to depend on their cellular origin, the underlying mechanism of formation, the presence of PS-binding proteins such as lactadherin that may artifactually shield PS from detection in our analyses, and, importantly, pre-analytical conditions such as collection, handling and storage.[27,28,33–35] Therefore, the detection and characterization of MVs based on PS exposure need to be reconsidered.

The biogenesis of exosomes begins with the inward budding of small parts of the plasma membrane, containing several antigens exposed on that outer membrane. These small intracellular vesicles form the early endosome. Then, formation of intraluminal vesicles (ILVs) by inward budding of the limiting membrane of endosome occurs. Once the endosome contains ILVs, it is called a multivesicular body (MVB; Fig. 1).[6] ILVs have a cytosolic-side inward orientation and thus expose the extracellular domains of transmembrane proteins. Four different mechanisms may contribute to protein sorting towards ILVs: (1) mono-ubiquitination and the endosomal sorting complex required for transport (ESCRT) machinery that facilitates the trafficking of ubiquitinated proteins from endosomes to lysosomes via MVBs, (2) association of proteins with detergent-resistant membrane domains or lipid rafts, (3) higher-ordered protein oligomerization, and (4) ceramide-dependent segregation into endosomal microdomains.[36–39] In fact, several proteins involved in the biogenesis of exosomes have been used to identify exosomes. Examples of such proteins are ESCRT-associated proteins such as PDCD6IP (Alix) and tumor susceptibility gene 101, tetraspanin molecules (CD9, CD63 and CD81) and heat shock protein 70.[20,40–42] The MVBs fuse with either lysosomes for cargo degradation or with the plasma membrane to secrete the ILVs as exosomes. The concentration of calcium ions within the MVBs also plays a role in secretion of exosomes.[43]

Because vesicles which are indistinguishable from exosomes may also be directly budded from the plasma membrane,[3,6] and because at least part of the MVB membranes may be deep invaginations of the plasma membrane, it is unclear whether ILVs, exosomes, and MVs are truly separate entities. So to summarize, to which extent EVs contain truly distinct types of vesicles requires further investigation, and at present no tools are available to purify a single type or population of vesicle based on size or density.[3]

EVs expose tissue/cell type-specific marker proteins of their parent cell.[3,4,44] When a sufficient number of such marker proteins are exposed, the cellular origin of a vesicle can be determined by e.g. flow cytometry using antibodies directed against such marker proteins. This is illustrated in Table 2, in which a shortlist of commonly used marker proteins is summarized for analysis of vesicles in human blood (CD: cluster of differentiation).

4. Sources of EVs in human body fluids

The numbers, cellular origin, composition and functional properties of EVs are not only disease (state) dependent, but also depend on the body fluids being studied. The major populations of EVs in a body fluid usually reflect the cells that are present in that particular body fluid and that surround the body fluid. Examples of the latter are vesicles from synoviocytes which are present in joint (synovial) fluid, and vesicles from endothelial cells (ECs) in blood. We will briefly summarize the cellular origin presence of EVs in blood, urine, saliva, cerebrospinal and synovial fluids in the following paragraphs.

In peripheral blood of a healthy subject, platelets and erythrocytes are the major sources of EVs, but in certain disease states such as sepsis, cardiovascular disease (CVD), or cancer, also MVs from monocytes, granulocytes, lymphocytes, ECs, and cancer cells can be present.[45] Peripheral blood also contains exosomes,[46] although the cellular origin of these vesicles is unknown.

Table 1
The size distribution of EVs.

Type of vesicles	Size (nm)	Detection	References
Microvesicles (microparticles)	20–50	TEM	138
	100–1000	TEM	20
	40–70	TEM	139
	200–800	TEM	140
	180 (mean)	AFM	128
	10–475 (mean 67.5)	AFM	125
	30–90 (mean 50)	AFM with microfluidics	135
	100–500	TEM	22
Exosomes	40–100	TEM	20
	30–100	TEM	141
	50–100	NTA	130

TEM (transmission electron microscopy), AFM (atomic force microscopy), FCM (flow cytometry), NTA (nanoparticle tracking analysis).