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REVIEW

Extracellular vesicles in physiological and pathological conditions

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A B S T R A C T

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.

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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).

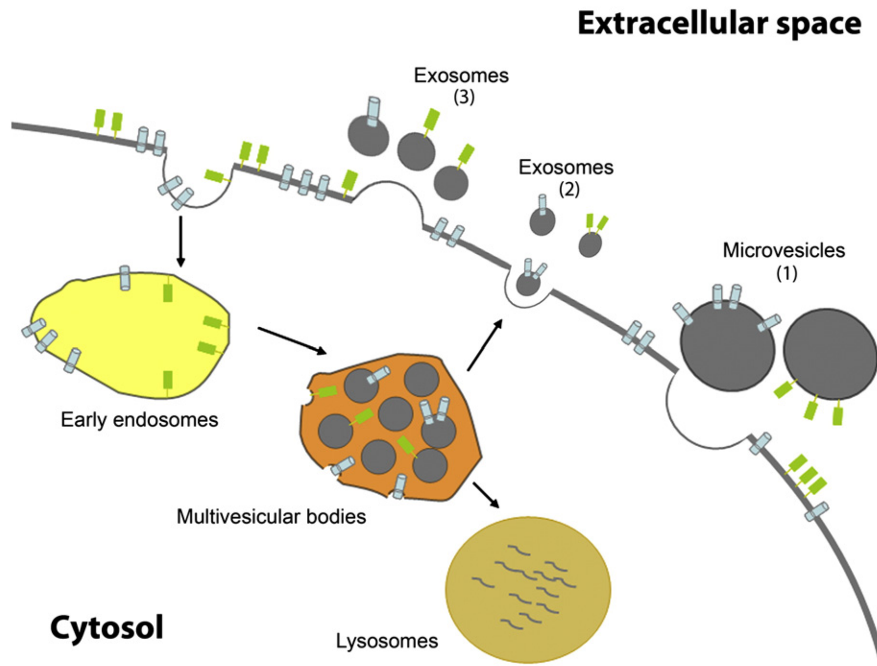

Fig. 1. Formation and shedding of extracellular vesicles. Microvesicles (MVs, 1), also called microparticles, are formed directly from the cell membrane by a shedding process of which the exact molecular mechanisms are largely unknown. Specific targeting of membrane proteins and lipids to the MV is known to occur. For the formation of exosomes, invagination of small parts of the cell membrane, with specific membrane protein components incorporated, starts the formation process.[6] The small vesicles are taken up by this endocytosis process into early endosomes. The proteins are then packaged into intraluminal vesicles (ILVs) upon inward budding of the membrane of the endosome, transforming the endosome into multivesicular bodies (MVBs). When proteins are destined for degradation, MVBs fuse with the lysosomal membrane and release ILVs into the lysosome for degradation. Alternatively, MVBs fuse with the plasma membrane and ILVs are released into the extracellular space as exosomes (2). Exosomes (3) may also be formed directly by outward budding of plasma membrane, thus resembling the formation of MVs.[3] Please keep in mind that there is no consensus whether endosomes are intracellular organelles or deep invaginations of the plasma membrane. When endosomes would be deep invaginations, the consequence is that ILVs would be extracellular and thus are indistinguishable from exosomes. In fact, then the term "ILVs" would be redundant.

Urine of healthy humans and amniotic fluid both contain significant numbers of exosomes or exosome-like vesicles.[47] These exosomes expose CD24 and aquaporin-2, therefore, are likely to originate from kidney cells[48] and from epithelial cells facing the renal tubule lumen.[49] Urine contains also larger vesicles, but thus far the characterization of these two types of vesicles in urine has been problematic.[50]

In saliva from healthy individuals, the larger vesicles, MVs, are derived mainly from epithelial cells and granulocytes, whereas the smaller vesicles, i.e. exosomes or vesicles resembling exosomes, are mainly from epithelial cell origin.[51]

Cerebrospinal fluid also contains EVs.[52] In vitro, various types of brain cells such as astrocytes, microglia, oligodendrocytes and neurons release exosomes.[53] The source of the EVs in cerebrospinal fluid, however, is presently unknown.

Synovial fluid of rheumatoid arthritis (RA) patients and patients with other types of arthritis contain MVs.[18,54] Most of these MVs originate from cells associated with inflammation, such as monocytes and granulocytes. In addition, synovial fluid also contains vesicles from synovial fibroblasts.[55] Taken together, every body fluid has a clearly distinct vesicle profile.

Table 2
Antibodies for staining microvesicles derived from different cell types.

Cellular origin	Cell surface marker
Erythrocyte	CD235a
Lymphocyte	CD3, CD4 and CD8
Neutrophil/granulocyte	CD66b, CD66e
Monocyte	CD14
Platelet	CD41, CD42, CD61
Endothelial cell	CD105, CD144, CD62e

5. Functions of EVs

In the following paragraphs, an overview will be presented of the cellular functions, which are summarized in Fig. 2.

5.1. Angiogenesis

EVs have pro- as well as anti-angiogenic properties.[30,56–62] Angiogenesis involves the formation and growth of new blood vessels to provide expanding tissues and organs with oxygen and nutrients, and concurrently remove the metabolic waste.[63]

Cultured ECs release MVs containing metalloproteinase proteins MMP-2 and MMP-9.[64] These endothelial-MVs (EMVs) promote matrix degradation, thereby promoting the formation of new blood vessels. Also MVs from platelets (PMVs) promote proliferation, survival, migration, and formation of capillary-like structures of ECs in vitro.[59] PMVs also induce angiogenesis in vivo because subcutaneous injection of PMVs promotes the development of endothelial capillaries in mice, and injection of PMVs in the ischemic heart muscle of rats increases revascularization.[60] Both processes are apparently mediated by vascular endothelial growth factor (VEGF), which is secreted upon platelet activation and seems to be associated with the PMVs. This also holds true for other growth factors, such as basic fibroblast growth factor and platelet derived growth factor.[60] However, because isolated fractions of PMVs may still contain low levels of growth factors that have become released by platelets during blood collection and handling, one has to be careful with the interpretation of these results.

Induction of angiogenesis by PMVs or other vesicles may also support tumor angiogenesis and metastasis. For example, binding of PMVs to metastatic lung cancer cells triggers the expression of matrix metalloproteinases (MMP-9, MMP-2 and MMP-14), VEGF,

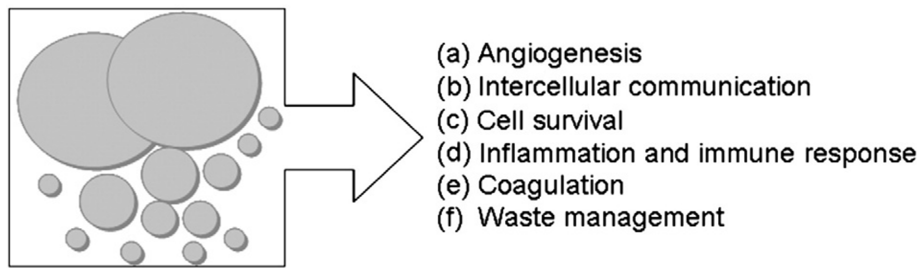

Fig. 2. Functions of extracellular vesicles.

interleukin-8 (IL-8) and hepatocyte growth factor.[65] In addition, also cancer cells release exosomes which promote tumor angiogenesis. Glioblastoma tumor cells release exosomes containing mRNA and miRNA involved in remodeling the tumor stroma and enhancing tumor growth.[30] These glioma-derived exosomes are also enriched in angiogenin, IL-6 and IL-8, all of which have been implicated in glioma angiogenesis and increased malignancy.[30]

Besides pro-angiogenic features, EMVs also inhibit angiogenesis as they can stimulate the production of endothelial reactive oxygen species (ROS).[66] Lymphocyte-derived MVs generated after actinomycin D treatment in vitro decrease nitrite oxide (NO) and increase ROS production by stimulating phosphatidylinositol 3-kinase, xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase pathways.[56,58] Thus, reduced NO and increased ROS productions inhibit angiogenesis.

5.2. Intercellular communication

EVs can transfer biomolecules to recipient cells e.g. adhesion receptors or ligands, cytokines, and genetic information, and therefore are capable of changing the composition and function of recipient cells. For example, PMVs can transfer the platelet fibrinogen receptor (integrin αIIbβ3) to cancer cells, thereby increasing the ability of the cancer cells to adhere to ECs in vitro.[65,67] One has to bear in mind, however, that in vivo the situation may be far more complex because such vesicles may also inhibit the interaction between cancer cells and ECs.

Patients with stage 3 or 4 melanomas have increased levels of phosphorylated MET, a receptor tyrosine kinase, in tumor exosomes, and circulating bone marrow progenitor cells from these patients also show an increased expression of phosphorylated MET compared to cells from healthy volunteers.[68] In a mouse melanoma model, tumor-derived exosomes promote tumor cell proliferation by transfer of MET to bone marrow cells.[68] Thus, tumor-derived exosomes are likely to transfer MET and educate bone marrow progenitor cells to support tumor growth and metastasis in vivo.

Tumor exosomes transfer mutant epidermal growth factor receptor (EGFRvIII) RNA into platelets. Nilsson et al.[69] showed that platelets, after incubation with vesicles from EGFRvIII-positive glioma cells, contain EGFRvIII RNA. In addition, they showed that EGFRvIII RNA was detectable in platelets from 80% of the EGFRvIII-positive glioma patients, but absent in platelets from healthy individuals. The presence of tumor-associated messages is apparently not unique for platelets from glioma patients, because platelets from prostate cancer patients—but not from healthy controls—contain RNA encoding the prostate cancer marker PCA3. However, one must bear in mind that platelets and vesicles overlap in size (diameter), and isolation and purification of either platelets without contaminating vesicles or vesicles without contaminating platelets is and will likely remain a tremendous challenge. This may lead to misinterpretation of results on the exact origin of certain components. Moreover, isolated vesicles also contain DNA, which further complicates analysis and interpretation of results.

Transfer of receptors by EVs can also support intracellular signaling. Human umbilical vein ECs produce exosomes that contain Delta-like 4 (Dll4), a notch ligand that is up-regulated during angiogenesis. D114 is transferred between ECs by exosomes in vitro and in vivo, suggesting that such exosomes are indeed capable of transferring Delta like/Notch signaling to recipient cells.[70]

5.3. Cell survival

After treatment with chemotherapeutic drugs, tumor cells release vesicles which contain the corresponding drugs. Experiments with cisplatin[10] and doxorubicin[11] on cultured resistance cancer cell lines confirm drug accumulation and expulsion in shed vesicles. Although these studies show that the release of vesicles may support tumor cell survival by removing the chemotherapeutic drug, the relative contributions of exosomes to reduce the intracellular drug concentration, however, is thought to be modest.[71] Alternatively, MVs can transfer multidrug transporters, such as P-glycoprotein (P-gp), between cells. MVs released from drug-resistant cancer cells in vitro transfer functional P-gp to drug-sensitive cells.[72] To which extent such mechanisms contribute to drug resistance in vivo, however, is still unknown, and there may be other mechanisms via which vesicles contribute to tumor progression. For example, MVs from human mesenchymal stem cells (MSCs) enhance the survival of cisplatin-induced acute kidney injury in a mouse model by about 80% by increasing the expression of anti-apoptotic genes and down-regulating the expression of pro-apoptotic genes.[73]

5.4. Inflammation and Immune response

EVs can affect or enhance autoimmunity and inflammation. Synovial fluid of RA patients contains strongly coagulant and pro-inflammatory vesicles which are mainly of leukocytic origin.[54] Such EVs trigger autologous fibroblast-like synoviocytes to produce and secrete inflammatory mediators including monocyte chemoattractant protein-1, IL-8, IL-6, RANTES (regulated on activation, normal T cell expressed and secreted), ICAM-1 (Intercellular Adhesion Molecule-1) and VEGF.[54] Although PMVs were also reported to be present in synovial fluid, there is no consensus on this matter yet.[18,74] PMVs can also activate monocytes via the RANTES pathway, thereby inducing monocyte migration and recruitment to sites of inflammation.[75]

MVs from neutrophils trigger secretion of transforming growth factor β1, a potent inhibitor of macrophage activation, by human macrophages, and thus elicit an anti-inflammatory activity.[76] These MVs also contain the anti-inflammatory protein annexin 1,[77] and such vesicles inhibit the inflammatory response of macrophages to bacterial lipopolysaccharide.[76]

PMVs orchestrate immune responses by delivering CD154, also known as CD40 ligand or CD40L, to initiate and propagate the adaptive immune response via $CD4^+$ T cells.[78] Also tumor-derived exosomes can modulate the immune response by affecting the differentiation of antigen presenting cells, such as dendritic cells (DCs). Differentiation of monocytes to DCs is impaired by tumor-derived exosomes isolated

from plasma of patients with advanced melanoma, and these exosomes also promote the generation of a myeloid immunosuppressive cell subset ($CD14^{+}HLA\text{-}DR^{-/low}$).[29] In addition, exosomes from tumor cells can also down-regulate the immune response against the tumor by inducing apoptosis of activated T cells via the Fas/Fas ligand pathway. Wieckowski et al.[79] demonstrated that EVs from tumor cells but not EVs from DCs isolated from sera of head and neck squamous cell carcinoma and melanoma patients are enriched in Fas ligand. These EVs induced the proliferation of $CD4^{+}CD25^{+}FOXP3^{+}$ T regulatory cells and suppressed $CD8^{+}$ effector T cells in vitro. The suppression effect is mediated by Fas/FasL interactions. Thus, tumor-derived vesicles may contribute to tumor growth and development by interfering with the anti-tumor immune response via various mechanisms.

5.5. Coagulation

Tissue factor (TF) initiates coagulation. TF is not expressed and produced by cells within the blood under physiological conditions, but is constitutively expressed and produced by extravascular cells such as smooth muscle cells. Under pathological conditions, however, sepsis, ECs and monocytes, and perhaps neutrophils, can produce coagulant TF.[80–85] Reports of the presence, cellular source and coagulant activity of TF in blood are controversial. In 1999 Giesen et al.[86] demonstrated the presence of TF antigen and coagulation activity on monocytes, neutrophils, and cell-derived vesicles (also named 'blood-borne TF') in blood and plasma of healthy individuals. However, others showed that the concentration of coagulation active TF either in blood or plasma from healthy individuals does not exceed 20 fmol/l.[87] Moreover, it seems unlikely that such concentrations of vesicle-exposed coagulant TF can be present in vivo under normal conditions because in vitro the addition of (sub)picomolar concentrations of active TF induces the clotting of blood or plasma within minutes.[88,89] In fact, the presence of detectable levels of coagulant TF in blood has been associated with intravascular bleeding and thrombosis. Blood from a patient with meningococcal septic shock, who suffered and probably also died from disseminated intravascular coagulation, contained a large number of monocyte-derived vesicles exposing highly coagulant TF.[45] Furthermore increased levels of coagulant TF exposed on circulating vesicles are present in blood from cancer patients who developed venous thromboembolism (VTE), suggesting that such vesicles may contribute to thrombotic events in such patients. One must bear in mind that TF can also be present in a non-coagulant form on vesicles.[13,80,90] This is likely to be the main form of TF in the circulating blood. In contrast, vesicles exposing highly coagulant TF are present in human wound blood, where they are likely to play a physiological role in hemostasis.[91,92]

In contrast to blood, saliva and urine of healthy humans contain high numbers of vesicles exposing coagulant TF. Addition of saliva shortens the clotting time of autologous plasma and whole blood.[51] EVs isolated from saliva expose TF and initiate TF/factor VII-mediated coagulation, illustrating that saliva and urine, but not blood, contain vesicles exposing coagulant TF under physiological conditions.

MVs exposing coagulant TF have been reported in various pathological conditions such as sickle cell disease (SCD), acute coronary syndrome (ACS), essential thrombocythemia and cancer, but often the results from such studies are difficult to compare to each other. For example, plasma from SCD patients was reported to contain endothelial- and monocyte-derived MVs exposing TF, and these MVs were shown to be procoagulant.[93] In contrast, we detected only platelet and erythrocyte-derived MVs in plasma of SCD patients, and the procoagulant state was associated with activation of factor XI and not with extrinsic coagulation activation.[94] The isolation conditions of vesicles in the two studies, however, were markedly different, and are likely to affect the results.

In most but not all studies, elevated levels of MVs of endothelial origin are reported in plasma from ACS patients compared to non-ACS patients.[95,96] To which extent these endothelial MVs contribute to the hypercoagulable status of these patients, however, is unknown.

MVs isolated from blood of patients with essential thrombocythemia, a chronic myeloproliferative disease that is characterized by an increased risk of both arterial and venous thrombosis, are mostly derived from platelets and ECs. The MVs in these patients are thought to contribute to the hypercoagulable state that is observed in vivo.[97]

Plasma from patients with certain types of cancer contains higher numbers of vesicles than plasma from healthy subjects.[13,14,98] Furthermore, MVs exposing coagulant TF in blood of cancer patients have been associated not only with thrombosis but also with disease progression.[13,15] Interestingly, in some cancer patients with a detectable level of coagulant TF present within the blood, a minor fraction of MVs exposes the epithelial marker, MUC-1.[13] To which extent these MUC-1-expressing vesicles, i.e. vesicles likely to originate from the tumor, are exposing coagulant TF and to which extent such vesicles are associated with development of VTE, however, remain to be determined.[99] Furthermore, tumor cells may elicit a host response that leads to expression of TF by monocytes and possibly ECs, and to the shedding of MVs bearing TF. Recently, in a study comprising over 200 cancer patients, we found a subpopulation of vesicles in one patient exposing TF, VE-cadherin (CD144) and E-selectin (CD62e), both specific markers of endothelial origin. How much TF exposed by this subpopulation is coagulant or how TF contributes to coagulation activation in vivo has not been investigated yet (A. Kleinjan, MD, personal communication). One has to bear in mind that TF can also induce angiogenesis and transmembrane signaling, each processes important for cancer growth and development. To which extent vesicle-exposed TF contributes to such functions in cancer patients is unknown.

It is still unknown whether exosomes are coagulant. This is a relevant question because most vesicles present in body fluids are within the size range of exosomes rather than of MVs, and thus may have a relatively large contribution to coagulation because formation of tenase and prothrombinase complexes requires a membrane surface which both MVs and exosomes could provide. The membrane surface has to expose negatively charged lipids such as PS to enable the formation of the coagulation factor complexes and the PS can be detected by binding of annexin V. Heijnen et al.[20] showed that only a relatively low number of exosomes, supposed to originate from platelets, bound annexin V. Furthermore, MVs but not exosomes bound factor X and prothrombin in this study. This would indicate that exosomes are not a mainly determinant in the propagation of the coagulation process once the coagulation system has been activated. In contrast, Davila et al.[100] showed that exosomes, defined as vesicles with a diameter of less than 100 nm, contribute to the overall procoagulant activity of tumor cell derived vesicles. They showed that approximately 20% of the TF coagulant activity was still present after filtration through a 0.1 μm filter, which would indicate a role for exosomes in coagulation activation. Unfortunately, they did not investigate whether filtration enables removal of all vesicles larger than 0.1 μm, or whether larger vesicles are fragmented by such a procedure, making the distinction between exosomes and small MVs uncertain.

5.6. Waste management

Vesicles act at two levels regarding waste management. Vesicles can contain redundant intracellular components, thus acting as cellular waste disposal bags by their extrusion from the cell. In turn, such vesicles may be removed from the circulation by phagocytosis by other cells. It is tempting to speculate that EVs containing cellular waste are especially equipped to facilitate their clearance, e.g. by exposing PS, thereby becoming easy targets for phagocytes. There is evidence that the spleen is involved in the clearance of MVs in vivo.[100] Thirty minutes after injection of PS-exposing MVs from breast or

pancreatic cancer cell lines into mice, both TF antigen and TF activity decreased by 72% and 90%, respectively, becoming undetectable 2 h after injection. Already 5 min after injection, the TF antigen was detectable in the spleen. In contrast, in splenectomized mice most of the human TF antigen was still detectable 30 min after injection, and 30% of the splenectomized mice did not survive 2 h after injection. In humans, clearance of circulating vesicles exposing coagulant TF is extremely fast and efficient. We showed that human wound (pericardial) blood from patients undergoing open heart surgery contains exceptionally high levels of coagulant TF-exposing vesicles that trigger coagulation in vitro[91] and thrombus formation in vivo.[92] When this wound blood is retransfused, the TF-coagulant activity becomes undetectable in peripheral blood already after 20–30 min, revealing that also in humans clearance of vesicles must be very efficient.[101]

In pathological conditions, the waste management may not function properly. This could happen because of the failure of the phagocytes to recognize the danger signal[102,103] or because these phagocytes are impaired (apoptotic/necrotic).[104–106] The consequence is that EVs containing redundant and unwanted biomolecules are not rapidly cleared from the circulation. Thus, these EVs are likely to play a role in the pathological conditions. Monocytes are phagocytes which expose a PS-specific receptor that recognizes PS-exposing vesicles.[107] In an in vitro study, human monocytic leukemia cells (THP-1 cells) showed signs of apoptosis or possibly even necrosis after incubation with PS-exposing PMVs containing caspase 3.[106] This study supports the notion that decreased clearance of vesicles from the circulation may be detrimental.

6. Potential biomarkers and novel therapies

EVs are potential biomarkers for detection of diseases. Total numbers and/or numbers of certain subsets of EVs in body fluids may be used to predict the presence of a disease, or a risk factor of developing a disease. Recently, increased numbers of several types of EVs were shown to increase the Framingham risk score (FRS), a risk assessment tool to estimate a patient's 10-year risk of developing CVD.[108–110] These results are promising and imply that more prospective studies are needed to further investigate the prognostic value of EVs in individuals at risk for CVD.

In cancer patients with VTE, the coagulant activity of TF associated with MVs isolated from platelet-poor plasma is markedly increased compared to the cancer patients without VTE.[13,98] These findings suggest that MVs associated with coagulant TF in cancer patients may predict thrombotic events in patients at risk of developing VTE.

EGFRvIII promotes the expression of the proangiogenic protein IL-8 through the NF-κB pathway.[62] EGFRvIII mRNA was present not only in resected glioma tissue but also detectable in exosomes isolated from serum of 7 out of 25 glioblastoma patients.[30] Thus, measuring EGFRvIII mRNA in vesicles may provide clinically relevant information on tumor presence, tumor progression, and response to therapy. Not only blood or fractions thereof, but also other body fluids may be a useful source of vesicular biomarkers. For example, aquaporin-2, exposed by exosomes isolated from urine, may be a biomarker for renal and systemic disease.[50] Exosomes isolated from urine were shown to contain the mRNA encoding two known prostate cancer biomarkers, PCA3 and TMPRSS2: ERG, and both mRNAs can be transferred to platelets.[69] Thus, extraction of mRNA from urine or platelets may provide a useful means for prostate cancer diagnosis.

Vesicles also offer therapeutic applications. For example, the adhesion of hematopoietic stem–progenitor cells (HSPC) to the endothelium is significantly improved in the presence of PMVs, thereby supporting engraftment after stem cell transplantation in lethally irradiated mice.[111] MVs derived from MSCs may provide a future (adjuvant) therapy for acute renal injury[112] because intravenous administration of MSC-derived MVs improves the recovery of glycerol induced-acute renal injury in SCID mice.[113] Exosomes from IL-10-treated immature DCs suppress inflammatory and autoimmune responses.[114] This type of exosome may therefore become a suitable therapy for arthritis. Another interesting clinical application is exosome-based immunotherapy. The initial studies by using DC-derived exosomes ("dexosomes") loaded with tumor peptides showed that "dexosomes" are capable of priming cytotoxic T cells and inducing tumor rejection in mice.[115] Dexosomes also promote NK cell activation in immunocompetent mice and NK cell-dependent anti-tumor effects.[115] Based on these results, clinical trials are ongoing.[116] There are several strategies to use exosomes as a (therapeutic) vaccine. Tumor-derived exosomes carrying tumor antigens and plasmacytoma cell-derived exosomes may be used to induce tumor-specific immunity and thus to prevent tumor development.[117]

7. Preparation and measurement of EVs

Despite the extensive studies on EVs, until now there are no protocols available for standardized collection, isolation and storage of EVs. Such standardized protocols are important to be able to compare results between laboratories. Despite the fact that blood is probably our most complex body fluid, EVs present in or isolated from blood or fractions thereof have been most extensively studied so far. Although there are several recommendations regarding the collection of blood with regard to EVs,[118] for other body fluids no protocols are available. In most studies EVs have been isolated from body fluids by differential centrifugation.[3,47] Differential centrifugation involves multiple sequential centrifugation steps where in each step the centrifugal force is increased to separate smaller and less dense components from the previous step. Another type of separation by means of centrifugation is density-gradient ultracentrifugation, which separates vesicles based on density.[20,119] Although different types of vesicles have been distinguished based on density,[3,20,41] differences in density are likely too small to allow full separation of EV species. Differential centrifugation and density-gradient centrifugation protocols are unlikely to isolate only a single type of vesicle. Immunoaffinity-based assays, usually coated with a specific CD-antibody, are also used.[84,120] Theoretically, this method isolates only one subpopulation of vesicles. Unfortunately, in daily practice successful isolation and purification of a single population with an acceptable recovery by this technique are usually very difficult. Ideally, EVs are measured directly in freshly collected samples, but in a clinical setting this is hardly feasible at present. When samples are frozen and thawed before analysis, concentrations and exposure of PS can markedly increase in samples containing PMVs.[35,118]

As EVs may expose one or more surface antigens of their parent cell, the cellular origin of EVs can be assessed by using antibodies directed against such cell-type specific surface antigens. Flow cytometry (FCM) is still commonly used to estimate the number of EVs. Due to the fact that the refractive index of vesicles is low, only the larger vesicles will be detected as single vesicles and the smaller vesicles will be detected only as a swarm.[121] Thus, FCM will underestimate the number and concentration of vesicles. Although many researchers use annexin V to identify or isolate MVs, PS exposure by MVs is still ambiguous because exposure of PS can be due to isolation and handling procedures such as centrifugation and storage.[33,35] Furthermore, the binding of annexin V to MVs depends on the calcium concentration and the membrane PS content,[33,122] and staining of PS-exposing MVs with lactadherin, a milk fat globule-epidermal growth factor VIII (MFG-E8), may be more sensitive to small changes in PS exposure than annexin V.[34,123] Because the binding of lactadherin to PS is calcium independent, lactadherin can be used to detect PS-exposing MVs directly in citrate- or EDTA-anticoagulated plasma samples, whereas PS detection by annexin V is calcium dependent and can therefore not be performed in those materials.

Other techniques such as TEM,[20–22,40] capture assays[22,84,124] and atomic force microscopy (AFM)[23,125–127] can also be used in combination with specific antibodies. However, the specificity, affinity, and whether the antibody tends to form aggregates, are all important considerations in selecting the antibody of choice.[118,128]

As regards techniques such as NTA,[129–133] AFM[125,127,134,135] and RPS,[121] single EVs can be detected directly in body fluids or buffers. Based on data obtained by these techniques, EVs in solution are reported to be spherical and to have diameters ranging between 20 and 600 nm, with a mean diameter of 50 nm.[21,125,132] But again, things are complicated. One has to keep in mind that plasma also contains high concentrations of lipoprotein particles, and techniques such as NTA or RPS cannot distinguish between EVs and lipoprotein particles. The body fluid containing EVs, the pre-analytical conditions of body fluid collection and sample preparation, and the methodology used to measure the EVs all considerably influence the number and size distribution of EVs.[35,118] Interestingly, by using AFM combined with microfluidics, Ashcroft et al.[135] showed that the size distributions of CD41-exposing vesicles in fresh plasma before and after isolation are comparable, indicating that the size distribution was unaffected by the isolation procedure used in that study.

Recently, a novel high resolution FCM-based method was developed to detect single exosome-sized particles based on fluorescence. Although this methodology offers the opportunity to detect single exosome-sized vesicles directly in solution, unbound antibody has to be removed from vesicles using gradient centrifugation, making this technology not or hardly useful in a clinical setting.[136,137]

8. Conclusions and future directions

The underlying mechanisms of the formation of EVs are still largely unexplored, and the distinction or isolation of purified EV species is still a goal to be attained. Nevertheless, the formation and release of EVs seem to relate to cellular homeostasis by balancing intra- and extracellular signals. Clearly, EVs are likely to contribute to physiology and pathology. There is still no consensus on EV classification which is likely related to the lack of sensitive methodologies on the detection of EVs. Currently, new technologies which provide sensitive detection and reliable measurements of EVs are being developed. These new technologies as well as the preparation of EVs from body fluids also need to be standardized to make the measurements of EVs feasible in the clinical settings. In the near future, EVs may serve as potential clinical biomarkers for diagnosis and prognosis, and therapy of certain diseases.

Practice points

- All human body fluids including blood, urine, saliva, mother milk, and cerebrospinal and synovial fluid contain surprising numbers of extracellular vesicles (EVs) which are now thought to contribute to both physiology and pathology.
- EVs carry biomolecules such as proteins, second messengers, and genetic information for delivery and transfer only to dedicated target cells, and therefore are capable of changing the composition and function of target (recipient) cells.
- Increased total numbers and/or numbers of certain subsets of EVs in body fluids may be used to predict the presence of a disease, or a risk factor of developing a disease.
- EVs also offer therapeutic applications, i.e. tumor- and dendritic cell-derived exosomes to induce tumor-specific immunity and thus to prevent tumor development.

Research agenda

- The underlying mechanisms of the formation of EVs are still largely unexplored.
- There is still no consensus on EV classification which is likely related to the lack of suitable isolation/purification protocols of single type of EVs.
- There are no protocols available for standardized collection, isolation and storage of EVs.
- New technologies which allow sensitive detection and reliable measurements of EVs also need to be standardized to make the measurements of EVs feasible in the clinical settings.

Conflict of interest

None of the authors (YY, AS, RN) of this manuscript has a conflict of interest.

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