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Review

Exosomes and Ectosomes in Intercellular Communication

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Exosomes and ectosomes, two distinct types of extracellular vesicles generated by all types of cell, play key roles in intercellular communication. The formation of these vesicles depends on local microdomains assembled in endocytic membranes for exosomes and in the plasma membrane for ectosomes. These microdomains govern the accumulation of proteins and various types of RNA associated with their cytosolic surface, followed by membrane budding inward for exosome precursors and outward for ectosomes. A fraction of endocytic cisternae filled with vesicles — multivesicular bodies — are later destined to undergo regulated exocytosis, leading to the extracellular release of exosomes. In contrast, the regulated release of ectosomes follows promptly after their generation. These two types of vesicle differ in size — 50–150 nm for exosomes and 100–500 nm for ectosomes — and in the mechanisms of assembly, composition, and regulation of release, albeit only partially. For both exosomes and ectosomes, the surface and luminal cargoes are heterogeneous when comparing vesicles released by different cell types or by single cells in different functional states. Upon release, the two types of vesicle navigate through extracellular fluid for varying times and distances. Subsequently, they interact with recognized target cells and undergo fusion with endocytic or plasma membranes, followed by integration of vesicle membranes into their fusion membranes and discharge of luminal cargoes into the cytosol, resulting in changes to cellular physiology. After fusion, exosome/ectosome components can be reassembled in new vesicles that are then recycled to other cells, activating effector networks. Extracellular vesicles also play critical roles in brain and heart diseases and in cancer, and are useful as biomarkers and in the development of innovative therapeutic approaches.

Introduction

Until almost 30 years ago, membrane fragments observed in extracellular fluid were believed to result from apoptosis and other processes of cell death. This proposal has been increasingly questioned during the last two decades and is now known to be wrong. In fact, together with membrane fragments, extracellular fluid contains two types of extracellular vesicle (EV), exosomes and ectosomes, which act close to and also at considerable distance from their parent cells [1,2]. In order to focus your attention on these vesicles, keep in mind some problems that have led to difficulties in their identification and characterization. In the past, the term exosome was used for another type of vesicle that was released from the plasma membrane during the maturation of reticulocytes, and also for a peculiar molecular machine involved in RNA processing [3,4]. Moreover, growing evidence has shown that exosomes in extracellular fluid are composed of distinct subpopulations, including very small nanovesicles [5–8]. As for ectosomes, which are more variable in size than previously believed [5], they have been called by many other names, such as microvesicles, microparticles, and shedding vesicles, and these terms are still widely employed [1,2].

When they were first identified [9,10], the two types of EV were shown to emerge from membranes of different types, located at different sites within the cell (Figure 1). The exosome precursors, called intraluminal vesicles (ILVs), originate from the membrane of endocytic cisternae by inward budding of microdomains. Upon ILV accumulation, the cisternae become multivesicular bodies (MVBs) [1,2,9,10] (Figure 1). After persisting in the cytosol for variable periods of time, some MVBs undergo exocytic fusion with the plasma membrane, followed by release of their ILVs — now defined as exosomes — to the extracellular space [1,2,9,10]. Ectosomes, on the other hand, are rapidly generated at the plasma membrane [2,10]: cargoes are initially assembled at the cytosolic face, then differentiated membrane microdomains, marked by outward budding, appear at the cell surface, followed by vesicle fission and rapid release to the extracellular space [1,2] (Figure 1).

The different sites and mechanisms of their formation strongly suggest that exosomes and ectosomes differ from each other. To clarify the extent and properties of these differences, efforts were made to isolate pure fractions of the two EV types. For many years, however, procedures based on differential centrifugation, mostly employed for this task, resulted in the isolation not of pure fractions, but of various EV mixtures. Recently, differential centrifugation procedures have improved in combination with other techniques, such as ultrafiltration and immune-isolation [7,11,12]. Moreover, appropriate new techniques, such as mass spectrometry and nanoparticle tracking analysis, have been introduced to EV separation with positive results [13,14]. At present, therefore, the comparative knowledge of the two — not always pure — EV types is stronger and more convincing than years ago.

Despite their almost simultaneous discovery [9,10,15,16], studies of the two EV types have progressed differently for

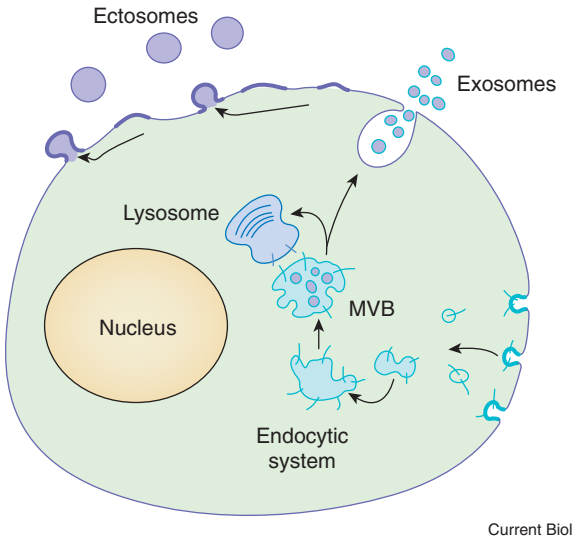

Figure 1. Generation and release of both exosomes and ectosomes.
The dependence of exosome generation on the endocytic system is illustrated at the center of the figure. A multivesicular body (MVB), generated from an endocytic cisterna by the accumulation of vesicles, exhibits small membrane curvatures corresponding to distinct microdomains. Concomitantly, specific proteins and nucleic acids (cargoes) from the cytosol accumulate at the intracellular surface of these microdomains. The subsequent inward budding of the microdomains is followed by their fission and the release into the MVB lumen of exosome precursors, the small (50–150 nm diameter) intraluminal vesicles (ILVs). These vesicles (light violet) are characterized by membranes and dense cargoes largely different from those of the endocytic system. Upon their generation, the MVB can proceed in two alternative directions (arrows), either towards lysosomes, followed by fusion and degradation of its components, or towards the plasma membrane, followed by the fusion (exocytosis, top right) and prompt discharge of its ILVs to the extracellular fluid. The released vesicles are recognized as exosomes. The assembly and release of ectosomes, marked here by horizontal arrows at the top middle and left of the cell surface, occur at the plasma membrane. In this case, the initial step involves the assembly of membrane microdomains larger than those of MVBs, distinct in composition from the originating plasma membrane of origin, and partially similar to the membrane of ILVs. Concomitantly, specific cargoes, composed of proteins, lipids and nucleic acids, accumulate at their cytosolic surface. The subsequent stages involve the rapid outward curvature and outward budding of the microdomain, and then fission and release to the extracellular fluid of ectosomes (violet), which at 100–500 nm diameter are distinctly larger than exosomes. A complex property of both exosomes and ectosomes, not shown in this figure, is the possible heterogeneity of both membranes and cargoes. This feature, discovered by comparative analysis of distinct cells, occurs also in single cells, especially following functional or pathological changes.

many years. Relevant studies of the cell biology and physiology of exosomes were first reported before 1990 and exhibited a continuous increase thereafter, reaching high values around 2008–2010. During this time, the interest in ectosomes remained limited. Subsequently, however, ectosomes started to attract interest and in 2011/2012, their studies, although still fewer, were comparable to those of exosomes [1,2]. During recent years, the studies of both EV types has continued to increase because, in parallel with providing important developments in biology and physiology, the studies have introduced substantial new insights into pathology and various diseases.

Relevant EV discoveries made in the past have expanded our knowledge of intercellular communication. As already reported and critically discussed in a number of comprehensive reviews, all cells are now known to communicate by the exchange of large molecules via EV traffic [1,2,17–19]. This review focuses instead on the recent progress made by studies of the biology and physiology of EVs and discusses the role of EVs in disease and the potential utility of EVs as specific biomarkers and as important tools for the development of innovative therapies.

Generation of Extracellular Vesicles

The generation of ILVs (diameter 50–150 nm) within MVBs (Figures 1 and 2) has been reported to depend primarily on the endosomal sorting complex required for transport (ESCRT), a molecular machine composed of four complexes of protein subunits that is active in local membrane remodeling during viral budding, cytokinesis, autophagy and other processes. In EVs, the ESCRT machinery acts in a stepwise manner together with associated proteins, such as Alix (Alg-2-interacting protein X) and ARRDC1 (arrestin domain containing 1) [17,18,20–22]. During exosome formation, the accumulation of distinct proteins, lipids and nucleic acids at the cytosolic face of endocytic membrane microdomains is driven by these associated proteins [17,18]. Concomitantly, the microdomains undergo inward curvature and budding, with the final fission and release of discrete ILVs into the MVB lumen (Figure 1). Some of the proteins that end up being contained within ILVs are ubiquitinated. The ESCRT machinery participates in the deubiquitination of some of the sorted proteins via its interaction with the protein tyrosine phosphatase HD-PTP [20], and this deubiquitination is necessary for exosome function. ILV generation depends not only on the ESCRT machinery but also on additional mechanisms: for example, the sphingomyelin-derivative ceramide participates in the inward curvature and budding of endocytic membrane microdomains, and the small GTPase Ral is involved in both the fusion of MVBs with the plasma membrane and the ensuing exosome release [2,17,18,23,24].

Less is known about the generation of ectosomes (diameter 100–500 nm) compared with that of ILVs. The assembly of ectosomes (Figure 1) requires the accumulation of cargoes, which are partially different from those of ILVs, at the cytosolic surface of specialized plasma membrane microdomains. The concomitant membrane dynamics involve the outward budding and fission of the corresponding microdomains. Such an effect may be due, on the one hand, to the rearrangement of the asymmetric membrane phospholipid layers induced by Ca^{2+}-dependent enzymes, flippases and floppases [2], and on the other hand, to the action of at least two ESCRT complexes that activate processes analogous to those occurring during ILV generation [2,17]. Additional factors that regulate shedding of ectosomes from the plasma membrane include the small GTPase Arf6, which is active in vesicular traffic, and the small GTPases of the Rho family, RhoA, Cdc42, and Rac1, which operate via the contraction of cortical actin beneath the plasma membrane [25–28]. In cells exposed to various types of stimulation, ectosome generation is often distributed across multiple, large areas of the plasma membrane.

Properties of Extracellular Vesicles

In addition to various molecules derived from MVBs and plasma membranes, EVs sort and accumulate additional molecules that are reported to be rare or even absent in other cellular structures.

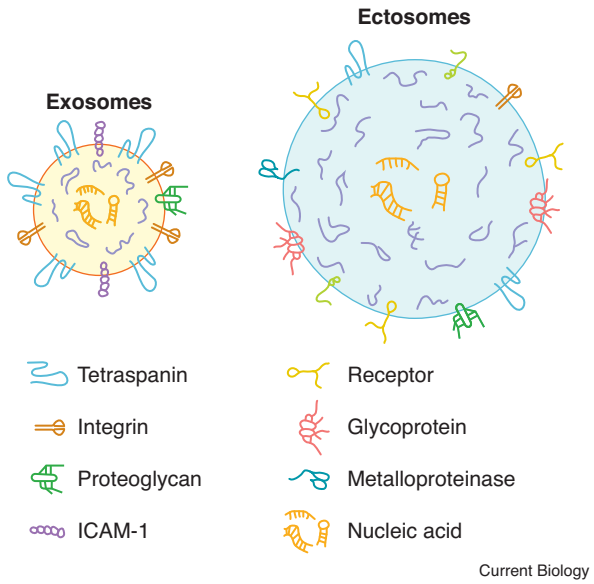

Figure 2. Structure and composition of exosomes and ectosomes. The comparative analysis of the two EV types shows that, in addition to their different sizes, both the membranes and cargoes of these EV types differ from each other, albeit only partially. In exosomes (yellow fill), the membranes are rich in tetraspanins, small transmembrane proteins that are critically important for trapping both membrane and luminal proteins. In contrast, the adhesion protein ICAM-1 appears to be present only in the exosome membrane. In ectosomes (pale blue fill), the membrane contains many additional proteins and is abundant in some receptors, glycoproteins and metalloproteinases. The cargoes trapped within the lumina of both EV types contain many proteins (purple strings). Among these proteins, those concentrated or highly concentrated in these lumina are considered to be typical of EVs; those of lower concentration might be typical of general cytosol trapped within EVs. The proteins near the EV surface might be anchored to the plasma membrane by myristoylation, palmitoylation or other sequences. At the center of the lumina of both EV types is depicted three types of nucleic acid (orange). (Additional images of EVs can be found in other papers [62,88,95].)

Because of these peculiar molecules and the mechanism behind their function, EVs are of particular relevance in cell physiology. The release of these vesicles from the surface, in fact, can induce changes in their parent cells. The subsequent interaction, fusion and integration of their membranes with membranes of target cells and of parent cells can affect the dynamics and functional activities of both cell types. Many other effects, such as the appearance of new activities, regulation of pre-existing functions, changes in protein turnover, and expression of new genes, can appear or be reinforced in target cells upon the discharge of EV cargoes. In many cases, however, the identification of discharged molecules, i.e. proteins, lipids and nucleic acids, is still limited, although this is not a result of insufficient knowledge about EV content. In fact, extensive results from proteomic analyses of EVs (for example [5,7,11,12,28]) have been used in the development of three online public databases — EVpedia (www.evpedia.info), ExoCarta (www.exocarta.org), and Vesiclepedia (www.microvesicles.org) — in which some non-protein EV molecules have also been reported [29–31]. However, in view of the problems still existing in EV studies, including the inadequate separation of exosomes and ectosomes by differential centrifugation [11,12] and the heterogeneity of EVs (see next section), these databases need to be considered with some caution [12,13].

Heterogeneity

Until recently, exosomes and ectosomes were presented as two distinct, homogeneous types of EVs, each exhibiting its own properties. This claimed homogeneity, believed for many years, was initially questioned approximately 10 years ago, when exosomes released from different cells began to be investigated in parallel. Up to now, exosome heterogeneity has been reported among numerous cell types, including endothelial cells, macrophages, hepatocytes, adipocytes, and smooth and skeletal muscle fibers. In these cells, heterogeneity affects not only luminal cargoes but also the EV membranes [32]. Until recently, heterogeneity was thought to occur only between exosomes from different cell types, but this limitation no longer exists. Heterogeneity also occurs among exosomes from a single cell type, when investigated under various developmental and functional states. Under these conditions, single cells could contain populations composed of mixtures of different exosome subtypes, carrying a few differently expressed proteins together with many homogeneous proteins [5,6]. Most often, heterogeneous protein cargoes are accompanied by mRNAs and miRNAs, which can also be heterogeneous [6,26]. Heterogeneity is not a property specific to exosomes; it has also been demonstrated for ectosomes [33] released from control and disease cells. In addition, heterogeneity of both exosomes and ectosomes has been reported in cancer cells [34]. In conclusion, heterogeneity is important in the complex physiology of the two EV types, impacting on the effects induced by membrane fusion and/or cytosolic discharge in both parent and target cells [5,6,32,34].

Membrane and Cargo Composition

Recent insights into EV composition have revealed that the two EV types share some properties and differ in many others. Exosome membranes have been investigated extensively and the lipid component is characterized by high concentrations of cholesterol and sphingomyelin, together with ceramide, a product of the latter that is important for the biogenesis of ILVs [1,2]. In addition, exosome membrane lipids include lysobisphosphatidic acid, an unconventional phospholipid absent in other cellular membranes that contributes to the accumulation of cholesterol [35]. Among exosome membrane proteins, clusters of tetraspanins together with other transmembrane proteins assemble into functionally active membrane structures, the so-called dynamic platforms. During EV generation, tetraspanins have been reported to interact with cytosolic proteins and possibly participate in the process of luminal cargo loading [13,36]. Additional tetraspanins, regulated by cholesterol [37] and operating independently of other proteins, appear to play a role in the trapping of surface and intracellular signaling proteins, such as E-cadherin, β-catenin and Wnt [38–41]. In addition to tetraspanins, the exosome membranes include: flotillin, PGRL (CD81 regulatory-like protein), and stomatin, which bind lipids; the adhesion proteins L1CAM (L1 cell adhesion molecule) and LAMP2 (lysosomal associated membrane protein 2); integrins; the enzyme alanyl aminopeptidase N [41]; and insoluble fibronectin, a surface glycoprotein [42] (Figure 2).

The accumulation of cargoes (Figure 2) begins at the start of ILV assembly and continues until fission. Some of these cargo proteins are found within vesicles at concentrations analogous to, or even lower than, those in the cytosol from which they are derived. Among these proteins are: cytoskeletal and associated

proteins, such as actin, vimentin, talin and annexin; a few chaperones, such as Hsp70 (a chaperone that protects the structure and function of proteins) and Hsc70; and a number of enzymes, such as phosphoglycerate kinase 1 (PGK1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Additional luminal proteins may be accumulated as cargoes via a cooperation between the ESCRT machinery and tetraspanins [38,39]. Various GTPases, such as Arf6, are involved in the regulation of the ILV assembly processes. Together with luminal proteins, the ILVs, via mechanisms including a cooperation between ESCRTs and tetraspanins, accumulate RNAs, such as miRNAs, and in addition include RNA-binding proteins as well as proteins that modulate RNA function, such as Argonaute 2 (AGO2) and Y-box binding protein 1 [43–46]. Non-coding RNAs and DNA sequences have been found amongst cargoes of the exosome lumen [43,45,47], although their mechanisms of accumulation are not clear.

Ectosome membranes have high levels of cholesterol, sphingomyelin, and ceramide [25,28,33,34], analogous to those of exosomes. Perturbations induced by Ca^{2+}-dependent enzymes can result in the reorganization of phospholipid bilayers, with translocation of phosphatidylserine from the inner leaflet to the outer leaflet, followed by functional changes of the plasma membrane and cytosol. Phospholipid changes are accompanied by changes in membrane proteins. In addition to the presence of integrins, tetraspanins and a few receptors at relatively low density, ectosomes contain other proteins, such as the matrix metalloproteinase MT1-MMP, two glycoprotein receptors (GP1b and GPIIb/GPIIa), the adhesion protein P-selectin, and the integrin Mac-1 [2,48]. In agreement with the EV heterogeneities already discussed, some of these proteins are not present in all ectosomes and are found only in subpopulations of ectosomes.

The ectosome lumen resembles that of the exosome in terms of the presence of particular cytosolic proteins, most of which have a cytoskeletal function. Heat shock proteins and several enzymes are present at concentrations analogous to those in the cytosol. In contrast, other luminal proteins accumulate at high concentrations by various mechanisms. Several proteins active in ectosome assembly are driven by the ESCRT machinery. Other proteins, however, operate not by specific sequence motifs, but by their direct interaction with the plasma membrane. These interactions are often mediated by protein anchoring, myristoylation, palmitoylation and sumoylation and by high-order polymerizations, concentrated in direct contact with the tethering microdomains. Additional proteins, directly bound by anchored complexes, are concentrated in deep areas of the ectosome lumen [49]. Rho and Ras GTPases are involved in the regulation of the assembly of ectosome cargo complexes. As in exosomes, RNAs are also abundant within ectosomes — mostly miRNAs but also mRNAs and non-coding RNAs [47].

Release of Extracellular Vesicles from Parent Cells

As already mentioned, exosomes and ectosomes are both released from parent cells to the extracellular fluid in response to appropriate triggers, for example, the activation of specific receptors or the presence of signaling factors, such as cyclic AMP and Ca^{2+}. Upon release, both types of EV start to navigate. The release of exosomes, both at rest and in response to appropriate stimulations, occurs at various times after the biogenesis of ILVs. In many cells, MVBs, which undergo exocytosis with the ensuing release of exosomes, are numerous and can therefore operate as stores that prolong the release, delaying the depletion of exosomes. For ectosomes, the situation is profoundly different: once generated by the accumulation of specific molecules at their plasma membrane microdomains, these vesicles are promptly released. The different properties of the two EV types could depend, at least in part, on the different timing of their release.

In response to appropriate stimulation, MVBs move towards the plasma membrane from the perinuclear cytoplasm, their intracellular location during resting conditions. Shortly thereafter, the MVBs undergo fusion by a process of exocytosis (Figure 1). Several G proteins — Ral1, Rab27a and Rab27b — participate in the control of both the intracellular translocation and the fusion of MVBs [23,50]. The sites of fusion, depending on the cells involved, can be found across the whole plasma membrane or concentrated at specialized sites. As with intracellular membrane fusions, MVB fusion is governed by a SNARE complex composed of an as yet unidentified P-SNARE within the MVB membrane and two Q-SNAREs (SNAP23 and syntaxin 1a) in the plasma membrane [51]. The exocytosis is followed by so-called MVB secretion, i.e. the release of exosomes to the extracellular fluid.

Compared with exosomes, ectosome release is much faster. From macrophages and microglial cells, the release occurs at a high rate even when the cells are at rest, although the release from many other cells, including neurons, is slower. Upon appropriate stimulation, ectosome release increases from all cell types. In neurons and other neural cells, such release is triggered by Ca^{2+} responses dependent on depolarization; in many other cells, the release is stimulated by ATP upon activation of its receptor P_2X7. Protein kinase C and ATP kinases are also involved. Together with local mechanisms necessary to initiate ectosome release, other processes occur in the cytoplasmic regions proximal to the microdomains. In the presence of RhoA, Rac, and Cdc42 [26], local ectosome release is favored by contraction of cortical actomyosin adjacent to the membrane microdomains [52]. The ensuing local outward curvature and budding of the plasma membrane are followed by the generation of spiral filaments by ESCRT-III and then by membrane fission induced by the Vps4 ATPase via a ring established at the budding neck. The ensuing membrane scission and the final pinching off result in the release of ectosome vesicles to the extracellular fluid [53–55].

Extracellular Vesicle Navigation, Interaction and Fusion

The events that take place after release of the vesicles primarily involve their membranes. Firstly, the vesicles experience a change of their environment, as they move from the cytosol and the surface of the cell to the extracellular fluid. Secondly, the vesicles then undergo interactions with the plasma membrane and endocytic membranes of target cells. Finally, the subsequent fusions between EVs and cell membranes correspond to the completion of the extracellular EV pathway.

Navigation

Upon their release from parent cells, EVs begin their navigation — a non-regulated process with several interesting consequences. First, a fraction of the vesicles remains intact for only a short

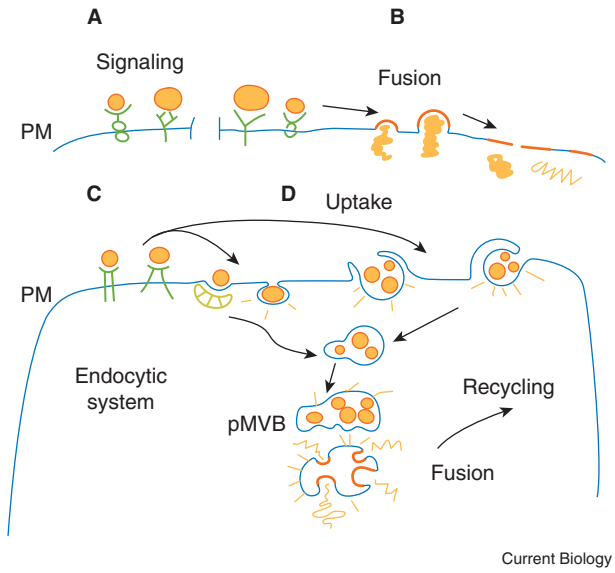

Figure 3. Interactions and fusions of EVs with target cells.
(A,B) The interaction of the two EV types with the plasma membrane (PM) of a target cell. (A) An exosome and an ectosome of different size are directly bound to cell surface receptors. This process, which does not occur frequently, is not followed by EV fusion but results in the activation of intracellular signaling. (B) EVs bind to receptors in a similar way as in (A), but here binding to receptors or other appropriate sites is followed by EV fusion with the plasma membrane, discharge of the luminal cargoes into the cytosol, and integration of the EV membranes with the surface of the cell. (C,D) Additional events follow the EV binding to the plasma membrane and take place in the endocytic system. The two forms of uptake on the left are mediated by clathrin and caveolae, and those to the right depend on phagocytosis and macropinocytosis. After uptake, intact EVs are loaded first within endocytic vesicles, then within cisternae. Accumulation within a pseudo-multivesicular body (pMVB) is followed by EV membrane fusion and discharge of cargoes to the cytosol. The EV membranes and cargoes could then undergo redistribution within the cell, with ensuing recycling of either exosomes or ectosomes assembled at MVBs or the plasma membrane, respectively. Recycled EVs are finally released to the extracellular fluid by the previous target cell that has now become the parent cell.

time before the membrane breaks down. Bioactive cargoes, such as interleukin-1β and several growth factors (TGFβ, FGF, VEGF and others), are released from the vesicles and become available for direct binding to their receptors on adjacent cells, and thus for the induction of specific responses [56,57].

Most EVs, however, are resistant to membrane breakdown and thus persist in the extracellular fluid, even for long periods of time. Their surface enzymes and other molecules induce some form of pre-binding effects in the surrounding cells, such as the digestion of extracellular matrices [58]. In addition, EVs tend to accumulate in the extracellular spaces proximal to intercellular junctions, moving through and between cells. By these movements, EVs can thus leave their initial fluid and move to adjacent areas of tissues [59] and, possibly, also to large bodies of fluid, such as blood serum, lymph and cerebrospinal fluid. Movement to the latter fluids is seen at high levels following injection of EVs into diseased mice (such as mice with cancer or kidney disease). Upon injection into healthy mice, EVs accumulate mostly in organs such as the liver [60,61].

The most interesting feature of navigating EVs is the recognition of their specific target cells. Examples of such recognition processes were observed over 10 years ago when platelet ectosomes in the blood were shown to bind monocytes and not neutrophils [62]. Since then, additional observations have been made concerning, for example: exosomes from cortical neurons binding other neurons, not glial cells; exosomes from oligodendrocytes binding microglia, not other types of brain cells; and ectosomes from mesenchymal cells binding mesenchymal cells, but not mesenchymal stem cells, endothelial cells or granulocytes [63–65].

Interaction

Upon recognition of their target cells, EVs (often investigated by optical tweezers [66]) establish interactions with their surface, followed in many cases by fusion with the plasma membrane or endocytic membranes [67] (Figure 3). In the first step, the EV–target cell interaction resembles the analogous interaction of host cells with herpesviruses, which are built around a core glycoprotein that acts as an ‘activator’ of their binding to the cell surface [68]. Recent studies have led to a greater understanding of this interaction process and shown that the same EVs can bind in different ways to various target cells. For example, when ectosomes released from microglia interact with other microglial cells, they start rolling over the surface until they reach sites appropriate for internalization. Upon astrocyte binding, however, the microglial ectosomes remain almost stationary [66,69]. Exosomes released from human fibroblasts are first recruited on filopodia, and then start surfing until reaching a ‘hotspot’ of internalization at the body of their target cells [69]. What are the mechanisms of these varied EV–cell interactions? EVs lack an ‘activator’ like that seen for herpesviruses; instead, interactions appear to be generated by the reciprocal binding of molecules present at the surface of the two interacting membranes. The nature of these molecules is still largely unknown. In EVs, such a role has been proposed for tetraspanins, integrins, proteoglycans and lectins, which are present at variable density on the membrane of both exosomes and ectosomes. In target cells the proposed binding could involve surface receptors and adhesion molecules, as well as laminin and fibronectin, two extracellular matrix proteins [67,69–71]. In some cases, EVs induce signaling upon their binding to receptors differently exposed at the surface of many target cells [72] (Figure 3A). In most other cases, however, EV signaling occurs only upon membrane fusion and is followed by the discharge of luminal cargoes into the cytosol of target cells [66,67,69–71] (Figure 3B).

Fusion

Before discussing the details of membrane fusions between EVs and target cells, the present knowledge of these processes is considered. In contrast to membrane fusions that occur inside cells, EV–target cell plasma membrane fusions are not governed by SNARE complexes. CD9, a tetraspanin exposed at the plasma membrane, participates in the fusion of muscle fibers [73]. Expression of CD9 in eggs was proposed to participate in their fertilization by sperm [74]. In view of the expression of tetraspanins in both types of EV, the possible participation of CD9 in their fusion cannot be excluded [75]. Additional studies have been carried out on proteins with similarities to proteins first identified in viruses [68,76], such as syncytin-1 and syncytin-2,

first discovered in placental trophoblasts and then found in a variety of other cell types [77,78]. Recently, these proteins have been reported to be present in exosomes and may be important for their fusion [79,80]. The membrane fusion governed by syncytins appears to begin with their high-affinity binding to MFSD2a or ASCT2, two transmembrane proteins expressed by target cells that are analogous to transporters for carbohydrates and neutral amino acids, respectively. Completion of fusion requires the insertion into the cell membrane of the hydrophobic sequences of syncytins, followed by lipid reorganization, protein restructuring, and membrane dimpling [80].

EV fusions can occur in different locations. Fusion with the plasma membrane is followed by the concentration of discharged cargo molecules into the cytosolic layer near the surface of the cell [17,80] (Figure 3B). Alternatively, after docking at an appropriate surface site of endocytosis, EVs are transferred to intracellular cisternae, where they start trafficking [80–84] (Figure 3C). The initial binding occurs at various endocytic sites (Figure 3C,D): some clathrin-dependent forms of internalization have been confirmed by the observation of EVs surrounded by coated pits [66,69,81]; dependence on some caveolae has been demonstrated by the co-localization of some of them with exosomes [66,69,71,82]. Macropinocytosis is also involved in EV uptake, concomitantly with other forms of endocytosis [65,84]. Finally, exosomes are internalized by phagocytosis and co-localize with lysosome markers such as LAMP-1 [85]. Following endocytic internalization, EVs move along the endocytic pathway until their fusion with an endocytic membrane [66,69,71]. Operationally, the fusion of EV membranes with either the plasma membrane or endocytic membranes induces the integration of its molecules at the same time as the discharge of cargoes into the cytosol.

Molecules of both integrated membranes and cargoes participate in EV recycling, i.e. in the assembly of new EVs destined to be released from the original target cell [1,2,6,17] (Figure 3D). In the latter processes, however, the molecules incorporated into EVs originate from different sources, such as from the previously fused vesicles or from local synthesis in target cells [83,84]. Compared with integrated EVs, recycling EVs have reduced homogeneity and belong to distinct subpopulations. The back-and-forth exchange of EVs therefore does not correspond to real ‘crosstalk’ but to complex bidirectional interactions of partially heterogeneous vesicles between functionally coordinated cell networks.

General Effects of Extracellular Vesicles

The effects of EVs on target cells, some of which have already been summarized in this review, as well as their general effects on organs, first described in various diseases (see, for example [27,34,60]), are relevant for many physiological and pathological processes. Since, however, many such effects are open to question, only a few of them will be mentioned here. In terms of general effects, EVs are likely to participate in the maintenance of cell homeostasis and in the regulation of cellular functions, although conclusive demonstrations are still limited [19,80]. In contrast, the role of EVs in intercellular communication, an extension of the effects induced by other processes, is fully established. Such communication is based on the traffic of proteins [6,13], RNAs (mRNAs, miRNAs and non-coding RNAs) [31,45,47,86], and DNA sequences [13,86]. The most relevant of these effects are those induced by miRNAs that in some cases affect cell survival, especially by changing the levels of components essential for life [45,86]. Another important series of EV effects concerns signaling in target cells. Such effects, induced by surface receptors and/or surface enzymes, are activated upon EV binding to the plasma membrane and by intracellular enzymes and factors, and are affected by cargoes discharged into the cytosol upon EV fusion [1,2,40,41,62]. Similar mechanisms can affect the regulation of gene expression by transcription factors, signaling proteins and many enzymes [40,51–55,87]. In specific tissues, some EVs control events based on intense recycling among new target cells [88]. Finally, EVs derived from stem or progenitor cells play important roles in the repair of various organs and in protection against diseases, such as diabetes [89].

Extracellular Vesicles and Disease

Interest in the role of EVs in disease is long standing. Research started a long time ago, in which some studies provided insights into the role of EVs in pathology (see, for example [25,34,39,42,51]), while others were useful for increasing our understanding of their role in cell biology and physiology. Recently, the interest in EV research has become increasingly focused on specialized medicine, although general associations of EVs with diseases are still being investigated. Based on these considerations, this section focuses on diseases of the brain and heart/blood vessels as well as on cancer, as these are diseases that are most frequently and seriously affected by EVs, and goes on to discuss the potential of EVs to act as biomarkers and the development of EV-dependent therapies.

Role of Extracellular Vesicles in Disease

An important role of EVs in cancer often depends on the transfer of components of oncogenic signaling pathways from one cell to another [25–27,90–93]. In particular, transfer of specific miRNAs by EVs through cell populations has been found to increase the resistance to widely employed chemotherapeutic drugs, coincident with a progressive increase of disease severity [26,94–96]. Analogous horizontal transfer of EVs, originating from healthy cells, was found to affect cancer by the development of metastases resistant to therapy [97]. EVs have critical effects also on neurodegeneration. In Alzheimer’s disease and amyotrophic lateral sclerosis, neurodegenerative effects were found to depend on the transfer of multiple molecules — miRNAs, gangliosides and proteins — by EVs from healthy cells [98]. Additional effects are induced by some EVs via the crossing of the blood–brain barrier, with the spreading of brain antigens to peripheral tissues. These events contribute to the development of immunological brain diseases, such as multiple sclerosis [99]. Both exosomes and ectosomes also participate in the communication between various types of cardiac cells [100], and some EVs affect atherosclerotic plaque development in vessels by inducing inflammation, proliferation, thrombosis and vasoactive responses [101].

Biomarkers and Therapeutic Agents

In addition to their toxic effects, EVs have prognostic and therapeutic roles in various diseases. As previously discussed, during various diseases EVs can accumulate in large fluids, especially in the blood serum. Analyses of these fluids can therefore reveal the presence of EVs in specific diseases as well as the presence

of some of their components, such as DNAs and miRNAs, which are important as biomarkers for disease diagnosis and personalized therapy [98–103]. In addition, the increase or decrease of these biomarkers can provide information about the state of the patients and can be used as prognostic indicators.

EVs engineered by multiple, largely innovative approaches are important in therapeutic approaches. The properties of these EVs hold tremendous promise because of their high stability in the circulation, their biocompatibility, their low immunogenicity and toxicity, and their possible targeting to desired organs, tissues or cells [91,92,104,105]. EV engineering has been achieved by different procedures. Viral and non-viral procedures have been employed to induce parent cells to secrete modified EVs or to manipulate EV cargoes [106,107]. Loading of drugs has been obtained by incubation, electroporation, transfection or activation of parent cells [107]. To obtain targeted therapeutics, specific peptides have been added to the surfaces of exosomes. Alternatively, specific genes or tumor-associated antibodies have been loaded within EVs [107]. Most results obtained by these approaches have been positive, with decreased tumor cell migration and proliferation, increased sensitivity to chemotherapeutic drugs and enhanced immune responses [108]. Examples of these developments, especially in cancers and neurodegenerative diseases, have appeared recently in the studies of functionally coordinated cell networks [109,110]. Ongoing active research will convert these results into established therapies for precisely identified and personally characterized diseases.

Conclusions

The discovery and characterization of EVs have contributed to the revelation of innovative properties in cell biology and physiology. The existence of the two EV systems, which are functionally analogous yet profoundly different in their origin, association, distribution, cellular lifetime, and mechanisms of release, was unexpected. The two EV types become apparently similar upon their release from parent cells, during a period that covers many steps, from their navigation and extracellular distribution to their interaction with target cells, fusion/integration with cell membranes, and discharge of cargoes, as well as their possible recycling. EV-dependent communication is a critical process for all cells. In contrast to signaling and exchange of small molecules, EV-dependent communication induces the exchange of large molecules: proteins, various types of RNA and, in some cases, of DNA. Intercellular communication was already mentioned in various sections of this review [13,31,45,47,81,91–93,100], but emphasis is given here to the reciprocal communications sustained by EV recycling, resulting in functionally coordinated cell networks that are critical in many cancers and participate in the spread of disease and in metastasis [111,112].

Additional properties unique in cell biology are the accumulation of molecules during EV assembly. Proteins and lipids that are rare or absent in other cell membranes accumulate in the microdomains destined to become EV membranes. Concomitantly, microdomains facilitate the accumulation of cargoes, composed of proteins and nucleic acids, at the cytosolic surface. Upon release and fusion, the properties of EV components induce significant changes in target cells, interfering extensively with the expression and levels of some proteins and other molecules. These properties are especially prominent in many cancer cells, where the numerous EVs are particularly rich in miRNAs involved in the control of various critical processes [86,95,98].

During the next few years, several important developments are expected to occur, reinforcing and expanding the present knowledge of EVs. Among these are the isolation and detailed characterization of heterogeneous EV subpopulations. By new procedures, various important results should become available, including the identification of various EV and sub-EV markers as well as the generation of new tools for the isolation of distinct types of RNA. Additional problems to solve include the recognition of EV target cells and the mechanism of EV targeting, fusion and membrane integration. Finally, the increased knowledge about various diseases, necessary for the progress of preclinical, prognostic, and therapeutic studies [90–112], could also contribute to further developments of clinical — and physiological — relevance.

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