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Review

Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials

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ABSTRACT

Background: Cells continuously secrete a large number of microvesicles, macromolecular complexes, and small molecules into the extracellular space. Of the secreted microvesicles, the nanoparticles called exosomes are currently undergoing intense scrutiny. These are small vesicles (30–120 nm) containing nucleic acid and protein, perceived to be carriers of this cargo between diverse locations in the body. They are distinguished in their genesis by being budded into endosomes to form multivesicular bodies (MVBs) in the cytoplasm. The exosomes are released to extracellular fluids by fusion of these multivesicular bodies with the cell surface, resulting in secretion in bursts. Exosomes are secreted by all types of cells in culture, and also found in abundance in body fluids including blood, saliva, urine, and breast milk.
Scope of review: In this review, we summarize strategies for exosome isolation, our understanding to date of exosome composition, functions, and pathways, and discuss their potential for diagnostic and therapeutic applications.
Major conclusions: Currently, the control of exosome formation, the makeup of the "cargo", biological pathways and resulting functions are incompletely understood. One of their most intriguing roles is intercellular communication — exosomes are thought to function as the messengers, delivering various effectors or signaling macromolecules between supposedly very specific cells.
General significance: Both seasoned and newer investigators of nanovesicles have presented various viewpoints on what exosomes are, with some differences but a large common area. It would be useful to develop a codified definition of exosomes in both descriptive and practical terms. We hope this in turns leads to a consistent set of practices for their isolation, characterization and manipulation.

1. Introduction

Cells are known to secrete a large variety of vesicles (along with macromolecules – usually in complexes – and smaller molecules like salts and cofactors) into the extracellular space. The vesicles are diverse and depend on the type and origin of the cells and their current state – for example, transformed, differentiated, stimulated, stressed. Subsets of these vesicles have been variously called exosomes, apoptotic blebs, shedding vesicles, microparticles, prostasomes, tolerosomes and prominosomes [reviewed in 1,2]. Most of these names relate to origin or presumed function, but the subset used in particular experiments are defined by physical characteristics reflecting the isolation and characterization protocols used. As a result, there is a big overlap between all these classes, making it difficult to precisely ascribe functions to each type.

Exosomes are the microvesicles that have received most attention over the past decade [3–5]. The term was first coined by Trams et al. in 1981 [6] for exfoliated vesicles from cell lines with ectoenzyme activity. Two years later, release of small vesicles and tubules from rat reticulocytes was described [7]. Shortly after that an electron microscopic study was published describing the exocytosis of approximately 50-nm bodies by sheep erythrocytes demonstrating both size and their initial genesis in endosomes to create MVBs [8]. Johnstone et al. [9] were the first to re-isolate these nanovesicles and show they retained multiple active enzymes. Although protein transport by exosomes was accepted by the research community, the transport of RNA was not shown until much later — Valadi et al. 2007 [10] is the first publication to definitively show that RNA was carried in exosomes as well. Searches for this research are obfuscated by the fact that in the late 1990s and early 2000s the term "exosome" was co-opted to name the mRNA-degradosome in eukaryotic cells. The current conceptualization of exosomes is that they are small (30–120 nm) vesicles containing nucleic acid and protein cargo. Not only are they secreted by all cell types in culture, they are also found

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to occur naturally in body fluids including blood, saliva, urine, and breast milk. Although the mechanism of exosome formation is defined to be through invagination into endosomes to form MVBs, the precise molecular mechanics for this process, as well as their composition, "cargo", and resulting functions are only beginning to be unraveled. Originally thought to be just "garbage bags" allowing cells to get rid of the unnecessary proteins, now exosomes are viewed, at least in part, as specifically-secreted vesicles enabling intercellular communication. There is an exponentially growing interest in the study of exosomes, from their function in the body to more practical applications — utilizing them for diagnostics and therapeutics development. Indeed, to date, over 1500 "exosome" papers are found on PubMed, most of which were published within the last 3 years, and the International Society of Extracellular Vesicles (ISEV) was recently established, with over 700 members joining instantly. Dissemination of the current knowledge outside this core group can only aid understanding of these microvesicles, and potentially help generate an increased general audience interest in the analysis and utilization of these fascinating nanoparticles. In this review, we summarize to-date knowledge on exosome composition, functions, in vivo pathways, strategies for their isolation, and discuss their potential diagnostic and therapeutic applications.

2. Defining exosomes

The variety of extracellular vesicles secreted by different cell types and inability to easily distinguish them with the currently available isolation and characterization procedures has led to some confusion regarding nomenclature. Their origin through multivesicular-endosomes (MVEs, but usually referred to as MVBs for multivesicular bodies) was initially used by Johnstone et al. to define exosomes. Although he was looking at exosomes formed during erythrocyte maturation [9], a similar formation pathway was reported for vesicles secreted by B-lymphocytes when Raposo et al. examined this process [11]. This is the defining pathway for exosome production in current parlance. Another type of secreted vesicular body is called the shedding microvesicle, as they are secreted by direct budding (shedding) of cytoplasmic contents from the plasma membrane. We will not discuss these further here — a concise review was written by Cocucci et al. [2]. The Raposo group pointed out the similarities between the aforementioned multivesicular bodies and lysosomes. This similarity was later investigated by the Simons lab [12], which showed that the sphingolipid ceramide plays a key role in the genesis of exosomal, but not lysosomal MVBs. However, experimental procedures used to purify all secreted vesicles must of course be based on extant properties such as the size, density and morphology and cannot discriminate their mode of origin. Indeed, the most rigorous protocols for isolation of exosomes are based on ultracentrifugation of the sample fluid after it has been cleared by a series of lower-speed spins. Pelleting at 100,000–110,000 $\times g$, followed by resuspensions and repelleting is the most-commonly used method. For purer preparations, some researchers use sucrose cushions or gradients, since exosomes float in sucrose solutions at a density that ranges from 1.13 to 1.19 g/ml [4,11]. Initial characterization of exosomes is typically based on the electron microscopy — as their small size, less than 130 nm, is below the resolution of the light microscope.

The size of exosomes is in part dictated by their origin. Since they are indeed vesicles, their minimum size is dependent on the structures of a lipid bilayer. A lipid bilayer has a thickness of about 5 nm, and the bilayer has enough stiffness that the smallest vesicle possible is on the range of 30 nm. Since they derive by budding off inside endosomes (200–500 nm), their maximum diameter realistically has to be on the order of 100 nm. The implication of this small size is that the "cargo hold" for these particles is on the order of 20–90 nm across, indicating an internal volume of 4.2–380 yl (10^{-24} l). This is comparable to the volume of a eukaryotic ribosome, calculated to be about 14.7 yl [13], so the total cargo per exosome is probably $\leq$100 proteins and $\leq$10,000 net nucleotides of nucleic acid. Standard negative staining methods for transmission electron microscopy (TEM) allow visualization of round vesicles with obvious lipid bilayers as well as some bodies with a characteristic cup-shaped morphology. These latter are thought to represent exosomes deformed during the preparative process, as they are not found when the more structure-preservative method of cryoelectron microscopy is used [14]. A representative standard TEM image of the exosome sample is shown in Fig. 1.

Besides a characteristic morphology, exosomes are thought to be somewhat unique in their protein and lipid composition, providing additional traits for their identification. Due to their endosomal origin, all exosomes contain membrane transport and fusion proteins (GTPases, Annexins, flotillin), tetraspannins (CD9, CD63, CD81, CD82), heat shock proteins (Hsc70, Hsp 90), proteins involved in multivesicular body biogenesis (Alix, TSG101), as well as lipid-related proteins and phospholipases [14,15] (Fig. 2). Although these proteins are routinely used as positive markers, there is wide variation across exosomes from different sources. Beyond these membrane-associated proteins, over 4400 different proteins have been identified in association with exosomes, usually by mass spectrometry, presumably serving as cargo for inter-cell communication [16]. Although much of this variation has to do with the cells of origin, Carayon et al. [17] reported that the protein repertoire in secreted exosomes also changed during erythrocyte maturation. The most widely used "markers" include tetraspannins, Alix, flotillin, TSG101, and Rab5b. Antibody-based techniques to detect these targets, such as Western or ELISA, are becoming popular for rapid confirmation of exosome presence. The same antibodies putatively could also be utilized for affinity purification of exosomes — as will be discussed in more detail below.

Besides proteins, exosomes are enriched in certain raft-associated lipids such as cholesterol (primarily B lymphocytes), ceramide (implicated in the differentiation of exosomes from lysosomes) other sphingolipids, and phospoglycerides with long and saturated fatty-acyl chains [12,18,19]. Laulagnier et al. [20] also found that, at least for mast and dendritic cells, there was an increase in phosphatidylethanolamines and that the rate of flipping between the two leaflets of the bilayer was higher than in cellular membranes. There are also indications that exosomes could serve to deliver prostaglandins to target cells [15].

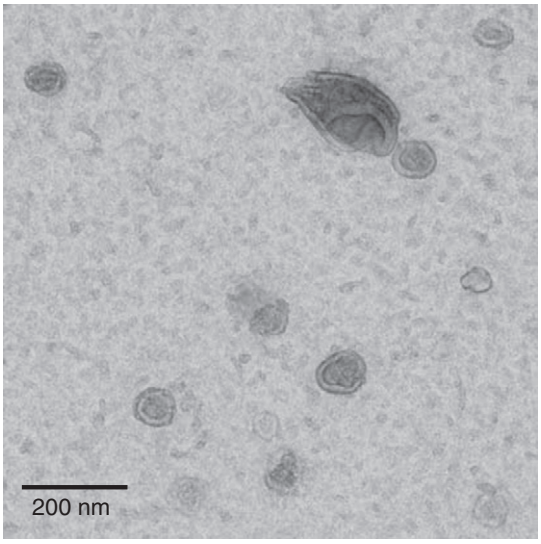

Fig. 1. A representative TEM image of the exosome sample. Exosomes isolated from the human blood serum by the ultracentrifugation protocol were analyzed at the Texas A&M Microscopy & Imaging Center.

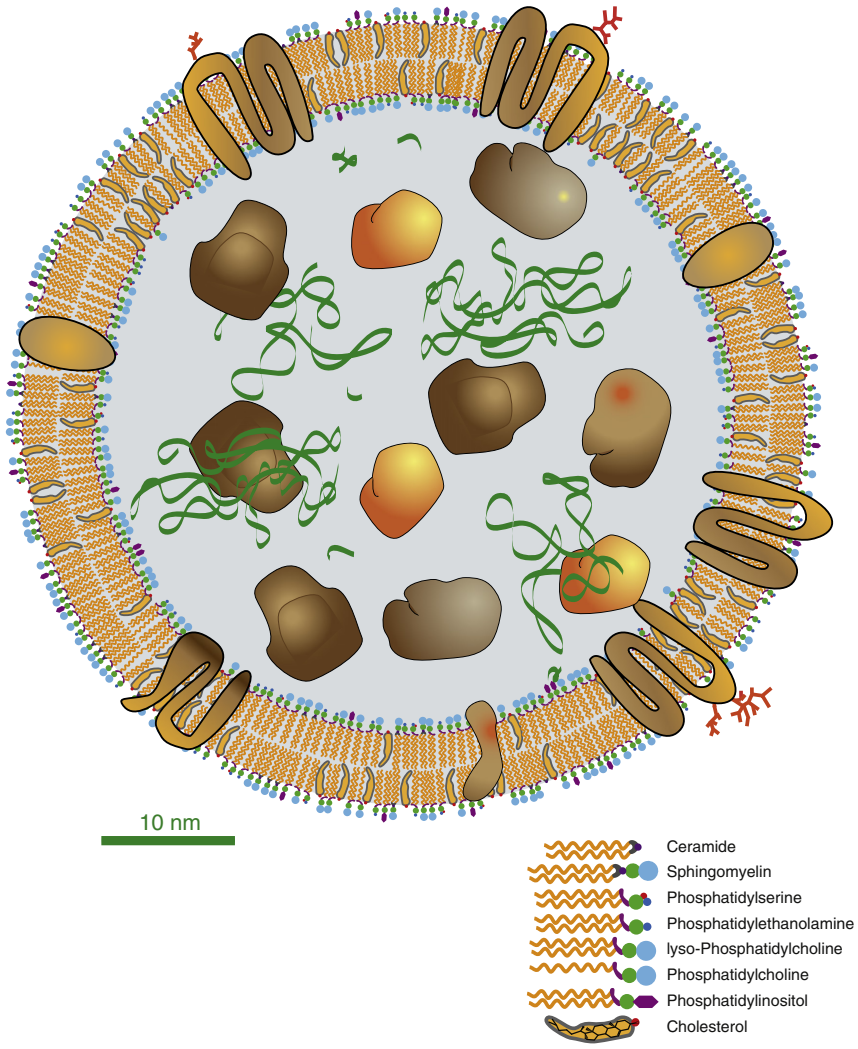

Fig. 2. Representation of a mid-size exosome, about 60 nm in diameter, with the relative size of the membrane and cargo (blobs = proteins, green ribbons = RNAs) drawn in proportion. Additionally, the lipids in the membrane, symbolized as shown in the key, are in the proportions as given for mast cell-derived exosomes by Lanlagnier et al. Red branches represent polysaccharide chains, and positions and relative amount are purely speculative.

Exosomes also bear saccharide groups on their outer surface. This was investigated recently by Batista et al. [21] and they found that these were enriched in mannose, polylactosamine, α-2,6 sialic acid, and complex N-linked glycans.

Exosomes have been reported to contain significant amounts of miRNA, other non-coding RNAs, as well as mRNA. Valadi et al. [10] reported that, although the RNA appeared to be mostly degraded to less than 200 nt fragments, some full-length molecules must also be present, since the extracted RNA could be used to generate identifiable full-length proteins using an in vitro translation system. Several papers indicate that the RNA "cargo" of exosomes is significantly different from the parental cell content, i.e. certain RNAs are present at significantly different levels compared to the total cell lysate from the originating cells [22–24]. This runs counter to several authors working with cancer cells, who have noted that the miRNA content for their originating cancer cells is similar to that found in circulating exosomes, and they have postulated the feasibility of using this as a basis for diagnostic markers [25,26]. Since the primary current procedure to verify the presence of exosomes is through EM, a costly and time-consuming process, there is clearly an urgent need to develop simpler, more molecule-based tools and protocols for confirmation of exosomal presence. MicroRNA (miRNA) may provide this marker, although it has yet to be determined if any RNA molecules can serve as reliable generic exosomal markers.

3. Isolation of exosomes

The accepted protocol for isolation of exosomes includes ultracentrifugation, often in combination with sucrose density gradients or sucrose cushions to float the relatively low-density exosomes [27] (Fig. 3). Isolation of membrane vesicles by sequential differential centrifugations is complicated by the possibility of overlapping size distributions with other microvesicles or macromolecular complexes. Furthermore, centrifugation to pelleting may prove insufficient means to separate vesicles based on their sizes. However, sequential centrifugations, when combined with sucrose gradient ultracentrifugation, can provide a high enrichment of exosomes.

Isolation of exosomes based on size, using alternatives to the ultracentrifugation routes, is an obvious option. Cheruvanky et al. [28] reported successfully purifying exosomes using ultrafiltration procedures that are less time consuming than ultracentrifugation, and do not require use of special equipment. Similarly, Bioo Scientific launched a kit (ExomiR) that essentially removes all cells, platelets and cellular debris on one microfilter and captures all vesicles bigger

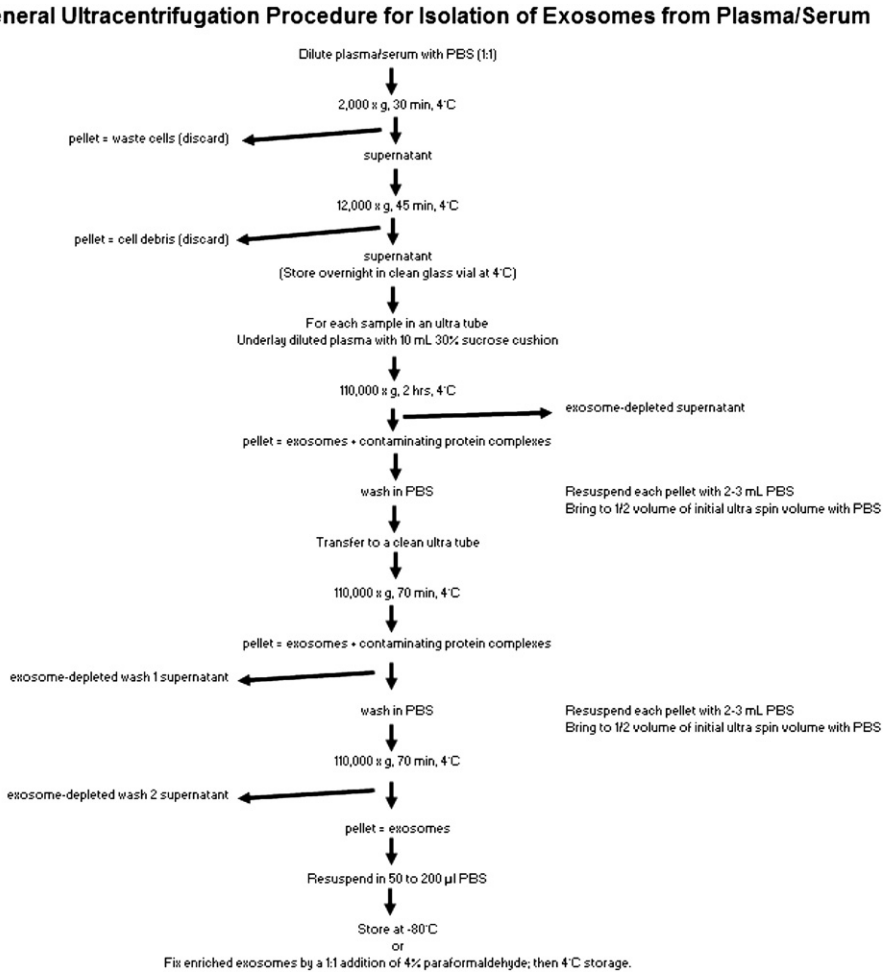

Fig. 3. The ultracentrifugation-based protocol for isolation of exosomes. A combination with sucrose density gradient or sucrose cushion is often included to float the relatively low-density exosomes.

than 30 nm on a second microfilter using positive pressure to drive the fluid. For this process, the exosomes are not reclaimed — their RNA content is directly extracted off the material caught on the second microfilter, which can then be used for PCR analysis [29]. HPLC-based protocols could potentially allow one to obtain highly pure exosomes, though these processes require dedicated equipment and are not trivial to scale up [30]. The complication is, both blood and cell culture media contain a large number of nanoparticles (some non-vesicular) in the same size range as exosomes. For example, Wang et al. [31] found that large number of miRNAs are contained within extracellular protein complexes rather than exosomes (biological roles for these are yet to be understood). As a consequence, the above methods are best described as allowing one to obtain exosome-enriched samples, rather than pure exosomes.

Volume-excluding polymers such as PEGs are routinely used for precipitation of viruses and other small particles [32–34]. This principle, or perhaps differential solubility in alternative solvents, could be used to precipitate exosomes (quite probably along with other macromolecular particles) from experimental samples. The precipitate can be isolated using either low-speed centrifugation or filtration. Recently, System Biosciences released a proprietary reagent named ExoQuick that can be added to serum, conditioned cell media or urine, and is claimed to precipitate the exosomes [35]. In our hands, although the process is very fast and straightforward, there is the inevitable lack of specificity toward exosomes, and the pellet from serum is rather difficult to resuspend.

In theory, a superior alternative for specific isolation of exosomes should be affinity purification with antibodies to CD63, CD81, CD82, CD9, EpCAM, and Rab5. These could be used by themselves or potentially in combination. For this application, the antibodies could be immobilized on a variety of media, including magnetic beads, chromatography matrices, plates and microfluidic devices [27,36]. HansaBioMed is offering an array of products called ExoTest kits – featuring anti-CD63, -CD81 or -CD9 antibodies immobilized on 96 well plates – for exosome capturing and characterization [37]. As with any young field, it has to be confirmed how well these systems work, and for researchers wanting a diverse exosome population, which of these proteins is(are) the best and most robust exosomal tag(s) for their needs.

In the same vein as antibodies, other affinity-capture methods could be used, such as lectins, which will bind to specific saccharide residues on the exosome surface. This strategy has been proposed by Aethlon Medical [38] using a proprietary lectin that targets mannose residues. The convenient feature of this procedure is easy elution/release of the captured exosomes by free alpha-methyl-mannoside. However this approach is not specific to exosomes as a number of cells contain mannose on their surface; and it has yet to be proven if all exosome types can be captured this way. Multiple types of lectins are available, and these can be carefully investigated to select the best options. Vn peptides were also recently reported to be capable of efficiently binding exosomes (Dr. S.Griffiths, pers. comm.). As in the case of lectins, little is known about the practicability at the moment, but the approach is very interesting and definitely worth investigating.

4. Exosomes biological functions

Multiple cell types have been described to release exosomes in extracellular medium in vitro, including: hematopoietic cells (B cells, T cells, dendritic cells, mast cells, platelets), intestinal epithelial cells, Schwann cells, adipocytes, neuronal cells, fibroblasts (NIH3T3), and numerous tumor cell lines. Exosomes are also found in vivo in many biological fluids including blood, urine, saliva, epididymal fluid, amniotic liquid, malignant and pleural effusions of ascites, broncho-alveolar lavage fluid, synovial fluid and breast milk. When they have been quantified, they are present in surprisingly high numbers; for example, blood serum contains about 3,000,000 exosomes per microliter (Fig. 4). All exosomes, whether secreted by cells in culture into their media, or by various organs into associated bodily fluids, have been suggested to participate in intercellular communication, either supporting or perturbing (in the case of cancer) different physiological processes [4,39,40].

First reports focused on the expulsion of proteins during the process of reticulocytes maturing into erythrocytes. These authors characterized the particles pelleted from the extracellular blood plasma at 100,000×*g* to be vesicular in nature and coined the term "exosomes" for them. Further analysis showed that the exosomes formed provided a major route for removal of plasma membrane proteins during the cell maturation process [9,41]. For some cells like these, exosome secretion is more like an excretion function — an efficient mechanism to get rid of unnecessary proteins and RNA. Exosomal release instead of lysosomal processing is beneficial to cells that don't have efficient degradation capability or are located toward a drainage system such as the tubules of the kidney or the gut [9,42]. In subsequent years more and more functions were discovered, and exosomes were found to be secreted by many types of cells not on this sort of maturation path.

Depending on their cell/tissue of origin many different functions have been attributed to exosomes. Exosomes were intensively studied as facilitators of the immune response [43] and the role of exosomes in antigen presentation has been extensively documented [44]. Roles for exosomes in programmed cell death, angiogenesis, inflammation, and coagulation were reported [45]. Exosomes have been implicated as morphogen transporters in the creation of polarity during development and differentiation [39].

Platelets secrete exosomes, in addition to shedding microvesicles, after activation [46]. In this case, the shed microvesicles are used to provide a larger surface for the prothrombinase complex, but the main function of the exosomes appears not to be in the coagulation reaction, but in some unknown function. Since they are carrying prostaglandins, they could be involved in the inflammatory response.

A role for exosomes in the migration of Dictyostelium cells by the secretion of chemo-attractant signals has been proposed [47]. This study shows that migrating cells accumulate multivesicular bodies and secrete vesicles at their trailing edge. These vesicles may form extracellular tracks, presumably allowing cells to follow a path left by a leading cell.

One group studied the levels of different microRNAs in the exosomes derived from human breast milk for several months of lactation [48]. Certain miRNAs, in particular miR-181a and miR-155, which are implicated in immune regulatory roles, were present at high levels in the first 6 months of lactation, but significantly reduced at later stages. It was proposed that the miRNAs contained within exosomes from breast milk can modulate the development of the infant's immune system.

More recent studies have demonstrated that exosomes are not only specifically targeted to recipient cells to exchange proteins and lipids or to trigger downstream signaling events, but also deliver specific nucleic acid cargo [10,49,50]. Exosomes' most unique function might be specific interaction with a target recipient cell, enabling cell–cell communication, putatively between widely separated locations in the body.

Mast cells secrete exosomes that contain mRNA from approximately 1300 genes (although how much of this is intact mRNA versus fragmented is still not clear) and small RNA, including >100 different microRNAs [10]. The transfer of exosomes to a donor cell showed that at least some mRNAs were full-length, as they were translated in the recipient cell.

Glioblastoma cells also secrete exosomes and microvesicles containing mRNA, miRNA and angiogenic proteins [23]. When taken up by host human brain microvascular endothelial cells, mRNA molecules were translated and tubule formation by the target endothelial cells was stimulated.

The spread of oncogenes by exosomes and microvesicles secreted by tumor cells has also been reported [51]. Aggressive human brain tumors (gliomas) often express a truncated and oncogenic form of the epidermal growth factor receptor, known as EGFRvIII. These authors showed that EGFRvIII can be 'shared' between glioma cells by intercellular transfer of microvesicles. EGFRvIII expression in indolent glioma cells stimulates formation of lipid-raft related microvesicles containing EGFRvIII. Microvesicles containing this receptor are then released to cellular surroundings and into the blood of tumor-bearing mice, and can merge with the plasma membranes of cancer cells lacking EGFRvIII. This event leads to the transfer of oncogenic activity to the target cells, including activation of transforming signaling pathways (MAPK and Akt), changes in expression of EGFRvIII-regulated genes (VEGF, Bcl-xL, p27), morphological transformation and an increase in anchorage-independent growth capacity. Thus, membrane microvesicles of cancer cells can contribute to a horizontal propagation of expression products of oncogenes and their associated transforming phenotype among populations of cancer cells.

Exosomes seem to also play a role in spreading pathogens such as prions and viruses from one cell to another [49,52]. Various pathogens are able to subvert the exosome pathway. Pegtel et al. [53] has shown that miRNAs secreted by Epstein Barr virus (EBV)-infected cells are transferred by exosomes and act in uninfected recipient cells. When infected with HIV, for example, dendritic cells, monocytes, macrophages and lymphocytes can produce both exosomes and HIV virions [54], and in many cases it's hard to discriminate them.

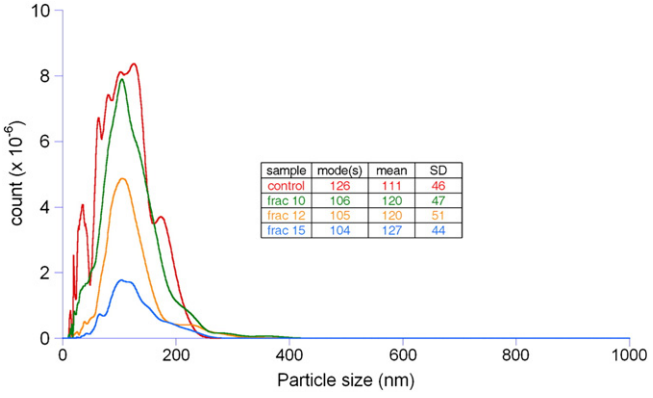

Fig. 4. Analysis of exosomes in liquid samples. Several samples of exosomes derived from blood serum were analyzed with the Nanosight LM10 instrument. The profiles are basically very-finely segmented histograms, indicating the number of particles per ml (in millions) for each size, in bins of 1 nm increment from 0 to 1000 nm (2000 nm on the actual instrument). Particles are tracked through light scattering from a laser source, and the paths calculated over time to determine their velocity due to Brownian motion. Their sizes can be calculated from this independent of density (see www.Nanosight.com for details). The samples above represent a control serum sample rendered cell-free as shown in the protocol in Fig. 3 (red), and the green, yellow, and blue lines are progressively lighter fractions through a sucrose gradient, sowing the more defined size of the particles in these preparations.

Interestingly, 10% of proteins are identical between HIV virions and exosomes issued from the same cell type. Also, exosomes compete with HIV for virus entry into endocytic compartments [55].

These studies suggest that exosomes are routinely used for intercellular communication. Traditionally, cell communication was classified as contact dependent (juxtacrine), paracrine, endocrine, exocrine or synaptic [56]. These modalities of cell–cell communication were thought to occur by receptor-medicated events, either by recognizing a component of an adjacent cell surface, a transmitter from a synaptic partner, or a hormonal molecule released by other cells at varying distances. In these last two cases of contact-independent communication, the historical understanding was that cells secrete signaling molecules that can passively diffuse into recipient cells due to their small size, or would be internalized via a specific receptor-mediated uptake. A new picture is now developing: that when a more complex "message" needs to be sent, cells use exosomes. These nanovesicles are essentially analogous to viruses — natural machines capable of traveling from one cell to another and easily unloading their contents across the cell membrane due to their unique characteristics.

An advantage of exosomes as mediators of intercellular communication is that the message can be targeted to specific, multiple locations. The delivery if multiple miRNAs through exosomes allows for rapid alterations in gene expression in the entire repertoire of targeted cells. The messages transmitted by this intercellular communication may include those for growth, division, survival, differentiation, stress responses, apoptosis, etc.

The details of the in vivo pathways from genesis in MVBs, through release to extracellular fluids, to their targeted internalization into recipient cells and release and utilization of cargo are all active areas of investigation. The packaging of specific cargo loads with appropriate destination tags itself is extremely intriguing and currently a black box. Since the RNA and protein cargo is encased in a membrane, they are impervious to circulating nucleases and proteases in the blood. It also appears that these vesicles are not ingested by macrophages leukocytes, presumably because they are recognized as 'self' by the immune system, as there seems to be a very long half-life in the bloodstream enabling communication between remote anatomical locations. Presumably sets of specific surface legends or adhesion molecules enable exosome specific targeting to a specific set of recipient cells, as has been exemplified in the immune system, between its components [40], and also in communication with the intestinal epithelium [57]. Given that exosomes are able to be endocytosed into the endosomal system of recipient cells, it seems likely that, following uptake, exosomes could fuse with the limiting membrane of endosomes to deliver their content into the host cell cytoplasm in a reversal of their formation process. It is still possible, though, that exosomes can directly fuse with the plasma membrane. Indeed, it is known that the fusogenic protein, CD9, is abundantly expressed in exosomes [58].

5. Exosomes in diagnostics

Over the last few years it has been discovered that all body fluids contain exosomes (e.g. blood, urine, saliva, milk), and because of their specific protein, RNA, and lipid content, exosomes may be useful for early diagnosis of various diseases. Minimally invasive diagnostics (based on analysis of blood) or non-invasive diagnostics (using urine and saliva samples) are superior alternatives to traditional needle or excision biopsies due to the reduced patient pain and inconvenience, and greater speed and lower cost of analysis.

In 2008, a number of groups reported bloodborne miRNAs as very valuable biomarkers. Since naked RNA is degraded rapidly in blood, it was apparent that this RNA must be protected by packaging into some sort of macromolecular complexes. Over the next two years, it became clear that a major portion of these complexes were exosomes.

Chen et al. [59] identified specific expression patterns of serum miRNAs for lung cancer, colorectal cancer, and diabetes, providing evidence that serum miRNAs contain fingerprints for various diseases. Two non-small cell lung cancer-specific serum miRNAs (miR25, miR223) were further validated in an independent trial of 75 healthy donors and 152 cancer patients. Mitchell et al. [60] reported serum levels of miR-141, a miRNA expressed in prostate cancer, can robustly distinguish patients with prostate cancer from healthy controls. The strong evidence that miRNAs in circulating exosomes are representative of those expressed in the originating tumor provides a compelling case for very early detection of the disease using exosomes as a screening tool. In all these cases, the cargo could be a byproduct of the cancer cells aberrant metabolism or could be involved in signaling remote cells as part of metastasis. In an example of diagnosing an abnormal but non-disease state, Gilad et al. [61] compared serum miRNA levels in pregnant and non-pregnant women. In sera from pregnant women, miRNAs associated with human placenta (miR526a, miR527, miR515-5p, miR521) were significantly elevated and their levels correlated with pregnancy stage.

Although blood serum is relatively-easily acquired, it still involves some limited invasiveness, and use of samples acquired through non-invasive procedures is preferable. Urine could be very useful source of exosomal markers of urogenital diseases. Urinary exosomes were reported to contain the mRNA encoding two molecules known to be over expressed in prostate cancer, PCA3 and a specific product resulting from a chromosomal rearrangement, the TMPRSS2:ERG fusion [62]. Aquaporins 1 and 2 have been also characterized as markers of renal ischemia/reperfusion injury and anti-diuretic hormone action [63].

Saliva is another fluid easily obtained by non-invasive means. Exosomes have also been found in human saliva, and these contain nucleic acid and protein that may serve as disease biomarkers [64]. Many of the markers are the same as those found in blood, and it seems to be informationally-rich enough to provide a portal to the whole organismal system. Thus, saliva-based diagnostics, in addition to assessing the health state of the oral cavity, also shows the potential to enable monitoring of systemic health [65].

Keller et al. [66] isolated exosomes from amniotic fluid, saliva, and urine by differential centrifugation on sucrose gradients, and investigated their diagnostic value. CD24 polymorphisms were selected as a model system for the diagnostic readouts. Two polymorphisms within the CD24 gene are known to modify disease risk and progression in multiple sclerosis (MS), systemic lupus erythematosus (SLE), giant cell arteritis, and chronic hepatitis B. A C→T SNP (rs52812045) results in an alanine (A) to valine (V) substitution. The CD24 V/V genotype is associated with faster disease progression. It was found that exosomal RNA is an efficient template for the typing of the CD24 SNP. It also allowed sex determination of the fetus based on the detection of the male specific ZFY gene product. Thus, in this case exosomes from all these body fluids contain RNAs which offer easy access to the transcriptome of the host organism, as well as minimally invasive early stage prenatal diagnostics.

Several companies have initiated development of the exosome-based diagnostics within the last three years. Caris Life Sciences is currently developing novel approaches for capturing and analyzing blood-based circulating microvesicles, primarily exosomes. In late 2010, they launched the Carisome® Prostate cMV 1.0 test, a sensitive diagnostic of prostate cancer based on analysis of exosomal proteins [67]. Exosome Diagnostics is developing biofluid-based molecular diagnostic tests for use in personalized medicine. Their main focus is on oncology diagnostics through an exosome-based technology platform [68]. Exosome Sciences is a wholly owned subsidiary of Aethlon Medical, Inc. They are creating diagnostic tools to detect and quantify exosomes in body fluids. The lead product, based on their variation of the ELISA procedure called ELLSA, has been validated to identify the presence of exosomes indicating HIV, tuberculosis, and

various forms of cancer [38]. HansaBioMed, a European collaborative company working with exosomes, are focusing on translational research in exosome-based diagnostics of cancer and neurodegenerative diseases. HansaBioMed performs discovery and pre-clinical development of new diagnostic tools using an ELISA platform called Exotest. They also produce CD63, CD9, CD81 antibodies immobilized on 96 well plates, which can be used for capturing, quantification and characterization of exosomes [37].

To summarize, there are a number of reports demonstrating that exosomes constitute a source of multiple markers of malignancy that could provide clinically useful information. Importantly, exosomes can be easily recovered in a noninvasive manner without the need for surgically obtaining a tissue sample. Several companies have initiated programs aimed toward exosome-based diagnostics, and initial results seem very promising.

6. Exosomes as therapeutics

Several Phase I studies with exosomes were completed in the 2000s. The first one [69; France] used vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes (DEX). DEX were generated containing functional MHC/peptide complexes capable of promoting T cell immune responses, including tumor rejection. They have established a GMP (Good Manufacturing Practice, necessary for manufacturing prescription-grade drugs) process to produce pharmaceutical-grade exosomes on a large scale. Through previous work, they had determined that they could attach peptides directly to exosomes post-purification by incubating them together in slightly acidic media, what they called “direct loading”. Exosomes purified from DC cultures derived from monocytes obtained by leukapheresis from the same patients were loaded with MAGE 3 antigenic peptides using this process, performing the direct loading at 10 and 100 μg peptide/ml of loading media. These exosomes were then used as vaccines. Fifteen stage III/IV melanoma patients received four exosome vaccinations. Loaded exosomes were separated from free peptide, and the amount of MHC antigen in these preparations was measured to determine the dose amounts. Two dose levels of exosomes, as determined by the levels of MHC class II molecules they carried (0.13 and 0.40×10^{14} molecules) were tested. Evaluations were performed before and two weeks after immunization. A continuation treatment was performed in 4 cases of non-progression. Following injection in patients of loaded DEX, an increase of NK cells number was observed and NKG2D expression was restored in NK cells and CD8T cells in some patients. There was no grade II toxicity and no maximal tolerated dose was achieved — indicating the safety of exosome administration.

A similar Phase I study [70; UK] used DEX immunotherapy in patients with advanced non-small cell lung cancer (NSCLC). Safety and efficacy of DEX loaded with the MAGE tumor antigens was tested in 13 patients with stage IIIb and IV NSCLC with tumor expression of MAGE-A3 or A4. Patients underwent leukapheresis to generate DC from which DEX were produced and isolated, then loaded with MAGE-A3, -A4, -A10, and MAGE-3DPO4 peptides. Patients received a total of 4 doses of DEX, one dose weekly. 9 of 13 patients completed therapy. Three formulations of DEX were evaluated, and all were well tolerated. The time from the first dose of DEX until disease progression was 30 to 429 days. Three patients had disease progression before the first DEX dose. Survival of patients after the first DEX dose was 52–665 days. DTH reactivity against MAGE peptides was detected in 3/9 patients. Immune responses were detected in patients as follows: MAGE-specific T cell responses in 1/3, increased NK lytic activity in 2/4. To summarize, large scale production of the DEX vaccine was feasible and DEX therapy was well-tolerated in patients with advanced NSCLC. Some patients experienced long term arrest of the progression of disease and activation of immune effectors.

Another Phase I study [71; China] used autologous ascites-derived exosomes (Aex) combined with the granulocyte–macrophage colony-stimulating factor (GM-CSF) in the immunotherapy of colorectal cancer (CRC). Aex were isolated from the ascites fluid by sucrose/D2O density gradient ultracentrifugation, and the 60–90-nm vesicles were immunoassayed and found to contain both the diverse immunomodulatory markers of exosomes as well as the ectopic carcinoembryonic antigens associated with tumors that were desired for the immunotherapy. 40 patients with advanced CRC were enrolled in the study, and randomly assigned to treatments with Aex alone or Aex plus GM-CSF. Patients in both groups received a total of four subcutaneous immunizations at weekly intervals. Both therapies were safe and well tolerated. Aex plus GM-CSF, but not Aex alone, were found to induce a beneficial response via production of antitumor cytotoxic T lymphocytes (CTL). Therefore, immunotherapy of CRC with Aex in combination with GM-CSF was shown to be feasible and safe.

A Phase II clinical trial was recently initiated with patients bearing inoperable stage III/IV non-small cell lung cancer (NSCLCa) after stabilization or regression with cycles of conventional chemotherapy. Dex vaccination with exosomes bearing IL-15Ra and NKG2D was used in association with cyclophosphamide dosing after platinum-based chemotherapy [72]. In this approach, cyclophosphamide allows the inhibition of regulatory T-lymphocytes, facilitates T cell priming mediated by Dex, and permits the restoration of T and NK cell functions in end-stage patients. The primary objectives of this study are clinical efficacy and safety of DEX.

In summary, exosomes that are appropriately generated have been shown to generate an immune response against tumors, thus holding great promise as a new therapeutic approach. Exosome-mediated immunotherapy is similar to cell therapy since they are both naturally occurring biological products. However, exosomes are more convenient to handle than cells, since they are not “alive”, but metabolically inactive vesicles. This makes them much more stable, allowing them to be stored at −80 °C for over two years without loss of their biological activities. Since they are static biochemical entities, after storage there is no need to expand exosomes, and they can be used directly, either as a standalone vaccine or in combination with other pharmacological agents. In addition, exosomes maintain their antigen presentation within a lymph node two times longer than an antigen presenting cell, which indicates they potentiate the immune response [73]. The only limitations at the moment are that the exosomal equivalents of immunological allotypes have not been defined at this point — all experiments conducted so far rely on exosomes prepared from the same patient they are used on. This is problematic for some patients to receive an adequate dose, as the yields of tumor antigen-loaded exosomes prepared from DC demonstrate rather large variations between individuals. Future experiments may determine compatibility groups, or even provide methods of generating synthetic exosomes de novo.

More recently, a completely different approach for treating cancer was proposed. It was reported that tumor-secreted exosomes are actually suppressing the immune response to the cancer [74]. Aethlon Medical developed and launched the Hemopurifier® medical device to selectively remove exosomes from the circulatory system, including tumor-secreted exosomes. Their postulate is that this will restore the immune system of the cancer patients [38]. The technology uses a large format flow-through canister that can work in an apheresis mode. Proprietary lectins attached to the canister bed matrix act as unique affinity-capture moieties for exosomes, targeting mannose residues on their surface. The company is planning to enter Phase I clinical trials in the near future.

As was mentioned above, exosomes aid in the removal of unneeded or harmful molecules from cells [9,75]. This is obviously useful for proteins without sequences for secretion, but interestingly, exosomes can also eliminate certain introduced drugs from cells, and the amount of drug removed by these exosomes has been reported to

enable the cell's resistance to these drugs [76,77]. Thus, exosomes are likely an additional route in the ability of some tumors to actively pump out various chemotherapeutic agents. Thus, pathways regulating secretion of exosomes from tumors might be novel targets for therapy.

Another very exciting application of exosomes for therapeutic development is their use as delivery vehicles for non-native therapeutics, including nucleic acids, proteins and small molecule drugs.

One group reported successful use of exosomes for delivery of short interfering RNA (siRNA) to the brain in mice [78]. Targeting was achieved by engineering dendritic cells to express Lamp2b, a membrane protein found in exosomes, fused to the neuron-specific RVG peptide3. This group loaded exosomes purified from the conditioned culture media of these cells with synthetic siRNA using electroporation. Intravenously injected RVG-p3-tagged exosomes delivered siRNA specifically to neurons, microglia, and oligodendrocytes in the brain, resulting in a specific gene's knockdown. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of BACE1, the therapeutic target in their mouse model of Alzheimer's disease.

Sun et al. reported that exosomes can deliver the anti-inflammatory agent curcumin to activated myeloid cells in vivo [79]. Monocyte-derived myeloid cells play vital roles in inflammation-related autoimmune/inflammatory diseases and cancers. This novel technology provides the means for anti-inflammatory drugs to be targeted to inflammatory cells, providing a method to overcome unwanted off-target effects that limit their utility. According to this report, not only the targeting is highly specific, but curcumin delivered by exosomes is more stable and higher blood concentrations are achieved.

To summarize, in the last decade several therapeutic approaches utilizing exosomes have been proposed and tested, and some of them have already progressed into Phase II clinical trials. Despite certain challenges that have arisen on the way, the field is rapidly progressing and evolving, and large-scale production of “synthetic” GMP grade exosomes, with the therapeutic cargo and targeting moieties of interest, might soon become a reality.

7. Conclusions

In the past few years, there has been an exponential increase in the number of studies aiming to understand the biology of exosomes as well as other microvesicles. The most concise definition as this juncture would be nanoparticles secreted by living cells into adjacent fluid that have a density of 1.12–1.20 g/ml in sucrose solution. As more characterization is performed, hopefully some widespread markers will be found that are indicative of the entire population (or at least expansive subsets of the entire population) and can be easily assayed. Every day we gain more knowledge on the mechanisms of their formation, secretion, pathways in vivo, internalization into recipient cells, and biological roles of their protein, nucleic acid and lipid cargo. The most intriguing interpretation of exosomes is that they are vesicular carries of the large molecules, such as RNA and proteins, which influence gene expression at separate, sometimes remote, anatomical sites. These fascinating vesicles, similar to viruses, are capable of traveling from one cell to another, easily passing their contents across the cell membrane due to their unique characteristics, and delivering the macromolecular message in a biologically active form. Despite this being a young area of research, it is clear that the obvious utility of these novel particles goes beyond basic research into applications in the diagnostics and therapeutics space.

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