

Extracellular vesicles: structure, function, and potential clinical uses in renal diseases

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Abstract

Interest in the role of extracellular vesicles in various diseases including cancer has been increasing. Extracellular vesicles include microvesicles, exosomes, apoptotic bodies, and argosomes, and are classified by size, content, synthesis, and function. Currently, the best characterized are exosomes and microvesicles. Exosomes are small vesicles (40-100 nm) involved in intercellular communication regardless of the distance between them. They are found in various biological fluids such as plasma, serum, and breast milk, and are formed from multivesicular bodies through the inward budding of the endosome membrane. Microvesicles are 100-1000 nm vesicles released from the cell by the outward budding of the plasma membrane. The therapeutic potential of extracellular vesicles is very broad, with applications including a route of drug delivery and as biomarkers for diagnosis. Extracellular vesicles extracted from stem cells may be used for treatment of many diseases including kidney diseases. This review highlights mechanisms of synthesis and function, and the potential uses of well-characterized extracellular vesicles, mainly exosomes, with a special focus on renal functions and diseases.

Key words: Extracellular vesicles; Cell biology; Kidney diseases; Diagnosis; Treatment

Introduction

The importance and role of extracellular vesicles, such as microvesicles and exosomes, are well documented in many diseases. Several studies have analyzed the effect of extracellular vesicles in cellular communication, but the different populations of extracellular vesicles are often presented in a confusing manner in the literature. Microvesicles are larger than exosomes (100-1000 nm) and are released into the extracellular space by outward budding of the cell membrane. On the other hand, exosomes are produced by a more complex inward budding of endosomes (1). Both microvesicles and exosomes are intercellular protein and RNA transporters and share other functions that are discussed in this review.

Other cellular vesicles include argosomes, blebs, and apoptotic bodies. Argosomes are exosome-like vesicles that contain morphogens, proteins that form a concentration gradient in the tissue, which is involved in signal transduction to convey cellular position during development in multicellular organisms. Argosomes differ from

exosomes mainly in their function. They were found to be released from the basolateral membrane of wing disc cells of *Drosophila melanogaster*. The spread of their morphogens through epithelium promotes development (2,3). It is suggested that they are involved in the direct transfer of materials between donor and recipient cells. Until recently, no standardized method of isolation or any specific marker for these extracellular vesicles was developed.

Apoptotic blebs are protrusive blisters formed when cellular plasma membrane delaminates from the cortical cytoskeleton, covering the entire surface of apoptotic cells. The formation of apoptotic blebs is a physical process that results from an increase in hydrostatic pressure following cellular contraction (4). This dynamic cyclic process of bleb formation and retraction can occur over sustained periods during the progression of programmed cell death. Apoptotic blebs become packed with cellular organelles and chromatin to form the basis of fragmentary membrane-clad apoptotic bodies. Some *in*

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in vitro studies have reported that inhibition of apoptotic blebbing significantly impaired corpse clearance by monocytes and macrophages (5,6). Apoptotic bodies are the final consequences of cellular fragmentation. They are 1000-5000 nm extracellular vesicles that contain intact organelles, DNA, and histones (7). Apoptotic bodies do not come under the scope of this review, but more information about isolation and characteristics can be found in Table 1, which summarizes the differences between well-characterized extracellular vesicles, exosomes, microvesicles, and apoptotic bodies.

There is increasing interest in the study of the importance and function of extracellular vesicles and this review analyzes the mechanism of production and function, and potential clinical uses of extracellular vesicles in renal diseases.

Exosomes: formation and release

Exosomes are secreted by many cell types (8-10), and have been isolated from several physiological fluids such as sperm (11), urine (12), plasma (13), and bronchial lavage fluid (14). They have a diameter of 40-100 nm, a homogeneous shape, with a density of 1.13-1.19 g/mL in sucrose, and can be sedimented at 100,000 g. Most exosomes have an evolutionarily conserved set of proteins, including tetraspanins, Alix, and Tsg101, and have specific proteins that reflect their cellular source (1) (Table 1). They are formed from multivesicular bodies (MVBs), which are intracellular endosomal organelles, characterized by multiple intraluminal vesicles enclosed within a single outer membrane. MVBs are formed from early endosomes, which as prelysosomal structures belong to the degradative endosomal pathway of internalized proteins. They are now known to be involved in numerous endocytic and trafficking functions, including protein sorting, recycling, transport, storage, and release. Actually, some authors recognize two types of MVBs, one in the degradative pathway and another in the exocytosis or recycling pathway (15).

Early endosomes can interact with the Golgi apparatus and the endoplasmic reticulum. Exosomes can be formed by endocytosis of the early endosome membrane,

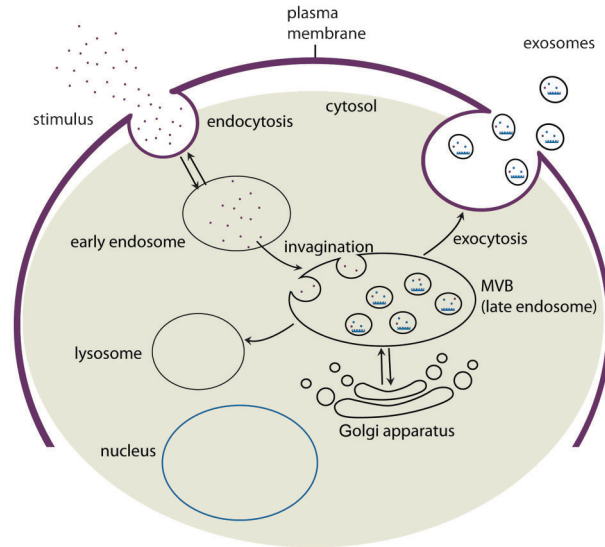


Figure 1. Early endosomes (EE) are formed by endocytosis following microenvironment modifications. The EE undergo maturation forming the late endosome or multivesicular body (MVB). During this process, EE communicate with the Golgi apparatus through bidirectional vesicle exchange. Inward budding of the membrane forms the intraluminal vesicles that will be released to the extracellular space as exosomes, or will fuse with lysosomes where an active degradation process will take place.

having a unique orientation of the involuted cytoplasmic side (16,17) (Figure 1). Generation of MVBs as well as secretion of exosomes are mediated through the concerted action of endosomal complexes required for transport (ESCRT complexes). These protein complexes are involved in the recognition of ubiquitinated cargo by MVBs, as well as the invagination of the MVB outer membrane (18,19).

The origin of exosomes suggests that their production is stimulated in response to alterations in the microenvironment. The formation of early endosomes and MVBs has been shown to increase upon signaling via growth factor receptors, suggesting that the cell adjusts exosome production according to its need (12,20).

Table 1. Main differences between extracellular vesicles, *in vitro* isolation methods, cargo, size, and function.

	Exosome	Microvesicle, ectosome, shedding vesicles	Apoptotic bodies
Size	40-100 nm	100-1000 nm	1000-5000 nm
Markers	CD63, CD9, Alix, TSG 101, HSP 70	Anexin V, Flotillin-2, selectin, integrin, CD40 metalloproteinase	Anexin V, DNA, histones
Isolation method	Immunoprecipitation (ExoQuick [®]), ultracentrifugation (100,000-200,000 g), ultracentrifugation with density gradient	Ultracentrifugation (10,000-60,000 g)	No standardized protocol
References	49,53	54	55

Microvesicles: formation, release, and main characteristics

Microvesicles are extracellular vesicles ranging in size from 100-1000 nm, with different shapes. Although some authors use the term microvesicles for extracellular vesicles in general, the main differences between exosomes and microvesicles depend on size, formation, and the secretion process. They are formed by regulated release of outward budding of the plasma membrane. Several authors use different markers for microvesicles like flotillin-2, selectins, integrins, metalloproteinases, and a high level of phosphatidylserine on the outer surface (Table 1). These extracellular vesicles can be isolated by ultracentrifugation (21), and it is not known if overlapping can occur between these two different populations of extracellular vesicles. There are some resemblances between them: both carry proteins, mRNA, and microRNA (miRNA) and are involved in cellular communication (22,23), possibly through the horizontal transfer of genetic material, directly stimulating the target cell by transferring receptors or proteins.

Extracellular vesicle functions

Cellular communication

Exosomes could be a vehicle by which cells communicate with each other. This finding is supported by experiments where exosomes modulated the function of specific cell lines but not others (24). In addition, exosomes produced by one type of cell can stimulate another specific cell line. For example, exosomes from human bone marrow mesenchymal stem cells can stimulate proximal tubular cells to proliferate through the transfer of hepatocyte growth factor-1 receptor mRNA, suggesting the horizontal transfer of genetic material (25).

Exosomes mediate communication between neighboring cells through a paracrine mechanism and also through long-distance targeting via the systemic circulation similar to hormones (i.e., an endocrine mechanism). This observation is supported by the fact that exosomes have been obtained from various biological fluids such as urine, blood, and breast milk (26). In addition, exosomes extracted from mesenchymal stem cells in culture were administered systemically to animals and protected them against acute kidney injury (AKI), induced ischemia/reperfusion (I/R) (27), and nephrotoxic antibiotics (28).

It was observed that about 60% of the proteins carried by these vesicles were obtained from exosomes of different cell lines (intestinal epithelial cells, T cells, B cells), suggesting that some proteins were constitutively present in these structures to mediate its functions (29). But also the presence of exosome proteins that were not present in the cytoplasm of the donor cell was observed, indicating that they were produced specifically for the mechanism of exosome-mediated communication or

other functions (29).

Exosomes carry bioactive lipids like phospholipases A2, C and D, and prostaglandins that may be involved in the clearance of exosomes (30). Interestingly, no DNA has been found in exosomes, and the RNA they contain does not exactly reflect the quantity of miRNA and RNA in the cells where they originated.

Supporting the communication function, exosomes not only have the capacity to carry a large cargo load, but can protect the contents from degradative enzymes like RNase, trypsin, or chemical substances, due to its bi-lipid membrane (31,32).

The recipient cell can take up this extracellular vesicle through different mechanisms. Exosomes carry membrane proteins that have binding affinity to ligands on the recipient cell membranes or the extracellular matrix, such as transferrin receptor, tumor necrosis factor receptors, lactadherins, integrins, and tetraspanin proteins (e.g., CD9, CD63, and CD81) (33). These membrane molecules may be involved in the homing of exosomes to a specific tissue or microenvironment (15).

Another mechanism of uptake involves fusion with recipient cell membrane, resulting in transfer of the contents of exosomes (mRNAs, miRNAs, proteins, and signaling molecules) or endocytosis (34-36). The mechanism by which these processes are regulated remains to be understood, but it is observed that exosome uptake correlated with intracellular and microenvironmental acidity (37).

Additionally, it was observed that the RNA carried by exosomes was taken up and translated in the recipient cell, demonstrating that they carry functional RNA (24). The findings support the hypothesis that exosomes can be secreted by a specific stimulus, transported to the recipient cell and taken up, and can stimulate the recipient cell.

Microvesicles formed by budding from the plasma membrane are also involved in intercellular communication. The main communication mechanism characterized in the kidney is mediated by microvesicles released from mesenchymal stem cells that reprogram injured kidney cells (38). But both of the extracellular vesicles, i.e., exosomes and microvesicles, share some characteristics such as the capability to reprogram the recipient cell.

Extracellular vesicles and kidney diseases

There are several functions suggested for the exosomes in kidney diseases. They may mediate the transfer of information during renal tubule hypertrophy after nephron loss. Enlargement of renal tubule cells, widening of the tubule lumen followed by an increase in single-nephron glomerular filtration rate, and the transport capacity for salt and water can be mediated by exosome signaling in downstream nephron segments (39). Proximal tubular cell proteins found in downstream segments of the nephron (collector ducts) were probably

transported by exosomes, demonstrating the communication function. The protein or RNA content in exosomes can reflect the cellular origin of the extracellular vesicle, and alteration in the microenvironment indicates an early phase of injury (40), emphasizing the diagnostic potential of extracellular vesicles in kidney diseases.

A number of studies have reported the regenerative potential of microvesicles derived from mesenchymal or endothelial progenitor stem cells against acute kidney injury induced by glycerol (38), cisplatin (41), ischemia, and reperfusion (27) in animals. It was reported that exosomes from mesenchymal stem cells protected rats against nephrotoxic drugs like gentamicin (28).

Extracellular vesicles are involved in the pathophysiological development of diseases. We observed that exosomes from hypoxic proximal cells carrying tumor growth factor- β mRNA could activate fibroblasts to proliferate and produce matrix proteins as another early mechanism of early fibroblast activation during fibrosis (20). Microvesicles also have an important function in the progression of diseases. The human renal carcinoma cells expressing CD105 release microvesicles that stimulate neoangiogenesis. This may be implicated in tumor progression, and in formation of a premetastatic niche in the lung (42). So extracellular vesicles are implicated in both the disease process and regeneration.

Extracellular vesicles as potential biomolecules in diagnostic tools

The potential of exosomes as diagnostic molecules is highly promising. Proteasome analysis of urinary exosomes has identified proteins from all segments of the nephron, including glomerular podocytes (podocin and podocalyxin), proximal tubules (megalin, cubilin, aquaporin-1, and type IV carbonic anhydrase), thick ascending limb of Henle (type 2 Na-K-2Cl cotransporter), distal convoluted tubule (thiazide-sensitive Na-Cl cotransporter), and the collecting duct (aquaporin 2) (12). They have been shown to reflect acute kidney injury and are candidate diagnostic markers (43). In another study, the excretion of exosomes containing aquaporin-1 protein was decreased in rats subjected to I/R (up to 96 h) but later returned to normal levels (after 480 h). The same result was observed in patients after renal allograft transplantation, suggesting that aquaporin-1 carried by exosomes in urine may allow early-to-late detection of renal cellular states after I/R-induced injury and the subsequent regeneration. So extracellular vesicles may be used to predict post-transplant AKI (delayed graft failure) (44).

In healthy humans, urinary exosome analysis has identified over 1000 proteins from different segments of the nephron, but, most importantly, 34 of them were implicated in many kidney diseases such as autosomal dominant polycystic kidney disease type 1 (polycystin-1), autosomal dominant and recessive nephrogenic diabetes

(aquaporin-2), antenatal Bartter syndrome type 1 (Na-K-2Cl symport), and Gitelman's syndrome (thiazide-sensitive Na-Cl cotransporter) (45). Nevertheless, additional studies are necessary to analyze these proteins in exosomes from patients with these diseases (12,45,46).

It was observed that circulating miRNA is decreased in patients with chronic kidney disease and is correlated with the decrease in estimated glomerular filtration rate. The authors suggest that total and specific miRNA can be used as biomarkers of uremia (47).

As observed for exosomes, microvesicles have been investigated for potential use as diagnostic tools. In tumors, microvesicles could be used to determine the status of the tumor and mRNA could indicate the origin of the microvesicle (48).

Exosomes as a drug delivery system

More recently, the property of exosomes as vehicles for intercellular communication has been exploited for the delivery of therapeutic small interfering RNAs (siRNAs) to the brain and to provide alternative routes for drug delivery systems, much as a 'Trojan Horse' (49). Through this strategy, the HIV retrovirus exploits the machinery of exosome genesis for intercellular communication in the host cell to perform virus assembly and the cellular spread of infection in the host, independently of the binding of virus envelope protein to the cell receptor (50). Development of such a delivery system for therapeutic purposes faces many challenges, including ethical and technical issues; for example, choice of the best gene transfection method to introduce the RNA/protein of interest (cargo) into exosome-secreting cells (virus, lipofection, electroporation), how to target the cargo into the exosomes, the best technique to load exosomes with the drug of interest (electroporation or lipofection), and the cost of the process are a few of the concerns that need to be addressed.

On the contrary, exosomes have many unique attributes that make them an excellent choice as a drug delivery system: both protein and genetic material can be loaded into exosomes, they are well tolerated by the human body, have a long circulating half-life, are capable of membrane penetration, have intrinsic homing ability, and are amenable to membrane modifications (51). If we consider mesenchymal stem cell exosomes and microvesicles, the therapeutic potential improves. Since they are easily accessible *in vitro*, mesenchymal stem cells have a large capacity for expansion, are nonimmunogenic, and have the intrinsic therapeutic property of reducing tissue injury, as highlighted by different authors (27,28,38,41,52).

Although research on the use of microvesicles/exosomes for drug delivery in kidney diseases is still in its infancy, one study analyzed the potential of chemically modified 143 miR transfected in THP-1 macrophages *ex vivo*, and microvesicles were isolated and injected into

xenografted nude animals. The microvesicles were localized in serum, tumor, and kidney of the host animal. These experiments indicate that the cargo of microvesicles can be modified to deliver miRNA, and probably mRNA and proteins, to the kidney of the host (52).

Although this mechanism has an important therapeutic potential, more studies are still necessary to sufficiently address a number of issues related to safety and ethics.

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Conclusion and future directions

The use of exosomes as diagnostic biomolecules in a wide range of diseases and as a therapeutic drug delivery system is highly promising. Nevertheless, more studies are required to understand the mechanism of exosome formation and release as well as their physiological and pathological functions in different organs and systems, including non-cancer-related kidney diseases.

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