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Exosomes and the kidney: prospects for diagnosis and therapy of renal diseases

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Exosomes are 40–100 nm membrane vesicles secreted into the extracellular space by numerous cell types. These structures can be isolated from body fluids including urine and plasma. Exosomes contain proteins, mRNAs, miRNAs, and signaling molecules that reflect the physiological state of their cells of origin and consequently provide a rich source of potential biomarker molecules. Aside from diagnostic uses, exosome-mediated transfer of proteins, mRNAs, miRNAs, and signaling molecules offer the promise that they may be used for therapeutic purposes. In this review, we integrate new knowledge about exosomes from outside the field of nephrology with recent progress by renal researchers in order to provide a basis for speculation about how the study of exosomes may affect the fields of nephrology and renal physiology in the next few years.

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Over the past several years, there has been increasing interest in the nephrology community in a newly recognized biological entity, namely, the exosome. Exosomes are small (40–100 nm) membrane-bound vesicles, secreted upon fusion of the limiting membrane of multivesicular bodies with the plasma membrane.[1] Besides the vesicular exosome, there is an entirely different structure in eukaryotic cells that is called an 'exosome', that is, the RNA–exosome, a multi-protein complex that degrades various types of RNA molecules.[2] Vesicular exosomes are also referred to as microvesicles in some works. As discussed in this article, vesicular exosomes may contain RNA exosomes. For simplicity, we use the term 'exosome' to refer to 'vesicular exosomes' throughout. They are present not only in urine[3] but also in a variety of other body fluids including blood plasma.[1] On the basis of protein mass spectrometry (MS) results, urinary exosomes appear to derive from each of the epithelial cell types facing the renal tubule lumen.[3] Similarly, exosomes in plasma most likely derive from the many cell types that face the vascular lumen, including various types of blood cells and endothelial cells. We have previously published reviews on the topic of urinary exosomes, focusing mainly on the isolation of urinary exosomes as the starting material for protein biomarker discovery experiments.[4–7] Here we take a broader view, with an attempt to integrate progress outside of the field of nephrology with recent progress by renal researchers to provide a basis for speculation about what impact the study of exosomes will have on the fields of nephrology and renal physiology in the next few years.

BIOLOGY OF EXOSOMES

In 1981, Trams *et al.*[8] proposed the term 'exosomes' for exfoliated membrane vesicles, appearing as large (500–1000 nm) and small (approximately 40 nm) vesicles, which they identified to be secreted by a variety of cell types. A few years later, Johnstone *et al.*[9] discovered that during reticulocyte maturation, specific proteins, including the transferrin receptor, are shed via secretion of <100 nm vesicles that they termed 'exosomes'. In the current literature, exosomes are defined as 40–100 nm vesicles that are secreted upon fusion of multivesicular bodies (late endosomes) with the plasma membrane. This fusion event results in the release of the intraluminal vesicles of multivesicular bodies, after

which they are termed 'exosomes', into the extracellular space.[1] Exosomes are known to be produced by many different cell types, including dendritic cells, B-lymphocytes, various stem cells, epithelial cells, and endothelial cells,[3,10–15] and can be isolated from cell culture supernatant, as well as from a variety of biological fluids, such as blood, urine, semen (prostasomes), amniotic fluid, and pleural fluid.[3,14,16–19] Multivesicular bodies are late endosomes that are populated with intraluminal vesicles by fusion of small cytoplasmic vesicles derived from early endosomes with the outer membranes of multivesicular bodies, followed by invagination of the recruited membrane, inward budding, and scission (Figure 1). These events are mediated through the concerted action of the so-called ESCRT complexes (endosomal complexes required for transport).[20,21] As vesicles bud inward, the lumina of these future exosomes capture a small portion of the cytosol, taking along a set of soluble proteins, mRNAs, microRNAs (miRNAs), and other cytosolic molecules. The orientation of the lipid membranes of exosomes is identical to that of cells; that is, integral membrane proteins are oriented such that the amino acid sequences facing the outside of the plasma membrane of cells also face to the outside of exosomes.[1] It has been proposed that in addition to random selection of a portion of the cytoplasm, proteins and RNA molecules may be selectively incorporated into exosomes.[22–24]

Besides exosomes, other types of microvesicles can also be isolated from cell culture supernatants and body fluids (reviewed by Camussi *et al.*[25]). These microvesicles are not derived from multivesicular bodies, but appear to be shed by the plasma membrane. Usually, these microvesicles tend to be larger in size (up to 1 μm), although smaller microvesicles, which fall in the range of exosomes, have been described.[26] In addition, it has been shown that there are microvesicles in urine that are derived from microvilli of podocytes.[27] Because of the overlap in size, microvesicles may be included among exosomes when they are isolated from urine.

Proteomic analyses show that many of the proteins detectable in exosomes are common to exosomes from all cell types.[3,13,28] These include ribosomal components, cytoskeletal proteins, small and heterotrimeric GTPases, tetraspanin proteins, and the components of the ESCRT complexes involved in forming multivesicular bodies. Furthermore, exosomes contain many cell-specific proteins. The incorporation of certain proteins into internal vesicles of multivesicular bodies is not a random selection of proteins expressed in a given cell type. For example, proteomic profiling of proteins in urinary exosomes revealed an abundance of integral membrane proteins targeted to the apical plasma membranes of epithelial cells, but a dearth of proteins associated with the basolateral domain.[3] Further evidence for selective protein sorting to exosomes comes from the observations in nonpolarized cells showing that particular proteins are enriched in exosomes compared with the whole cell. Such proteins include the transmembrane proteins CD55, CD59, CD63, CD81, CD82, the transferrin receptor, and phospholipase D2, as well as many soluble proteins such as certain heatshock proteins.[14,22,23,29,30] Ubiquitin- and lipid raft-associated protein sorting have been reported to be involved in this selective incorporation of proteins into exosomes.[28,31]

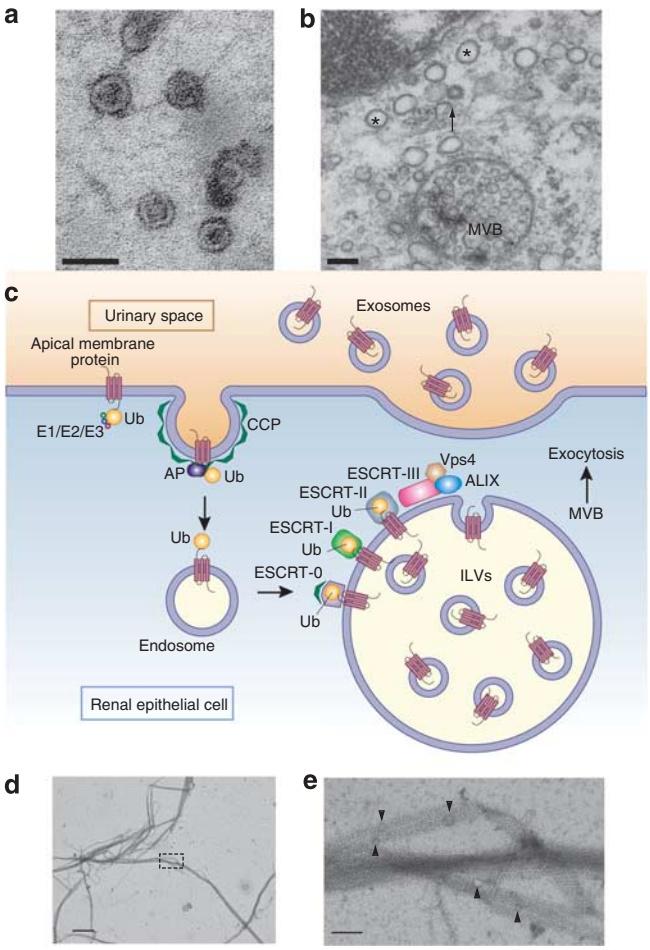

Figure 1 | Exosomes in urine. (**a**) Electron micrograph of negatively stained urinary exosomes (scale bar, 50 nm). (**b**) Electron micrograph of a renal inner medullary collecting duct cell (scale bar, 100 nm). Uncoated vesicles (asterisks) and coated vesicles (arrow) are indicated. MVB, multivesicular body. (**c**) Schematic of urinary exosome formation and release into the urine. AP, adaptor protein; ALIX, ALG-2 interacting protein X; CCP, clathrin-coated pit (clathrin molecules are shown in green); E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; ESCRT, endosomal sorting complex required for transport; ILVs, intraluminal vesicles; Ub, ubiquitin; Vps4, vacuolar protein sorting 4. (**d** and **e**) Electron microscope images of the 17,000 *g* pellets from pooled normal human urine. Tamm–Horsfall protein (THP) forms long polymeric filaments that are associated laterally to form rope-like structures (**d**, scale bar, 800 nm and **e**, depicting the dashed box in **d**; scale bar, 100 nm). The THP network depicted contains small (40–100 nm) vesicles compatible with exosomes (**e**, arrowheads).

PHYSIOLOGICAL ROLES OF EXOSOMES

Besides a likely role in elimination of excess or senescent proteins and lipids, there is considerable evidence that

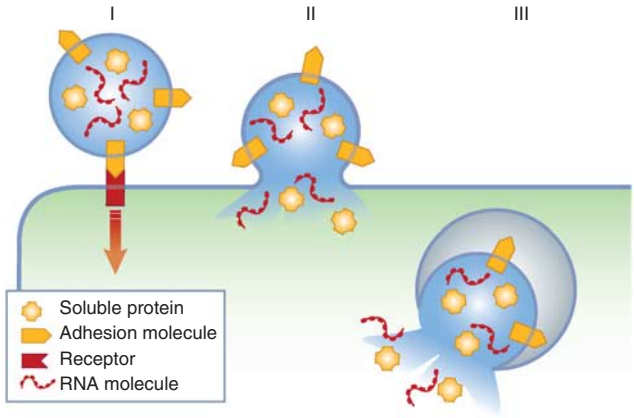

Figure 2 | Mechanisms for exosome-mediated signaling to target cells. Interaction of exosome surface proteins with adhesion molecules or receptors on target cells can initiate a downstream signaling cascade (I). Direct fusion of the exosome membrane with the target cell plasma membrane results in the release of content (RNA, proteins) into the cytoplasm of the target cell (II). Transfer of exosome content to the cytoplasm may also occur after endocytosis and subsequent fusion of the exosome membrane with the endosomal membrane (III).

exosomes can have roles in intercellular signaling in a cell-selective manner.[32–36] This role has been reviewed recently by Camussi *et al.*[25] in this journal. Exosomes may elicit effects on target cells by at least three possible mechanisms (Figure 2): (I) They can adhere with high specificity to the target cell surface (without membrane fusion) through adhesion molecules and receptors present on their surfaces, leading to receptor activation and downstream signaling in the target cell.[33,37] (II) Exosomes could hypothetically fuse directly with target cells, resulting in transfer of the contents of exosomes (mRNAs, miRNAs, proteins, and signaling molecules). (III) Another possibility that is better supported in the literature is that the contents of exosomes may incorporate into target cells after endocytosis of exosomes and processing in the endosomal pathway.[34–36,38]

These mechanisms have been demonstrated in exosomes secreted into the blood and extracellular fluid. If urinary exosomes can carry out intercellular signaling in the same way as exosomes secreted into blood and extracellular fluid, they could have important roles in renal physiology. For example, exosomes may mediate downstream information transfer in the process of renal tubule hypertrophy seen with nephron loss. In this process, increases in single-nephron glomerular filtration rate are matched by increases in the transport capacity for salt and water in downstream nephron segments, in association with marked enlargement of renal tubule cells and widening of the tubule lumen.[39] It is possible that such downstream information transfer is occurring in normal physiological states. Indeed, proteins that are nominally proximal tubule proteins have been detected in the renal-collecting duct, including the water channel aquaporin-1 (ref. 40) and the ammonium-generating enzyme glutaminase.[41,42] Such downstream information transfer may not always be beneficial. We speculate that Tamm–Horsfall protein (uromodulin), an abundant polymeric protein in normal urine, has a role to limit exosomal fusion in downstream nephron segments. Urinary exosomes are typically shrouded by large polymeric fibers formed from Tamm–Horsfall protein, which would prevent them from coming into contact with cell surfaces unless the polymeric network is locally dissolved.[43] If these speculations are true, they may provide a basis for understanding how mutations or deficiency of Tamm–Horsfall protein could cause renal disease[44,45] and could therefore warrant further investigation.

An additional way that urinary exosomes could have roles in kidney physiology is through actions of exosome-resident proteins in the renal tubule lumen. An example of this may be the demonstrated presence of abundant angiotensin-converting enzyme in urinary exosomes,[3,46] which could have a role in the well-known intraluminal renin–angiotensin system described by Navar *et al.*[47]

It is certainly possible that the main physiological role for urinary exosomes is the disposal of senescent proteins from cells, which may be a more efficient means of protein elimination than proteasomal degradation and lysosomal degradation.

Although the major focus of this article is on urinary exosomes, it is likely that exosomes secreted into the blood and extracellular fluid have roles in renal physiology and pathophysiology, especially among cell types with their plasma membranes in direct contact with the vascular compartment such as cells of the immune system and endothelial cells. Prime examples are the roles of exosomes and microvesicles in cell–cell communication in immune cell and stem cell signaling. For example, exosome-mediated communication is involved in the priming of CD8+ and CD4+ cells by antigen-loaded major histocompatibility complex class I and II molecules on exosomes, showing target cell-specific effects.[48,49] Receptor–ligand interaction between exosomes and cells are also essential to identify specific target cells, as demonstrated by the specific binding of dendritic cell-derived exosomes to activated, and not resting, T cells. The selection of the target cell is mediated by the interaction between inter-cellular adhesion molecule 1 on exosomes and its ligand, lymphocyte function-associated antigen 1, on activated T cells,[32] suggesting a mechanism by which, on a broader scale, exosomes may be targeted to specific cell types. In addition, it has been demonstrated that mesenchymal stem cells contain specific miRNA signatures, which are selectively incorporated and subsequently transferred to target cells.[50] Transferred miRNAs affect gene expression in target cells[51], demonstrating that besides transfer of proteins, exosomes can modulate the physiology of the target cell by transfer of RNA. Further examples for the role of micro-vesicle-mediated transfer of RNA include the modulation of stem cells and the stem cell niche, which could be a crucial stem in stem cell-mediated tissue repair (reviewed by Deregibus *et al.*[52] and Quesenberry *et al.*[53]), and potentially represent a system that is efficiently hijacked by tumors for

the stimulation of angiogenesis. Blood-borne exosomes may also be involved in angiogenesis, at least in tumors. Specifically, tumors promote their vascularization not only through the secretion of known angiogenic cytokines and growth factors, but also via exosomes.[38,54,55] On the basis of these observations and others, one could well imagine that blood-borne exosomes could have a role in various glomerulopathies in graft rejection, in hypertension, and in other kidney-related diseases.

EXOSOMES AS A SOURCE OF PROTEIN BIOMARKERS

Urinary proteomics studies have identified potential urinary biomarkers for several pathological entities, for example, acute kidney transplant rejection[56] and diabetic nephropathy.[57] Despite these and other successes, the number of kidney-derived proteins and peptides detectable in whole urine (or 'minimally processed' urine) by MS has been limited in part by the presence of filtered plasma proteins and very abundant kidney-derived proteins, especially Tamm–Horsfall protein or uromodulin. Abundant proteins compete with less abundant proteins for identification in the mass spectrometer. Consequently, we may be missing the biomarker candidates that would provide the best sensitivity and specificity for diagnosis of a given disease. One approach to enrichment of kidney-derived proteins has been the isolation of exosomes from urine.[3] Normal urine contains exosomes that derive from every epithelial cell type facing the urinary space (Figure 1), offering the potential to monitor physiological and pathophysiological changes throughout the nephron through the expedient of urine collection and analysis.

The advent of detailed protein sequence data from the human genome project and marked technological improvements in MS of proteins and peptides may lead to the discovery of even more protein biomarkers. It has become possible to identify and quantify literally thousands of proteins from a single sample using shotgun proteomics based on MS systems that combine liquid chromatography and tandem mass spectrometry (MS/MS). We have used liquid chromatography–MS/MS-based protein MS to carry out large-scale profiling of proteins present in urinary exosomes from normal humans[46] and have made the data available on a publicly accessible database (http://dir.nhlbi.nih.gov/papers/lkem/exosome/). This database provides a listing of 1160 proteins present in urinary exosomes and contains potential biomarker proteins that can be the basis of hypotheses regarding the mechanism of the disease.

A general analysis of urinary proteins by Adachi *et al.*[58] also detected large numbers of membrane proteins, presumably because of the presence of exosomes in the samples. About 3% of total urinary protein in samples from normal subjects is derived from exosomes.[59] Thus, isolating exosomes from urine provides a more than 30-fold enrichment of exosomal proteins, allowing proteins that are minor components of whole urine to be readily detectable immunochemically or by protein MS.

As noted above, exosomes are not uniquely found in urine and in fact have been identified in multiple body fluids including blood plasma, where they derive from reticulocytes, leukocytes, endothelial cells, and presumably other cell types that contact the intravascular space. Their presence in blood therefore offers an advantage for biomarker discovery in plasma, which is analogous to their advantage for biomarker discovery in urine. Specifically, the isolation of exosomes allows marked enrichment of biomarkers that may not be readily detectable in whole plasma or even plasma that has been stripped of its most abundant soluble proteins. Major efforts have been undertaken to define the proteomes of plasma-derived exosomes and exosomes from other body fluids, aiming especially at the discovery of novel biomarkers for prevalent diseases such as cancer and atherosclerosis.[16,18,60–62] A general database of exosomal proteins called 'ExoCarta' can be found at exocarta.ludwig.edu.au/.[63]

What disease processes in the kidney would be the best targets for exosome-based biomarker discovery? The answer, we believe, is 'those diseases that require clinical decision making, that is, currently non-optimal or too slow with current diagnostic methodologies'. Therefore, there may be renal diseases that are prevalent and have large negative impacts on length or quality of life, but are not good targets for urinary exosome-based biomarker discovery because the addition of a new biomarker would not significantly influence clinical decision making. On the basis of these considerations, one example of a good target for biomarker discovery in urinary exosomes may be the decision-making process encountered in renal allograft patients who experience an increase in serum creatinine levels. The discrimination between rejection and kidney injury, as well as the discrimination between different mechanisms of rejection, is generally addressed through renal biopsy for which a full battery of analyses generally requires many hours or days. Here, a rapid immunological test could speed the initiation of appropriate therapy. Another prime target is early diagnosis of acute kidney injury in surgical and intensive care settings. In studies reported thus far, several potential markers have been identified including KIM1,[64] HSP72,[65] Klotho,[66] IL-6,[67] NGAL,[68] L-FABP,[69] netrin-1,[70] or fetuin-A.[71] Among these, only the study identifying fetuin-A as a potential acute kidney injury biomarker was conducted using exosomes as starting materials. Exosome analysis may also be useful for classification of other disease processes involving the renal tubule, such as polycystic kidney disease,[72] lysosomal storage diseases (for example, Nieman–Pick disease and cystinosis), and transporter mutations (such as Gitelman and Bartter syndromes[46]). Urinary exosome analysis may also be useful in the detection and classification of liver damage, which can secondarily affect the kidney.[73] In addition, it has been proposed that analysis of urinary exosomes could be performed in patients with hypertension,[74] possibly to find biomarkers to predict which drugs will be the most effective in lowering the blood pressure in a given patient (personalized medicine). Multiple transcription factors have been

found in urinary exosomes and their analysis has been proposed as a means of noninvasively detecting and monitoring various glomerular diseases including focal segmental glomerulosclerosis.[75] Furthermore, it has been proposed that exosome analysis of urine may provide better ways to monitor responses to the treatment of prostatic cancer.[76] The above list of biomarker targets is not exhaustive, and other prime clinical decision-making processes that are amenable to urinary exosome-based biomarker discovery may be readily apparent to the reader.

Genetic diseases may also be diagnosable through urinary exosome analysis. Looking towards the future, the continual improvement in mass spectrometers is making it more and more feasible to use MS in *de novo* sequencing mode to screen for mutations and polymorphisms that affect the primary sequence of proteins. Thus, although significant strides are currently being made with regard to DNA sequencing using so-called 'deep-sequencing technologies', MS may provide an alternative way to discover sequence variations in proteins that appear in the urinary exosomal proteome.

EXOSOMES AS A SOURCE OF RNA BIOMARKERS

Besides proteins and peptides, exosomes contain mRNA and miRNAs.[1,28,34,77–79] Such RNAs are potentially useful as disease biomarkers. Although efficient exosome isolation protocols have been introduced for urinary RNA analysis,[79] most studies of urinary RNAs thus far have bypassed exosome isolation, opting for direct analysis of mRNA levels using RT-PCR in sediments from whole urine, which undoubtedly contains RNA from both exosomes and whole cells. An example is a recent study showing increased glycoprotein B7-1 to nephrin mRNA ratios in urinary sediments from patients with minimal change disease compared with focal segmental glomerulosclerosis.[80] Another recent example is the finding that urinary granzyme A mRNA levels can potentially distinguish patients with cellular rejection from those with acute kidney injury.[81] Exosome isolation can potentially increase the sensitivity and precision of urinary mRNA analysis.

MicroRNA profiling can also be used to identify potential biomarkers. Initially, exosomes from tumors were investigated for the presence of biomarkers, and in 2008, Skog *et al.*[38] discovered that mRNA encoding a specific variant of the VEGF-receptor (VEGFvIII) predicts a better treatment response in the treatment of glioblastoma. Furthermore, miRNA signatures of circulating exosomes may serve as a useful tool for the diagnosis of lung cancer and ovarian cancer,[82,83] and recently a method for the isolation of mRNA and miRNA for diagnostic purposes from urine exosomes were developed.[79] Even without enrichment by exosome isolation, the abundances of several miRNAs (miR-200a, miR-200b, and miR-429) were found to be decreased in urinary sediments from patients with immunoglobulin A nephropathy, and the degree of reduction correlated with the severity of the disease.[84]

It may be possible to increase both the sensitivity and the specificity of RNA biomarker approaches through the enrichment of exosomes specific to the given cell type. Flow cytometry approaches for the latter task are under development.[85,86]

EXOSOMES AS POTENTIAL THERAPEUTIC AGENTS

The finding of mRNAs and miRNAs in exosomes and evidence for a role for exosomes in cell–cell communication (reviewed above) foreshadows an important new direction, that is, the use of exosomes as delivery vehicles for therapeutics. The concept is that RNA-bearing exosomes can potentially deliver their contents to specific target cells in order to transiently correct dysregulated processes.

Already, several researchers have preliminarily explored the possibility of using exosomes as therapeutic delivery vehicles. In 1998, Zitvogel *et al.*[87] proposed the use of exosomes in the immunotherapy of cancer, showing that exosomes derived from tumor peptide-pulsed dendritic cells injected into tumor-bearing mice resulted in eradication or reduced growth of the tumor. More recently, others have pioneered the application of exosomes in cancer treatment.[88,89] Two phase I clinical trials studied injection of antigen-loaded exosomes from autologous dendritic cells into patients with melanoma or lung cancer and demonstrated feasibility and safety of exosome-based therapy, although the effects on reduction of disease progression were only minor.[90,91] Similar approaches have the potential for treatment of renal cancers.[92]

In 2007, Valadi *et al.*[34] demonstrated that exosomes are able to transfer miRNAs from their cell of origin to target cells. Besides miRNAs, pre-miRNA could be identified in mesenchymal stem cell-derived exosomes.[93] Functionally, this offers cells the possibility to increase (mRNA) or reduce (miRNA, pre-miRNA) protein expression levels in specific target cells. Transfer of mRNA and miRNA molecules to target cells can influence their function, which may be the mechanism by which endothelial progenitor cell-derived exosomes stimulate angiogenesis in endothelial cells.[77]

Another potential use of exosomes is as vehicles for the delivery of specific antigens. This approach has been applied for vaccination against severe acute respiratory syndrome, using exosomes containing the severe acute respiratory syndrome S protein[94] and against *Toxoplasma gondii*, using antigen-containing exosomes.[95] Both vaccines showed positive results, displayed as higher levels of neutralizing antibodies and, in the *T. gondii* study, there was a reduction of disease severity in mice.

Exosomes have been reported to be the active component in the conditioned medium of mesenchymal stem cells that display cardioprotective effects by reducing cardiac infarct size after experimental ischemia–reperfusion.[96] Cardiomyocyte progenitor cell-derived exosomes may also have this potential.[12] A role for exosomes may be found in the paracrine effects that have been observed in experimental stem cell therapy. For example, in experimental stem cell

therapy of acute kidney injury, mesenchymal stem cells have been shown to improve recovery in part through paracrine factors derived from secreted exosomes.[97,98] In experimental stem cell therapy of experimental glomerulonephritis in rats (anti-Thy1.1 glomerulonephritis), Kunter *et al.*[99] found a benefit that they attributed to paracrine factors from the injected mesenchymal stem cells rather than from the cells themselves. Conceivably, exosome secretion is involved in these observed paracrine effects.

For many kidney-related diseases, a prime target for potential exosome-based therapy are endothelial cells, which have essential roles in regulation of blood pressure, local regulation of blood flow, regulation of blood clotting, and clearance of plasma lipids. Failure of these processes is responsible for a large fraction of common chronic diseases that affect the kidney, including atherosclerosis and hypertension. Because the endothelial cells face the blood compartment, they might be considered 'low-hanging fruit' for potential exosome-based therapies, as the problem of targeting is largely obviated.

On the basis of the above observations and additional ongoing research, we conclude that exosomes have considerable promise for treatment of a variety of renal diseases. To succeed, however, there is a need to develop methods for efficient isolation of exosomes of appropriate composition, allowing targeting to specific cell types and allowing transfer of selected biomolecule cargos. This can only be achieved through further basic research based on the following questions: (1) How do multivescular bodies select biomolecules for inclusion in their intraluminal vesicles? (2) How do exosomes interact with cell surfaces of some cells and not others? (3) What is the mechanism of fusion of the limiting membrane of exosomes with plasma membranes?, and (4) How are exosomes in plasma normally cleared and how can this process be selectively delayed for therapeutic exosomes?

CONCLUSION

Our objective in this short review has been to provide a brief synopsis of knowledge about exosomes with a view toward future exploitation in the diagnosis and therapy of kidney diseases and kidney-related diseases. Nephrologists are in a continual search for new tools to improve their ability to rapidly and accurately diagnose renal disease via noninvasive methodologies. The advent of sensitive and accurate MS and genomics techniques has facilitated this quest, and it is likely that in the near future several exosomal biomarkers will come into play in clinical practice. Current efforts to replace ultracentrifugation with more efficient exosome isolation methods, such as filtration,[100,101] size-exclusion chromatography,[102] and affinity methods,[103] can be expected to lead to more practical protocols for the profiling of exosomal proteins and RNAs. The potential use of exosomes as therapeutic vehicles is based on the fact that exosome secretion and reuptake can move molecules (and information) between cells, processes that can be interrupted or modified by design. Possibly, in the coming years, we can extend this frontier beyond its current main focus in the areas of oncology and immune diseases to achieve new ways to treat kidney diseases and kidney-related diseases.

DISCLOSURE

All the authors declared no competing interests.

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