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Review Article

Exosomes and the kidney: Blaming the messenger

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SUMMARY AT A GLANCE

Enormous interest has focused on exosomes, the small membrane-bound vesicles released into the extracellular environment by many cell types, since the realization that they play important functional roles across many aspects of cell biology and the potential to use their unique protein and RNA content as signatures in biomarker research.

ABSTRACT:

Exosomes are membrane-bound vesicles of endosomal origin, present in a wide range of biological fluids, including blood and urine. They range between 30 and 100 nm in diameter, and consist of a limiting lipid bilayer, transmembrane proteins and a hydrophilic core containing proteins, mRNAs and microRNAs (miRNA). Exosomes can act as extracellular vehicles by which cells communicate, through the delivery of their functional cargo to recipient cells, with many important biological, physiological and pathological implications. The exosome release pathway contributes towards protein secretion, antigen presentation, pathogen transfer and cancer progression. Exosomes and exosome-mediated signalling have been implicated in disease processes such as atherosclerosis, calcification and kidney diseases. Circulating levels of exosomes and extracellular vesicles can be influenced by the progression of renal disease. Advances in methods for purification and analysis of exosomes are leading to potential diagnostic and therapeutic avenues for kidney diseases. This review will focus on biophysical properties and biogenesis of exosomes, their pathophysiological roles and their potential as biomarkers and therapeutics in kidney diseases.

Intercellular communication is vital for the regulation and coordination of many different processes within multicellular organisms. Extracellular membrane-bound vesicles are emerging as a novel and significant mechanism of cell signalling and communication. Exosomes are a specific subset of membrane-bound vesicles of endosomal origin, which are released into the extracellular environment by many cells from different tissues and organs. Exosomes exist in a wide range of biological fluids, including blood and urine. The ubiquitous nature of exosomes has highlighted them as significant vehicles of cellular communication, with many important biological and pathophysiological implications.

MOLECULAR AND BIOPHYSICAL PROPERTIES OF EXOSOMES

Exosomes are defined as small vesicles between 30 and 100 nm in diameter, consisting of a limiting lipid bilayer, transmembrane proteins and a hydrophilic core containing proteins, mRNAs and microRNAs (miRNA). They are distinguished from other microparticles by their size and the fact that they are formed intracellularly within multivesicular endosomes (multivesicular bodies; MVB), while microvesicles (100 to 1000 nm in diameter) are shed from the plasma membrane surface[1] (see Table 1).

Exosomes contain a defined set of proteins, which varies according to the cell of origin.[6] Common components of exosomes are proteins involved with endosomal trafficking, membrane trafficking and fusion proteins, tetraspanins (CD63, CD81, CD9, CD82), heat shock proteins (HSP70, HSP90), metabolic enzymes, adhesion molecules, signal transduction proteins, lipid rafts and cytoskeletal proteins, in addition to cell type-specific proteins, such as major histocompatibility complex (MHC) class I and II, α-synuclein, and the A33 antigen.[6] Exosomes have a specific lipid composition distinct from their parental MVB, although they do reflect

Table 1 Characteristics of exosomes, microvesicles and apoptotic bodies

	Exosomes	Microvesicles	Apoptotic bodies
Size	30–100 nm	100–1000 nm	Up to 4000 nm
Formation and release	Formed intracellularly within multivesicular bodies	Shed from plasma membrane surface	Cellular breakdown Release from cellular blebs during apoptosis
Isolation and detection	Ultracentrifugation, electron microscopy, western blotting, mass spectrometry, nanoparticle tracking analysis	Differential centrifugation, flow cytometry, electron microscopy, western blotting, mass spectrometry, nanoparticle tracking analysis	Flow cytometry using e.g. FITC-conjugated annexin V antibody, electron microscopy
Markers	Alix, TSG101 and the tetraspanin proteins CD81 and CD9	Integrins, selectins, markers of parental cells	Genomic DNA and intact organelles, externalized phophatidylserine
References	[1–4]	[1–5]	[2–4]

their cell of origin, and can also contain bioactive lipids such as prostaglandins, which may contribute to their function.[7]

Exosomes contain mRNAs and miRNAs, and RNA profiling of exosomal fractions has identified significant differences to parental cellular RNA.[8,9] Both mRNAs and miRNAs present in the exosomal fraction maintain their function when transferred to other cells,[8,10] demonstrating that exosomal RNA transfer may be an important route for epigenetic signalling between cells. However, recent studies suggested that many extracellular miRNAs may not be contained within exosomes, but can be complexed with circulating Argonaute-2 or other ribonucleoprotein complexes.[11–13]

EXOSOMES BIOGENESIS AND RELEASE

Exosomes are formed by the intraluminal budding of late endosomal compartments to create MVB, containing intraluminal vesicles.[14] As vesicles bud inward, the lumina of these future exosomes capture a small portion of the cytosol, taking along a cargo of soluble proteins, mRNAs, miRNAs and other cytosolic molecules. Fusion of the limiting MVB endosomal membrane with the plasma membrane releases the intraluminal vesicles into the extracellular environment,[14] whereafter they are known as exosomes (Fig. 1). The fusion of MVB with the plasma membrane and subsequent release of exosomes is a constitutive process in most cell types,[15] although it is also subject to regulation by a variety of stimuli. Exosome release from MVB has been demonstrated to be regulated by endosomal and vesicular trafficking proteins,[16,17] Rab small GTPase family members,[18,19] ceramide[20] and calcium.[18]

Exosomes are emerging as a part of the cellular response to a range of different stresses. Increased exosome release has been reported in hypoxia,[21] acidic pH[22], heat shock[23] and oxidative stress.[24] Significantly, p53 has been implicated in regulating exosome release,[25] further providing support to the idea that exosomes may act as a intercellular signals to communicate during cellular stress.

EXOSOMES PURIFICATION, DETECTION AND CHARACTERIZATION

Exosome isolation protocols vary depending on the biological fluid of origin, but generally involve serial centrifugation at low speed, followed by ultracentrifugation at 100 000 *g* to pellet exosomes.[26,27] Alternatively, exosomes can be isolated by immunocapture or size exclusion methods.[26,28] Filtration and microfluidics approaches have been developed,[29,30] but have yet to be widely adopted. Recently, a proprietary method of exosome isolation called Exoquick™ (System Biosciences, Mountain View, California, USA) has been made commercially available.[31] Exosomes have densities between 1.10–1.21 g/mL, and this characteristic is often exploited for further purification, either by sucrose density gradients or flotation on sucrose/deuterium oxide cushion.[26,27,32] Velocity gradients can also be used, especially in order to distinguish between viral and exosomal vesicles.[33,34] A comparison of different methods showed that circulating exosomes isolated by Exoquick™ precipitation produce exosomal mRNA and miRNA with greater purity and quantity than ultracentrifugation.[35]

The morphology and size of exosomes were first characterized by electron microscopy (see Fig. 2), and further characterization of exosomes has traditionally relied upon biochemical methods such as immunoblotting, mass spectrometry, 2-DIGE and microarrays, although atomic force microscopy and dynamic light scattering technologies have also been used. The ExoCarta and vesiclepedia databases provide a comprehensive record of exosomal protein, RNA and lipid profiles (http://www.microvesicles.org).[36] Detection and quantification of exosomes currently relies upon indirect methods such as immunoblotting of exosomal proteins, activity of exosomal enzymes,[37,38] exosomal protein quantification,[23] fluorescent labelling of exosomes[39,40] or antibody-specific bead-coupled approaches.[19,28] Recently, nanoparticle tracking analysis using the Nanosight microscope (NanoSight Ltd., Amesbury, Wiltshire, UK) has been demonstrated to allow both characterization of exosome size, as well as direct quantification of exosomes.[41,42]

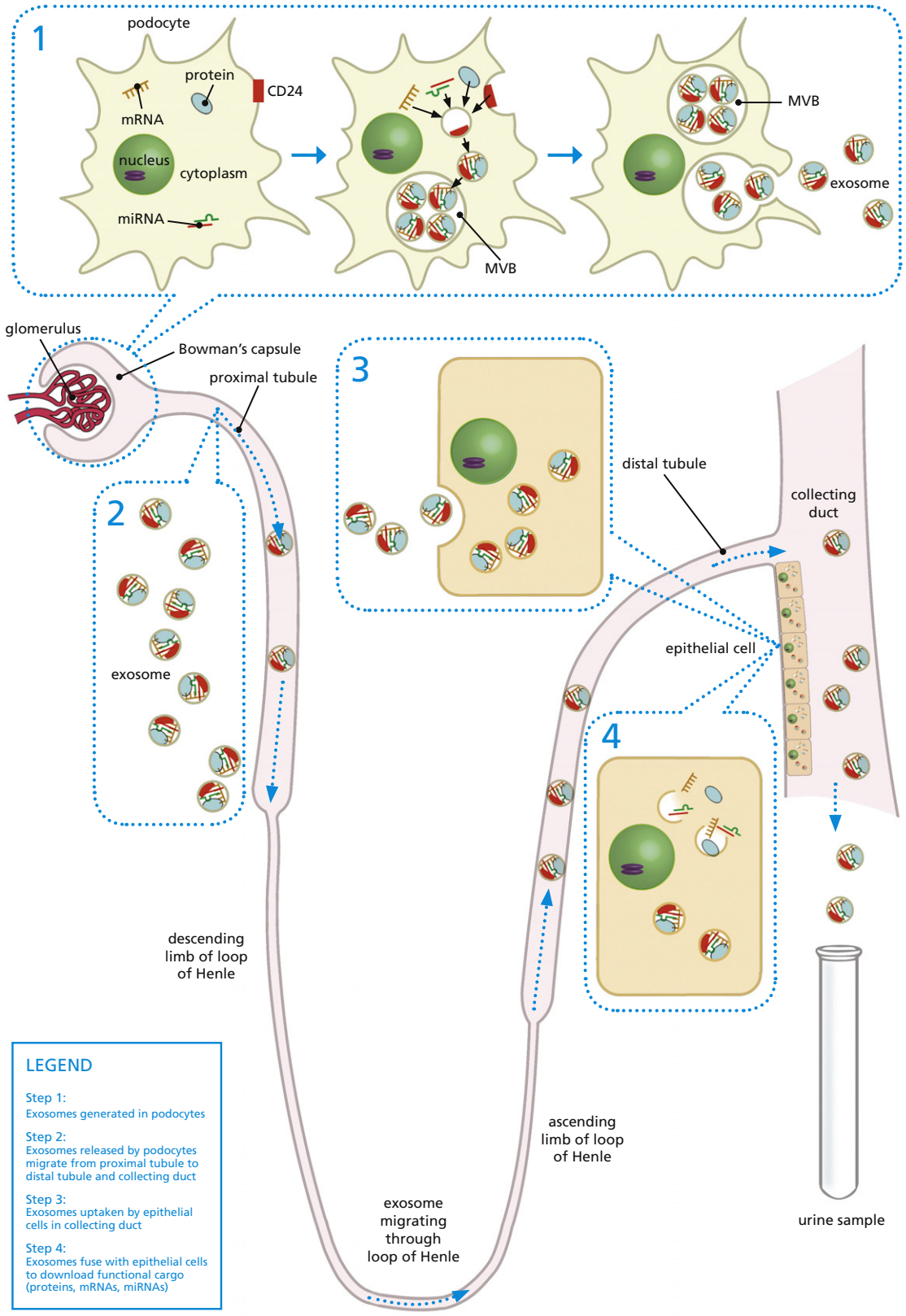

Fig. 1 Schematic representation of biogenesis of exosomes by cells and their content of proteins, mRNAs and microRNAs. The figure illustrates an example of the release of exosomes by cells such as the podocyte, their passage through the renal tubule and their uptake and influence on recipient cells such as the epithelial cells of the collecting duct and appearance in urine. MVB, multivesicular body.

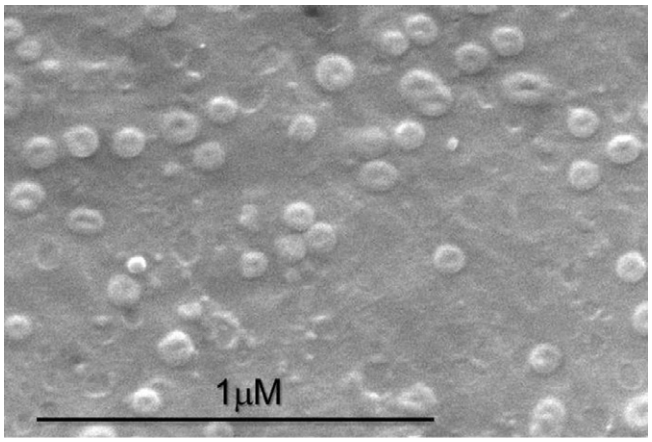

Fig. 2 Urinary exosomes visualized by scanning electron microscopy. Exosomes were purified by ultracentrifugation from urine and then visualized by scanning electron microscopy at 80 000× magnification. Multiple small spherical vesicles, many with a small central depression and with a mean diameter of approximately 100 nm, can be seen.

There are particular considerations required in the purification and storage of urinary exosomes. Tamm-Horsfall protein (uromodulin) can form fibrillary aggregates in urine especially at low temperature which can entrap exosomes and prevent their efficient isolation and purification by centrifugation. The entrapment can be eliminated by using the reducing agent dithiothreitol (DTT).[43] Currently, there is no standard protocol for collection, processing and storage of urine samples that will allow correct, comparable and reproducible urinary exosome analyses. Protease inhibitors and storage at −70°C gave a better recovery of urinary exosomes than at −20°C.[44] Nephrotic urine contains a large amount of proteins that tend to be retained after ultracentrifugation, which can affect the detection of exosomal proteins. Recent studies have demonstrated that ultracentrifugation followed by size exclusion chromatography can enrich and purify exosomes in nephrotic urine sample.[45]

BIOLOGICAL ROLES OF EXOSOMES

Despite being first described in the early 1980s,[46,47] exosomes garnered minimal scientific attention as their role was considered little more than to discard unwanted cellular components, until the 2000s. As a result, their biological and physiological roles are still being discovered. Currently, exosomes are known to play significant roles in intercellular communication, non-classical protein secretion, immunomodulation, pathogen biology and cancer progression.

Intercellular communication

Intercellular communication was previously thought to be limited to cell-to-cell adhesion contact (gap junctions) or secreted signals such as hormones, neurotransmitters, and cytokines released from cells and acting in an autocrine or paracrine manner. Exosomes can mediate a novel intercellular communication mechanism. They can be transported between different cells and adhere to target cells with high specificity via receptor or adhesion molecules but without membrane fusion leading to receptor activation and downstream signalling. Alternatively, exosomes can fuse with target cells or be incorporated by target cells via endocytosis.[10,48] Transferred RNAs can affect protein production and gene expression in target cells.[49] The exosomal lipid bilayer protects proteins, mRNAs and miRNAs from degradation, which may make this intercellular communication pathway more reliable in comparison with free floating proteins and RNAs and enable targeted delivery of a higher concentration of messenger.

Non-classical protein secretion pathway

A physiological role for exosomes was first described in the maturation process of erythrocytes from reticulocytes.[14,50] It is known that transferrin receptors are lost during this maturation process. Using labelled transferrin or antibody against the transferrin receptor, and electron microscopy, it was demonstrated that after endocytosis of the surface receptor and fusion of endosomes to form larger structures, budding occurred at the internal surface of the vesicles. MVB were then formed with the release of these small buds of ~50 nm diameter (intraluminal vesicles) into the main body of the vesicles. These MVB eventually fused with the cell membrane releasing the ~50 nm buds, now known as exosomes, into the extracellular milieu.[51] Exosome release allows maturing reticulocytes to shed obsolete membrane proteins and remodel their plasma membrane,[52] providing an alternative to lysosomal degradation. In addition to the secretion of unnecessary or damaged proteins, exosomes provide a non-classical secretion pathway for a wide range of physiologically relevant proteins, including β-catenin.[53]

Immune system functions

Exosomes released by immune cells play a wide range of important roles in the normal immune system,[54] as well as being involved with tumour immunomodulation.[55] The presence of functional MHC class II molecules in immune cell-derived exosomes highlights their role in antigen presentation.[56] Exosomes are capable of presenting pathogen-derived antigens[57] or exerting immunosuppressive or cytotoxic functions.[58] The functional effect of exosomes on immune cells may be exerted by exosomal miRNA transfer, as recently observed by T cells in response to antigen stimulation.[59]

Pathogen biology

Exosomes are exploited by pathogens as a means of intercellular spreading and communication. Exosomes are capable of

shuttling viral proteins which can promote pathogenesis or immune escape,[34] as well as functional viral miRNAs[49] and dissemination of HIV-1 infection.[60] The pathogenic prion protein has also been demonstrated to be packaged into exosomes.[61]

Roles in cancer progression

During tumour development, tumour cells interact with their surrounding microenvironment to promote their growth, survival and invasion. Tumour-derived exosomes are being described as important mediators of many of these processes, including tumour cell proliferation,[62] angiogenesis,[10] metastasis,[63,64] stromal remodelling[65,66] and immunomodulation.[55] In experimental models of renal cancer, cancer stem cell-derived vesicles appear able to contribute to triggering the angiogenic switch and promote metastasis.[67] Tumour-derived exosomes can suppress antigen-specific immune responses and dendritic cell maturation *in vivo*,[68] in addition to upregulating immunosuppressive cell differentiation and function, including regulatory T cells[69] and myeloid-derived suppressor cells.[16]

EXOSOMES IN BLOOD AND URINE

As described above, exosomes were initially identified in the loss of transferrin receptors, which accompanies maturation of reticulocytes to erythrocytes. Furthermore, evidence has since been obtained for the secretion of exosomes *in vitro* by a variety of other cells including lymphocytes, dendritic cells, mast cells, endothelial cells, platelets, and presumably other cell types that contact intravascular space.[60,64–68] In keeping with such release, more recent studies provided evidence for the presence of exosomes *in vivo* in the blood. Caby *et al*. examined plasma samples from healthy donors and successfully identified vesicles of 50–90 nm in diameter that have the molecular and biophysical properties of exosomes.[70] Besides blood, exosomes have also been detected in various bodily fluids such as urine, cerebrospinal fluid, saliva, breast milk, semen, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid and synovial fluid.[71]

The presence of urinary exosomes was verified when small vesicles (<100 nm in diameter) orientated 'cytoplasmic-side inward' were observed in normal urine with functions in urinary secretion of aquaporin-2 and other membrane-associated proteins[72] (see Fig. 2). The proteomic analysis of urinary exosomes identified proteins characteristically restricted in expression to renal epithelia of the glomerular podocytes, the proximal tubule, the thick ascending limb of Henle, the distal convoluted tubule and the collecting duct. Proteins from the transitional epithelium of the urinary bladder were also identified, suggesting urinary exosomes may be derived from cells throughout the renal tract.[72–74] Thus, analysis of urinary exosomes provides an attractive non-invasive means of acquiring information about the pathophysiological state of their renal cells of origin. CD24, a small but extensively glycosylated protein linked to the cell surface by means of a glycosyl-phosphatidylinositol anchor, has been reported to be a marker for urinary exosomes.[75]

EVIDENCE FOR PHYSIOLOGICAL AND PATHOLOGICAL ROLES IN THE KIDNEY

It was previously thought that the main physiological role for urinary exosomes is the disposal of senescent proteins from cells, which may be a more efficient way of protein elimination than proteasomal and lysosomal degradation,[76] similar to the process by which maturing reticulocytes shed obsolete membrane proteins and remodel their plasma membrane through the exosomal pathway.[52] However, increasing evidence is suggesting that urinary exosomes play a role beyond exocytic cell waste elimination.[75,77] Another possible role of exosomes in the urinary tract is to regulate the co-functioning between different parts of the nephron, through secretion and reuptake of their contents such as mRNAs and miRNAs that can affect the function of the recipient cell[73] (Fig. 1). Functional transfer of molecules such as aquaporin-2 between different renal cells has been described[78] and could mediate coordinate adaptation of nephron function.

The role of circulating exosomes in physiological messaging remains poorly defined, but pathophysiological roles have been increasingly explored. Endothelial dysfunction is thought to be the key event in the pathogenesis of atherosclerosis. Endothelial dysfunction is a systemic inflammatory process associated with increased adhesion molecule expression, loss of anti-thrombotic factors, increase in vasoconstrictor products and platelet activation. Activated platelets secrete two different types of membrane vesicles: microvesicles (100–1000 nm in diameter) shed from the plasma membrane, and exosomes which are released following fusion of MVB.[1] Microvesicles have protein content similar to the plasma membrane of activated platelets and have procoagulant and inflammatory functions.[79,80] In contrast, platelet exosomes only interact poorly with annexin-V and do not bind prothrombin and factor X. Platelet-derived exosomes are enriched in CD63, a tetraspanin protein also found on exosomes from other cell types.[81] Tetraspanin proteins have been implicated in adhesive as well as co-stimulatory and signalling functions. Platelet-derived exosomes may be released at sites of vascular injury and could well function in promotion of platelet and neutrophil adhesion.[1,82]

Endothelial dysfunction and vascular calcification is a significant risk factor for cardiovascular morbidity and mortality in patients with renal disease. *In vitro*, vesicles appear to be important in mediating vascular smooth muscle cell calcification.[83] In a recent study, it was found that phosphorylated fetuin-A is present in the calciprotein particles in serum of predialysis chronic kidney disease (CKD) patients. Increased calciprotein particle fetuin-A levels reflect an increasingly

procalcific milieu and are associated with increased aortic stiffness.[84] Increased levels of circulating microparticles (MP) or microvesicles have been detected in patients with CKD. Circulating levels of MP and microvesicles derived from endothelial cells correlate with arterial stiffness in haemodialysis patients.[85–87] It is unclear whether exosomes and/or other circulating MP may play an important role in transporting or promoting vascular calcification in CKD or in other calcification-associated diseases.

Nephrolithiasis is associated with the formation of calcium oxalate, calcium phosphate, cystine, struvite or urate crystals in the kidneys. *In vitro* studies have demonstrated that renal brush border-derived exosomes/microvesicles of ~100 nm in diameter can induce and promote calcium oxalate crystallization in nephrolithiasis.[88]

In transplantation, it has been shown that the exchange of exosomes between dendritic cells may constitute a potential mechanism by which passenger leukocytes transfer alloantigens to recipient antigen-presenting cells, leading to an increased generation of donor-reactive T cells.[89] On the other hand, other studies have found that dendritic cell-derived exosomes may induce tolerance rather than immune stimulation.[90] Engineering of dendritic cells to release tolerogenic exosomes could be useful to prevent/ameliorate transplant rejection.

ROLE AS BIOMARKERS IN RENAL DISEASE

Urine is the ideal biological sample for discovery of new biomarkers for kidney diseases because of the ease of non-invasive collection. Urinary exosomes are released from renal epithelial cells, including podocytes, renal tubule cells and the transitional epithelial cells lining the urinary drainage system. Isolation of urinary exosomes can identify their source and result in enrichment of low-abundance urinary protein, mRNAs, miRNAs and transcription factors that have potential pathophysiological significance.[73]

Exosome analysis may be useful for providing information with regard to kidney genetic diseases. Autosomal-dominant polycystic kidney disease (ADPKD) Types 1 and 2 are the most common genetic kidney diseases leading to renal failure. Polycystin-1 and -2 are the protein products of two genes mutated in ADPKD. These proteins are of low abundance or undetectable in kidney tissue homogenate, but easily detectable in urinary exosomes.[91,92] Immunoblot analysis of urinary exosomes was able to differentiate two different types of mutations for the thiazide-sensitive Na–Cl co-transporter of the distal convoluted tubule. This approach could have the potential to become a useful diagnostic tool to detect and sub-classify Gitelman's syndrome.[73] Similarly, immunoblotting of exosomes from urine samples of patients with a clinical diagnosis of Bartter syndrome type I showed absence of the sodium–potassium–chloride co-transporter 2 (NKCC2).[78]

It has been demonstrated that transcription factors can be detected and may be concentrated within urinary exosomes.[93] Using acute kidney injury (AKI) models (cisplatin and ischaemia-reperfusion) and podocyte injury models (puromycin-treated rats and podocin/Vpr-transgenic mice), elevated levels of activating transcription factor 3 (ATF3) were associated with AKI and Wilms Tumour 1 (WT-1) with early podocyte injury.[93] In a small number of patients, ATF3 was detected in urinary exosomes in patients with AKI but not in normal subjects or patients with CKD, and WT-1 in patients with focal segmental glomerulosclerosis (FSGS). Although further validation has not emerged, exosomal ATF3 may be a novel renal tubular cell injury biomarker for detecting AKI, and exosomal WT-1 might indicate podocyte injury.[93] Differences in the protein content of urinary exosomes from patients with early IgA nephropathy (IgAN) or thin basement membrane nephropathy have been reported.[94] Similarly, the presence of fetuin-A in urine exosomes has been reported as a predictive biomarker for AKI[95] and urinary exosomal aquaporin-1 was reduced in experimental ischaemia reperfusion injury.[96] Another recent observation of potential importance is the finding of high molecular oligomers of light chains only in urinary exosomes of patients with active amyloid light-chain amyloidosis and not in patients with other plasma cell dyscrasia-related kidney diseases.[97] While these preliminary studies are of interest, it has not been clearly established whether renal injury, ischaemia or proteinuria alter the actual numbers of exosomes liberated into urine and it is important to emphasize that all of these clinical studies have been limited to very small numbers of patients.

Exosomes contain mRNA and miRNAs. Although urinary mRNAs can be analysed directly from whole urine using reverse transcription polymerase chain reaction (RT-PCR), efficient urinary exosome isolation protocols might increase the sensitivity and specificity of urinary mRNA analysis.[98] This might be of relevance to recent studies that have found increased glycoprotein B7-1 to nephrin mRNA ratios in urinary sediments from patients with minimal change disease compared with FSGS[99] and to the finding that urinary granzyme A mRNA levels can potentially distinguish patients with cellular rejection from those with AKI.[100]

THERAPEUTICS

Harnessing exosomal delivery mechanisms to therapeutic ends could have far-reaching consequences. The exploitation of 'custom-made' exosomes as a delivery tool for pharmacological agents could allow the precise targeting of those molecules to certain cell types. Exosomes are potentially ideal gene delivery vectors. Their small size and flexibility enables them to cross biological membranes, while their bi-lipid structure protects the mRNA, miRNA and protein cargo from degradation, facilitating delivery to its target. A proof of

concept study has used modified murine exosomes to successfully deliver siRNA resulting in gene-specific silencing in the brain.[101]

For many kidney-related diseases a prime target for potential exosome-based therapy could be endothelial cells, which have essential roles in regulation of blood pressure, local regulation of blood flow, regulation of thrombosis and clearance of plasma lipids and are easily accessible to exosomes from the circulation. The artificial engineering of exosomes is a natural extension of the success of some liposomal therapies and can be used for delivery of specific RNAi molecules.[101] Furthermore, the purification and use of exosomes from particular cells or generated under certain stresses may be useful therapeutically. An example of this has developed from the interest in the mechanism underlying the potential of mesenchymal stem cells to promote tissue repair and mediate regeneration. Several studies have demonstrated that mesenchymal stem cells have the capacity to reverse acute and chronic kidney injury in different experimental models. These effects appear to be at least in part paracrine and can be largely mediated by the RNA cargo of exosomes and/or microvesicles.[102,103]

A potential approach to cancer immunotherapy based on exosomes has arisen from initial studies showing that dendritic cell-derived exosomes loaded with tumour peptides are capable of priming cytotoxic T cells. This can then mediate the rejection of tumours expressing the relevant antigens in mice.[104] These exosomes also promote natural killer (NK) cell activation in immunocompetent mice and NK cell-dependent anti-tumour effects. Based on these results, clinical trials are in progress. Vaccination strategies could also be envisioned using exosomes from tumour cells that carry tumour antigens. By using different sources of tumour exosomes such as plasmacytoma-derived exosomes, several groups have shown that exosomes can induce tumour-specific immunity in animal models,[105] prevent tumour development and are a potential strategy for future therapeutic tumour vaccination.

Exosomes released from cancers contain oncoproteins and miRNAs which may promote cancer progression. A novel technology which consists of immobilized affinity agents in the outer-capillary space of hollow-fibre plasma separator cartridges that integrate into standard dialysis machines has been devised. This technology is currently being evaluated for its efficacy for capturing exosomes secreted by cancer cell lines and present in biological fluids from cancer patients[106] and could potentially be applied to other situations such as atherosclerosis in which circulating microvesicles might have pathogenic roles.

FUTURE CHALLENGES

While there is an increasing appreciation of the existence and potential functions of exosomes and other vesicles, some very fundamental questions remain. Are there distinct cell-specific types or families of exosomes with well-defined sizes, cargos and differing functions? How is exosomal cargo modified? What are the physiological and pathological stimuli to their production, release and uptake? What are their physiological signalling roles in the circulation and urine? What receptors or other mechanisms define their target cells? What is the effect of renal function and disease on the levels and nature of circulating and urinary exosomes? Addressing these questions should provide new insights in the intercellular communication mechanism and enable a more sophisticated translation of the use of exosomes as novel biomarkers and therapeutic intervention strategies.

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