Urinary exosomes: is there a future?

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Tiny vesicles called ‘exosomes’, recently discovered in normal urine [1], provide a non-invasive means of acquiring unique information about the physiological or pathophysiological state of their renal cells of origin. Exosomes are delivered to the urine from all renal epithelial cell types. Consequently, analysis of urinary exosomes may provide a source of protein biomarkers for diseases involving glomerular podocytes, the various renal tubule segments or the transitional epithelium lining the urinary drainage tract [1]. Here, we discuss possible applications of urinary exosome analysis, as well as barriers to the development of practical clinical tools for exosome analysis.

Exosomes originate as the internal vesicles of multivesicular bodies (MVBs) in cells (Figure 1). They were first described as products of circulating blood cells [2], such as erythrocytes and lymphocytes, and are probably formed by most cell types throughout the body. In the kidney, exosomes are released to the urine by fusion of the outer membrane of the MVBs with the apical plasma membrane (Figure 1). Proteomic analysis of urinary exosomes using tandem mass spectrometry approaches [1] revealed membrane proteins from each cell type facing the urinary space. In addition, the lumens of exosomes contain many cytosolic proteins that are entrained when the exosomes are formed in the MVBs. Thus, urinary exosomes can provide the investigator with a sampling of membrane and cytosolic proteins from each renal epithelial cell type. Through the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS), more than a thousand proteins have been detected (Gonzales, Pisitkun and Knepper, unpublished data). In urine from normal subjects, urinary exosomes account for ~3% of the total urinary protein [3]. Hence, when exosomes are isolated, their constituent proteins are enriched >30-fold, enhancing the detectability of rare proteins that may have diagnostic value.

**How could analysis of urinary exosomes be used in clinical research?** One ambitious application is large-scale biomarker discovery, such as those currently being pursued in blood plasma or serum [4]. The object is to identify surrogate biomarkers that can be used for clinical decision making. According to current guidelines [4], discovery experiments would be done on samples from a relatively small number of extremely well-characterized patients (along with appropriately chosen control subjects). With a particular clinical decision process in mind, LC-MS/MS can be used in the quantitative mode to profile the entire proteome of the isolated exosomes. The objective is to choose a combination of proteins whose measurement can be hypothesized to provide the required specificity (lack of false positives) and sensitivity (lack of false negatives). The hypotheses (i.e. biomarker combinations) from discovery studies can then be tested in validation studies (clinical trials) with large numbers of patients (typically >1000). Because LC-MS/MS analysis is expensive for such large studies and because LC-MS/MS quantification is difficult, validation studies can be expected to depend on development of assays using immunochemical methods carried out on a large scale using automation. The ultimate objective is to develop robust immunochromatography assays that can be provided at the marketplace at a relatively low cost owing to ‘economy of scale’.

**What are examples of clinical decision-making processes that could benefit from urinary exosome analysis of surrogate biomarkers?** One potential application is in autosomal dominant polycystic kidney disease (ADPKD) [5]. Early identification of patients who will eventually develop renal cysts progressing to renal failure would allow early intervention with one or more of the therapeutic approaches currently under investigation, e.g. V2 receptor antagonists [6]. Identification of readily measurable urinary exosomal biomarkers that would accomplish this task would be of clear benefit. Another possibility is in the setting of renal transplantation when a rising serum creatinine level demands a rapid answer to the question of acute kidney injury versus rejection. A rapid urinary assay for a set of predictive exosome biomarkers could provide guidance in the interval prior to the reading of the biopsy, and may allow a faster choice of the optimal therapy. Clearly, there are many other possibilities and it is likely that the nephrologist-reader already has one or two in mind.
Use of exosome analysis in biomarker discovery and validation studies is a daunting task. However, there are some much more straightforward potential applications of exosome analysis that may prove to be of considerable value, particularly in the area of genetic mutation analysis. Tandem mass spectrometry can be used for determination of the primary sequence of urinary proteins for the identifications of mutations and polymorphisms. New tandem mass spectrometers that have recently reached the market have superior abilities to carry out de novo sequencing of proteins. A computational strategy for doing de novo sequencing has been reported by Bandeira et al. [7] and involves digestion of the target proteins with a battery of proteases followed by computer analysis of the resulting spectra to assemble the mass spectral data in complete (or near complete) protein sequences for particular protein targets.

An alternative approach to identification of mutations in the coding sequence of genes would take advantage of the recent discovery that exosomes contain mRNA [8]. When exosomes are formed by invagination of the outer membrane of multi-vesicular bodies, they not only entrain cytosolic proteins but also mRNA. We have recently demonstrated that urinary exosomes likewise contain mRNA from their cells-of-origin (Gonzales, Pisitkun and Knepper, unpublished data). In principle, mRNAs for candidate proteins expressed in renal epithelial cells could be subjected to RT-PCR followed by sequencing of the DNA products to identify specific mutations.

Despite the potential benefits of urinary exosome analysis, there are barriers and limitations that must be dealt with. Foremost among these is the challenge of quantification. Ideally, we would like to know the absolute rate of excretion of each protein and compare excretion rates among patients and controls. (Note: Simple measurement of the concentration of individual proteins in urine is of limited use because variation in water excretion would be the main determinant of the concentration of a given biomarker candidate.) Such an approach requires quantitative urine collections, which are difficult to accomplish in most patient populations. Normalization by urinary creatinine concentration is a traditional substitute as a normalizing variable, although creatinine excretion rate varies widely among humans, potentially masking true changes in the excretion of individual protein markers. Alternatively, the excretion of specific exosomal proteins can be normalized by the excretion of exosomal markers such as Alix or TSG101 that are found in all exosomes [3]. Studies are needed to evaluate alternatives among normalizing factors.

Currently, the most effective method for exosome isolation is ultracentrifugation [1]. However, this method requires expensive equipment that is not always available in clinical environments. Alternative approaches using ultrafiltration [9] are promising but tend to retain and concentrate soluble proteins in urine in addition to exosomes. These contaminating proteins can compete with exosomal proteins for identification by LC-MS/MS and therefore may reduce the sensitivity of the discovery process. However, this is less of a problem in immunochemical analysis if the antibodies used are specific.

Another barrier to exosomal analysis is the knotty problem of Tamm-Horsfall protein (uromodulin) excretion. The kidney excretes large amounts of Tamm-Horsfall protein every day. This protein is a member of the zona pellucida protein family and tends to form vast fibrillar aggregates in urine, particularly at low temperature. These aggregates can entrain the exosomes and prevent their efficient isolation and purification by centrifugation methods [10]. Thus, effective means are needed to eliminate the Tamm-Horsfall protein from urine samples or at least to prevent the aggregation process.
Residual renal function: the delicate balance between benefits and risks

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Introduction

Residual renal function (RRF) facilitates the achievement of dialysis adequacy in stage 5 chronic kidney disease (CKD-5) patients [1]. It facilitates patients’ acceptance of renal replacement therapy (RRT) in minimizing dietary and fluid restriction. RRF has been confirmed recently to be a significant determinant of morbidity and mortality in dialysis patients [2]. In addition, the preservation of RRF is also highly suitable in CKD-5 patients to enhance removal or degradation of middle and large uraemic toxins implicated

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Is exosome analysis a potentially useful tool for physiological studies? We have demonstrated that all of the predominant apical Na+ transporters expressed along the renal tubule are detectable in urine via exosome isolation [11]. Furthermore, the water channel aquaporin-2 is likewise detectable [12]. Do the rates of excretion of these transporters provide a measure of the rate of their production in the kidney? Probably not, since the rate of excretion of a given protein is the difference between the rate of its production and the rate of its degradation in the cell. Unless we know the rate of degradation, we cannot draw conclusions about production rate.

Like any new tool, exosome analysis has advantages and disadvantages that need to be explored prior to any exosome analysis project. However, given the non-invasive nature of the sample collection procedure and the large amount of information potentially available from such studies, it seems likely that exosome analysis will play a role in nephrology research in the future. With this possibility in mind, we have created internet sites to provide technical protocols (http://intramural.nih.gov/research/uroprot/) and listings of proteins already found in exosomes (http://dir.nhlbi.nih.gov/papers/lkem/exosome/index.htm) for use by researchers.

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