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Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome

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Urinary microvesicles, such as 40–100 nm exosomes and 100–1000 nm microparticles, contain many proteins that may serve as biomarkers of renal disease. Microvesicles have been isolated by ultracentrifugation or nanomembrane ultrafiltration from normal urine; however, little is known about the efficiency of these methods in isolating microvesicles from patients with nephrotic-range proteinuria. Here we compared three techniques to isolate microvesicles from nephrotic urine: nanomembrane ultrafiltration, ultracentrifugation, and ultracentrifugation followed by size-exclusion chromatography (UC-SEC). Highly abundant urinary proteins were still present in sufficient quantity after ultrafiltration or ultracentrifugation to blunt detection of less abundant microvesicular proteins by MALDI-TOF-TOF mass spectrometry. The microvesicular markers neprilysin, aquaporin-2, and podocalyxin were highly enriched following UC-SEC compared with preparations by ultrafiltration or ultracentrifugation alone. Electron microscopy of the UC-SEC fractions found microvesicles of varying size, compatible with the presence of both exosomes and microparticles. Thus, UC-SEC following ultracentrifugation to further enrich and purify microparticles facilitates the search for prognostic biomarkers that might be used to predict the clinical course of nephrotic syndrome.

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Urine is an ideal biological sample for the discovery of new biomarkers because of the ease and noninvasive nature of collection. In addition to soluble plasma proteins, urine also contains microvesicles such as exosomes and microparticles that may constitute a rich source of intracellular renal biomarkers.[1–3] Urinary exosomes are membrane vesicles with a diameter of 40–100 nm secreted by tubular cells and podocytes.[2,4] They are formed by fusion of endosomes with the outer membrane of multivesicular bodies (MVBs) and subsequent internalization into the MVB by membrane invagination.[2] When MVB fuse with the apical membrane, the internal vesicles enter the tubular lumen as exosomes.[5] In contrast, microparticles are membrane-shed vesicles with a size range between 100 and 1000 nm.[3] Microvesicles not only contain membrane-bound proteins, but their lumina also contain cytosolic proteins that become trapped during invagination into the MVB or budding from the plasma membrane.[6] Microparticles can be released by a variety of conditions, including cell activation, oxidative stress, and apoptosis.[7]

The formation and excretion of microvesicles is theorized to take place in every segment of the renal tubulus. Thus, analysis of microvesicles should provide information about the pathophysiological state of the entire renal tubule.[2,4] Recent studies have shown that microvesicles can be recovered from urine by ultracentrifugation or ultrafiltration techniques.[1,2,8] However, these studies focused predominantly on patients with normal urine. Little is known about the efficiency of these methods in isolating microvesicles from patients with nephrotic-range proteinuria. Nephrotic urine contains a large amount of highly abundant proteins that tend to be retained by ultrafiltration and to a lesser extent also by ultracentrifugation.[4] These highly abundant proteins interfere with microvesicular protein identification by proteomic techniques and complicate the search for prognostic biomarkers that might be used to predict the clinical course of the nephrotic syndrome. In this study, we have performed a comparison of different methods to isolate microvesicles from urine of patients with a nephrotic syndrome.

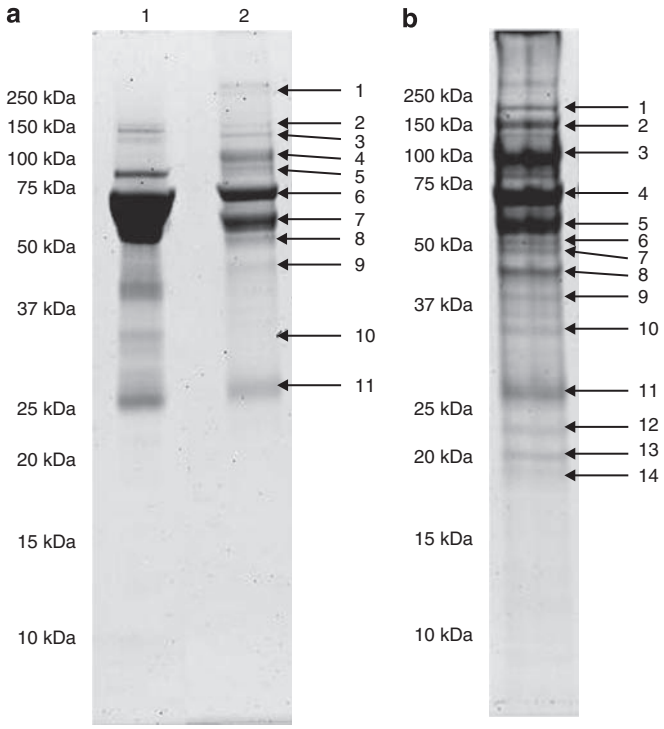

Figure 1 | One-dimensional gel electrophoresis showing the difficulty of isolating microvesicular proteins from nephrotic syndrome. Isolation by (**a**) the nanomembrane concentrator. *Lane 1*, retentate; *lane 2*, proteins remaining on the nanomembrane after the retentate was removed and washed with Laemmli buffer; and (**b**) the ultracentrifugation method. The abundant protein at 73 kDa was identified as albumin. Spot numbers refer to the numbers in Supplementary Table S1, where mass spectrometric data are presented. The urine sample was obtained from a patient with membranous nephropathy (protein concentration of 3.7 g/l; protein excretion 5.2 g per 24 h).

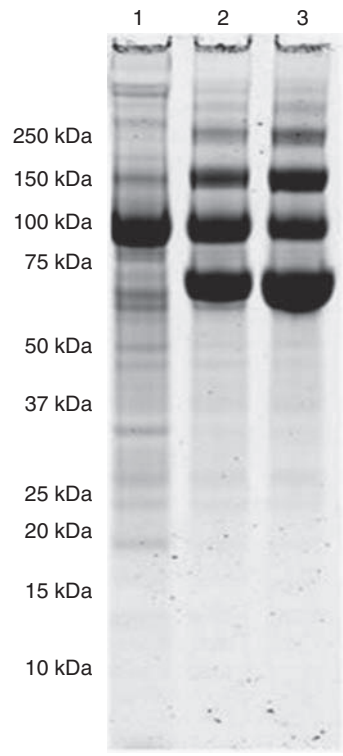

Figure 2 | One-dimensional gel electrophoresis of urine from a normal control showing coprecipitation of albumin with exosomes after ultracentrifugation. *Lane 1*, normal urine; *lane 2*, normal urine with 0.4% bovine serum albumin (BSA) added; *lane 3*, normal urine with 1% BSA. Ultracentrifugation of urine with nephrotic-range concentrations of albumin resulted in coprecipitation of albumin (lanes 2 and 3 at 73 and 150 kDa, respectively) with the exosomes.

RESULTS

Isolation of microvesicles by ultracentrifugation and nanomembrane ultrafiltration

Isolation of microvesicular proteins from nephrotic urine using the ultracentrifugation or the ultrafiltration method proved to be very difficult (Figure 1). Highly abundant proteins, especially albumin and α-1-antitrypsin, were present in large amounts after ultracentrifugation or nanomembrane ultrafiltration. These highly abundant proteins limited the detection of microvesicular proteins (Supplementary Table S1). Only two large membrane-associated proteins, aminopeptidase N and nebulin, could be identified after ultracentrifugation (Supplementary Table S1). We observed a similar interference with microvesicular protein detection after ultracentrifugation of urine from a healthy volunteer with 0.4 or 1% bovine serum albumin added. Addition of this resulted in extra bands at 73 and 150 kDa, compatible with albumin, and disappearance of the lower molecular weight (LMW) bands (Figure 2). These observations strongly suggested that coprecipitation of highly abundant proteins with microvesicles into the pellet interfered with the identification of microvesicular proteins.

Isolation of microvesicles by ultracentrifugation followed by SEC

To separate highly abundant proteins from microvesicles, pellets obtained by ultracentrifugation were loaded onto a size-exclusion column. The chromatograms after size-exclusion chromatography (SEC) of nephrotic urine showed three fractions: (1) a high molecular weight (HMW) fraction corresponding to a molecular weight >670 kDa; (2) a LMW fraction corresponding to a molecular weight 10–670 kDa; and (3) a third fraction corresponding to a molecular weight <10 kDa (Figure 3). For proteomic analysis, the HMW fraction was resolved by one-dimensional SDS polyacrylamide gel electrophoresis (Figure 4a). Matrix-assisted laser desorption/ionization-time of flight-time of flight analysis (MALDI-TOF-TOF) of the HMW fraction identified proteins known to be present in microvesicles, including membrane-associated proteins (annexin A2/A5, aminopeptidase N, angiotensin-converting enzyme 2, aquaporin-1), extracellular proteins (vitronectin and clusterin), and galectin-3-binding protein, a protein involved in cell adhesion and a potent immune stimulator (Supplementary Table S2). In contrast, the LMW fraction only contained highly abundant proteins, but no microvesicular proteins (Figure 4b; Supplementary