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

Ilse M. Rood[1,3], Jeroen K.J. Deegens[1,3], Michael L. Merchant[2], Wim P.M. Tamboer[1], Daniel W. Wilkey[2], Jack F.M. Wetzels[1] and Jon B. Klein[2]

[1]*Department of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands and* [2]*Kidney Disease Program and Clinical Proteomics Center, University of Louisville, Louisville, Kentucky, USA*

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.

Kidney International (2010) **78,** 810–816; doi:10.1038/ki.2010.262; published online 4 August 2010

KEYWORDS: exosome; mass spectrometry; nephrotic syndrome; proteinuria; ultrafiltration

Correspondence: *Jeroen K.J. Deegens, Department of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: j.deegens@nier.umcn.nl*

[3]These authors contributed equally to this work.

Received 30 August 2009; revised 17 April 2010; accepted 26 May 2010; published online 4 August 2010

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.

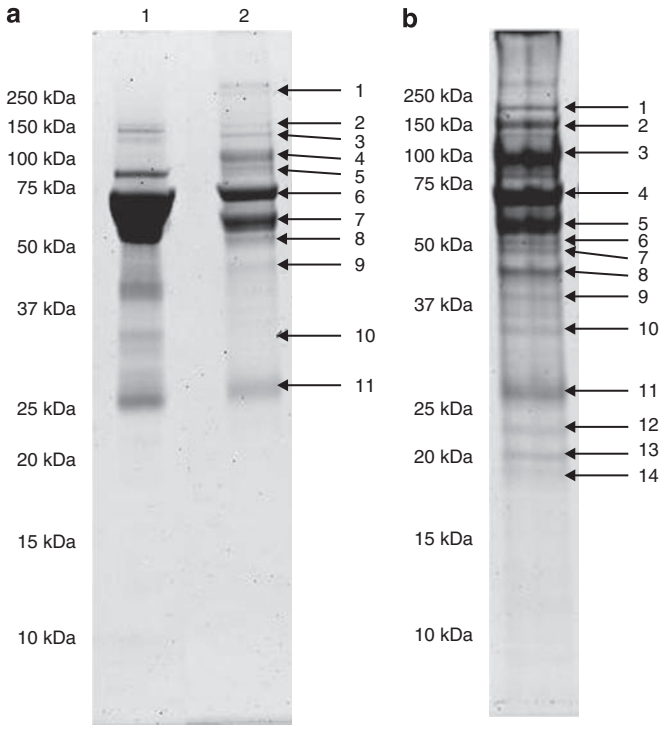

Figure 1 | One-dimensional gel electrophoresis showing the difficulty of isolating microvesicular proteins from nephrotic syndrome. Isolation by (**a**) the nanomembrane concentrator. *Lane 1*, retentate; *lane 2*, proteins remaining on the nanomembrane after the retentate was removed and washed with 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).

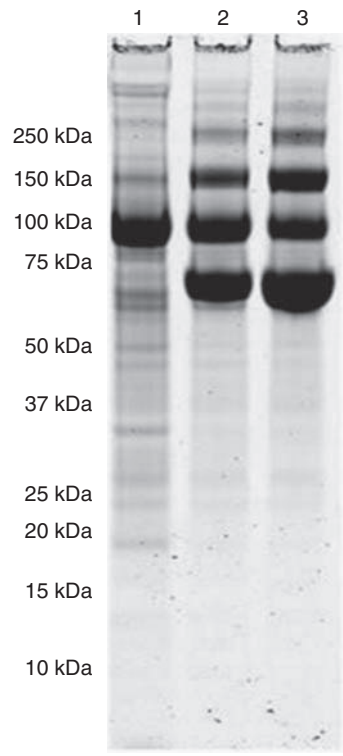

Figure 2 | One-dimensional gel electrophoresis of urine from a normal control showing coprecipitation of albumin with exosomes after ultracentrifugation. *Lane 1*, normal urine; *lane 2*, normal urine with 0.4% bovine serum albumin (BSA) added; *lane 3*, normal urine with 1% BSA. Ultracentrifugation 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

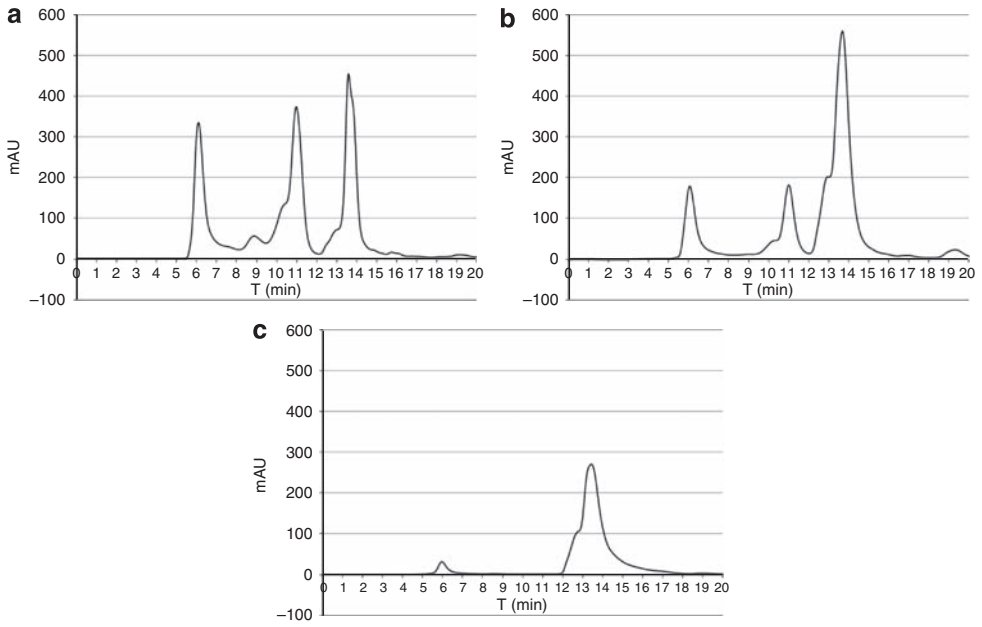

Figure 3 | Chromatogram obtained after size-exclusion chromatography. Chromatogram of (**a**) urine sample from a patient with membranous nephropathy (protein concentration 10.6 g/l; protein excretion 17.7 g per 24 h). (**b**) Urine sample from a patient with focal segmental glomerulosclerosis (protein concentration 7.3 g/l; protein excretion 7.2 g per 24 h). (**c**) Urine sample from a normal control. A lower molecular weight fraction with a retention time of 8–12 min (**a**) or 9–12 min (**b**) is present in the urine obtained from patients with nephrotic-range proteinuria.

Table S2). Of note, the LMW fraction was only present in urine from patients with nephrotic syndrome and was not observed in normal urine (Figure 3). Electron microscopy verified the presence of urinary microvesicles (Figure 5) in the HMW fraction. Quantitative analysis of electron micrographs of urinary microvesicles revealed that the size of these vesicles varied between 50 and 300 nm, compatible with both exosomes and microparticles.

Comparison of isolation methods by western blot analysis

We then compared the three methods for their ability to enrich for typical microvesicular proteins (aquaporin-2 (AQP2) and neprilysin) and depleting high abundant proteins (albumin) from nephrotic urine. Both AQP2 and neprilysin could be detected by western blot analysis (Figure 6) in the HMW fraction obtained after ultracentrifugation followed by SEC (UC-SEC), but not in the LMW fraction. In contrast, the HMW fraction contained lower amounts of albumin in comparison to the LMW fraction. Microvesicular markers were not detectable using ultrafiltration. A small neprilysin band was detectable using ultracentrifugation. Clearly, albumin was more abundant in samples obtained by ultracentrifugation or ultrafiltration (Figure 6).

Effect of DTT on removal of highly abundant proteins

To investigate the effect of dithiothreitol (DTT) on depletion of highly abundant proteins (albumin), we compared UC-SEC to ultracentrifugation followed by resuspension of the pellet in DTT. The HMW fraction obtained after UC-SEC still contained lower amounts of albumin in comparison to ultracentrifugation with DTT (Figure 7). Moreover, compared with ultracentrifugation with DTT, UC-SEC resulted in an enrichment of the podocytic marker podocalyxin (PODXL; Figure 7).

DISCUSSION

Urinary biomarkers can be derived from different protein sources, including soluble proteins, sediment proteins, and microvesicles.[5] It would be beneficial if candidate surrogate biomarkers for renal disease were not simply filtered serum proteins but rather cell-derived proteins that were involved in the disease pathogenesis or progression. To explore the different urinary proteomes, efficient isolation of fraction such as microvesicles requires minimal contamination by soluble proteins or sediment proteins for sensitive biomarker discovery. Previous studies demonstrated that ultracentrifugation and nanomembrane ultrafiltration can effectively isolate microvesicles, largely consisting of exosomes, from normal urine.[2,8] However, our study shows that isolation of microvesicles from nephrotic urine, by these established isolation methods, is limited due to interference by highly abundant soluble proteins. We noted that the large amount of protein present in nephrotic urine obstructed the nanomembrane during ultrafiltration. As a result, ultrafiltration efficiency was significantly reduced and soluble proteins were retained by the nanomembrane concentrator. Similarly, highly abundant soluble proteins were also present in large amounts in the microvesicular pellet after ultracentrifugation (Figure 1). Ultracentrifugation of normal urine with bovine serum albumin added in concentrations comparable to nephrotic urine showed that soluble proteins, not present in microvesicles (nonmicrovesicular proteins), can become

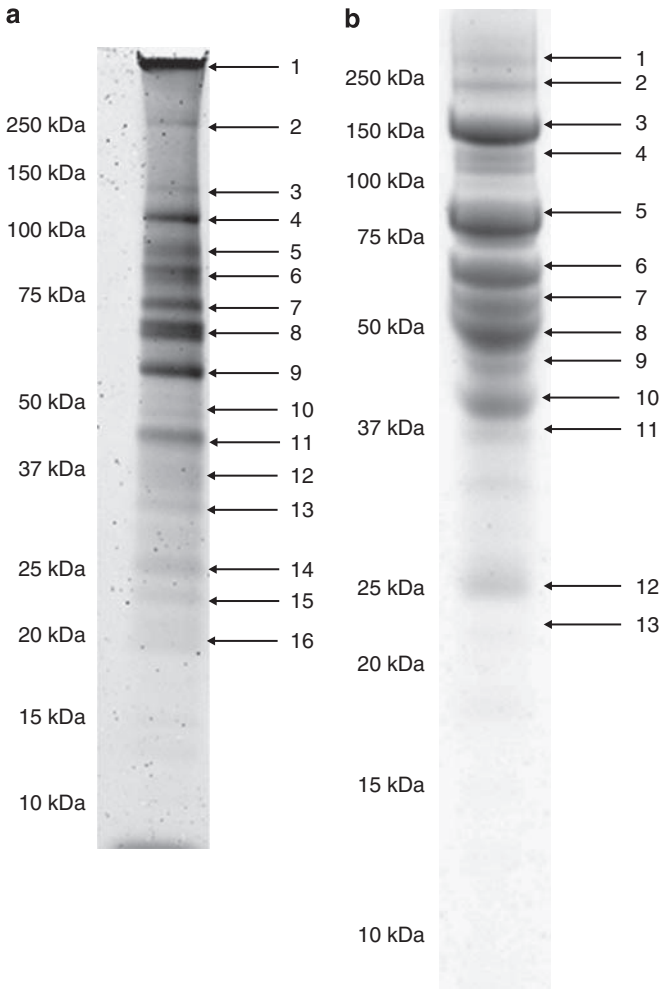

Figure 4 | One-dimensional gel electrophoresis of proteins isolated by ultracentrifugation followed by size-exclusion chromatography. (**a**) High molecular weight fraction (retention time 5–8 min). (**b**) Lower molecular weight fraction (retention time 8–10 min). Spot numbers refer to the numbers in Supplementary Table S2, where mass spectrometric data are presented. The urine sample was obtained from a patient with idiopathic membranous nephropathy (protein concentration 10.6 g/l; protein excretion 17.7 g per 24 h).

entrapped into the pellet and limit the identification of lower abundant microvesicular proteins (Figure 2). The exact mechanism resulting in entrapment of nonmicrovesicular proteins is not known. Soluble proteins could coprecipitate through specific or nonspecific mechanisms with microvesicles into the pellet. Indeed, highly abundant soluble proteins could also originate from within the microvesicles. Filtered proteins are reabsorbed along the entire nephron and accumulate in the cytoplasm of tubular cells. During their formation, microvesicles can incorporate these cytoplasmic soluble proteins in their lumina. Therefore, methods such as the one presented herein, which maintain the microvesicular structure and minimize the copurification of highly abundant nonmicrovesicular proteins, are vital to significantly improve detection of microvesicular proteins.

In dealing with complex protein samples, most of the current proteomic analysis methods are still limited with respect to their dynamic range and sensitivity.[9] One approach to enhanced detection and identification of microvesicular proteins is through use of the state-of-the-art mass spectrometric methods such as those demonstrated by Pisitkun and Gonzales.[2,10] A second approach to enhanced detection includes methods that also specifically reduce copurification of highly abundant proteins from urine samples. These methods would then also improve the ability of mass spectrometry approach to detect lower abundant proteins.[11] In this study, we demonstrate through enrichment of marker proteins neprilysin, AQP2, and PODXL that the existing ultracentrifugation method for urinary microvesicle isolation can be improved by application of SEC (Figures 6 and 7). The presented UC-SEC method also allowed for detection of microvesicular proteins such as membrane-associated proteins, antiapoptosis, and extracellular matrix proteins. It is our expectation that incorporation of this method ahead of direct analysis by liquid chromatography mass spectrophotometry methods would yield a more exhaustive list of known and possibly previously unrecognized microvesicular proteins that have not been described in normal urine.

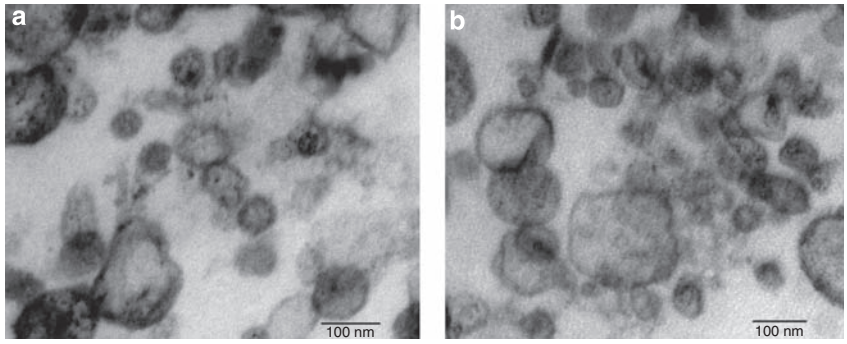

Figure 5 | Electron microscopic image of urinary microvesicles. Urinary microvesicles were obtained by ultracentrifugation followed by size-exclusion chromatography. (**a**) Sample from a patient with idiopathic membranous nephropathy (protein concentration 4.4 g/l; protein excretion 7.5 g per 24 h). (**b**) Sample from a patient with focal segmental glomerulosclerosis (protein concentration 6.0 g/l; protein excretion 19.4 g per 24 h).

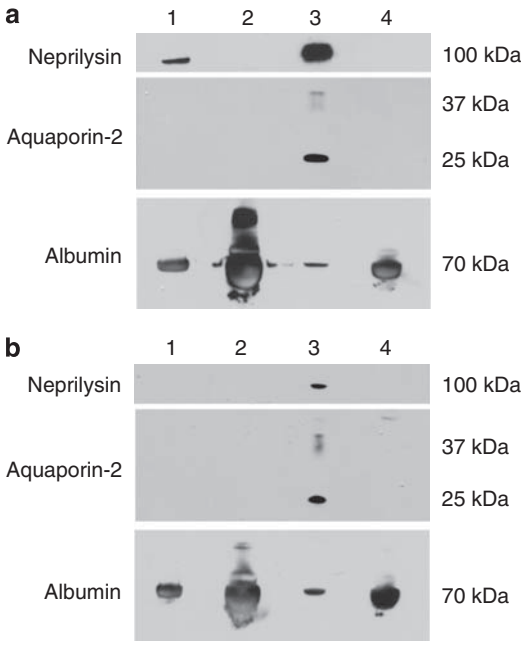

Figure 6 | Efficiency of urinary microvesicle isolation by ultracentrifugation (*lane 1*), nanomembrane ultrafiltration (*lane 2*), ultracentrifugation followed by size-exclusion chromatography (high molecular weight fraction, *lane 3*), and ultracentrifugation followed by size-exclusion chromatography (lower molecular weight fraction, *lane 4*). Western blot analysis was performed for neprilysin, aquaporin-2, and albumin. (**a**) Urine sample from a patient with idiopathic membranous nephropathy (protein concentration 4.4 g/l; protein excretion 7.5 g per 24 h). (**b**) Urine sample from a patient with focal segmental glomerulosclerosis (protein concentration 6.0 g/l; protein excretion 19.4 g per 24 h).

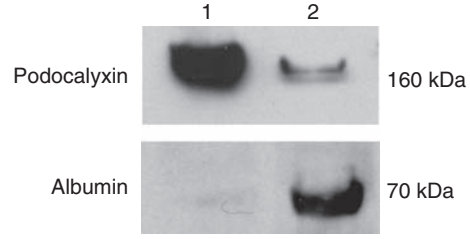

Figure 7 | Efficiency of urinary microvesicle isolation by ultracentrifugation followed by size-exclusion chromatography (high molecular weight fraction, *lane 1*), ultracentrifugation followed by removal of Tamm–Horsfall protein from the 200,000 *g* pellet with dithiothreitol (DTT) and reultracentrifugation (*lane 2*). Western blot analysis was performed for podocalyxin and albumin. Pooled urine sample from three patients with idiopathic membranous nephropathy (median protein concentration 4.6 (range 3.1–4.6) g/l; median protein excretion 5.0 (3.5–6.3) g per 24 h).

To optimize the identification of exosomal proteins in normal urine, earlier studies used DTT to remove the high abundant protein Tamm–Horsfall protein (THP) from exosomes obtained from the high-speed pellet at 200,000 *g*.[2,10] DTT denatures the zona pellucida domains in the THP, thus inhibiting aggregation and entrapment of exosomes by polymeric THP networks and removal of THP in the UC sample supernatant. Theoretically, highly abundant proteins could also become entrapped in the polymeric THP network containing microvesicles. We performed an immunoblot analysis after reduction of the 200,000 *g* pellet with DTT and reultracentrifugation. A direct comparison of the UC-SEC method with the ultracentrifugation with DTT reduction step showed that the UC-SEC approach is more efficient in the removal of albumin and enrichment for the microvesicular protein PODXL (Figure 7).

Recently, it was demonstrated that exosomes in normal urine can become entrapped in the polymeric THP networks present in the low-speed 17,000 *g* pellet.[12] The yield of exosomes was increased by addition of DTT to the low-speed pellet at 17,000 *g*. This modification resulted in a more complete retrieval of exosomes in the high-speed pellet of normal urine. To achieve a more complete retrieval of microvesicles from nephrotic urine, addition of DTT to the 17,000 *g* pellet can serve as a useful extension of our UC-SEC method.

On initial analysis, our data appear to differ with previous studies reporting successful identification of potential microvesicular biomarkers in nephrotic urine using ultrafiltration and ultracentrifugation. Cheruvanky *et al.*[8] isolated exosomes using the nanomembrane ultrafiltration method. They were able to identify PODXL in urine from patients with focal segmental glomerulosclerosis and nephrotic-range proteinuria by western blot analysis. In a second study, the same group used the ultracentrifugation method to detect a transcription factor, Wilms Tumor 1, in nephrotic urine from patients with focal segmental glomerulosclerosis.[13] Still, we do not believe that our results contradict those of previous studies. In agreement with our findings, Gonzales *et al.* also report that ultrafiltration tends to retain and concentrate soluble proteins in urine in addition to exosomes, reducing the sensitivity of the discovery process.[4,8] This study also shows that western blot analysis can identify microvesicular proteins, such as neprilysin and PODXL, in nephrotic urine using ultracentrifugation. However, our study shows that highly abundant soluble proteins can interfere with detection of microvesicular proteins as reflected by the significantly smaller neprilysin and PODXL band detected after ultracentrifugation in comparison with UC-SEC and the absent AQP2 band after ultracentrifugation. Therefore, the difference between previous studies and our study most likely reflects differences in the abundance of microvesicular proteins. If present in high concentrations, microvesicular proteins can still be detected by western blot analysis using ultracentrifugation and ultrafiltration, despite interference by highly abundant soluble proteins. In contrast, lower abundant microvesicular proteins are not detected in nephrotic urine by these isolation methods.

In normal urine, many of the microvesicles were extremely small.[2] Electron micrographic images of nephrotic samples revealed microvesicles that varied in size. A substantial part of these microvesicles was >100 nm. In addition to exosomes,

cells can also release other forms of membrane vesicles in the extracellular environment. These vesicles are larger than exosomes and are often referred to as microparticles (100–1000 nm). These microparticles bud directly from the plasma/apical cell membrane after stress, activation, or apoptosis.[3,14] The formation of microparticles can be stimulated by inflammatory stimuli including cytokines or activated complement.[15] Both in human proteinuric disease and in experimental models evidence of complement activation can be detected on the apical surface of the renal tubular cells.[16–18] Similarly, protein components can stimulate tubular cells to secrete a large number of cytokines.[19,20] Thus, stimulation of tubular complement and cytokine production by proteins could explain the presence of microparticles in nephrotic urine.

In conclusion, we present a method that improved the isolation of microvesicles from nephrotic urine. Although UC-SEC is more time consuming compared with ultracentrifugation or ultrafiltration, this method allows for identification of lower abundant microvesicular proteins in nephrotic urine without interference by highly abundant proteins. These improvements in microvesicular isolation should facilitate the identification of biomarkers in renal diseases associated with a nephrotic syndrome.

MATERIALS AND METHODS

A full description of the methods is provided in the Supplementary information.

Urine collection and storage

Urine samples from patients with idiopathic membranous nephropathy and focal segmental glomerulosclerosis were collected at the Radboud University Nijmegen Medical Center. These patients had a nephrotic syndrome defined as proteinuria >3 g per 24 h and serum albumin <3.0 g/dl. Urine samples from healthy volunteers were collected at the University of Louisville. Collection and storage was performed as described by Zhou *et al.*[21]

Sample preparation and isolation methods

Urine samples (45 ml) were extensively vortexed immediately after thawing.[21] Thawed samples were filtrated through chromatography paper followed by differential centrifugation at 17,000 *g* for 15 min at 4 °C to remove urinary sediment. Three different methods to isolate microvesicles from the 17,000 *g* supernatant were evaluated.[2,8] (1) Ultracentrifugation: The 17,000 *g* supernatant was centrifuged at 200,000 *g* for 110 min at 4 °C. The resulting pellet was resuspended in 50 µl of isolation solution (250 mM sucrose per 10 mM triethanolamine).[2] In one of the collected urine samples, the resuspended pellet was incubated with the reducing agent DTT and the ultracentrifugation was repeated as above. (2) Nanomembrane ultrafiltration concentrator: The 17,000 *g* supernatant was diluted to 0.5 g/l to reduce obstruction of the nanomembrane by highly abundant proteins and added to Vivaspin 20 polyethersulfone nanomembrane concentrators (Sartorius, Goettingen, Germany) (molecular weight cut-off 100 kDa) to collect the microvesicles.[8] After centrifugation of urine at 3000 *g*, the retentate was removed from the concentrator and an equal volume of preheated 2 × Laemmli buffer was added. The nanomembrane was subsequently washed to remove remaining proteins that adhered to the membrane by adding two volumes of preheated 1 × Laemmli buffer with 10% β-mercaptoethanol into the concentrator and shaking the concentrator at room temperature. (3) Ultracentrifugation followed by SEC: Ultracentrifugation was performed as described under (1). The resulting pellet was resuspended in phosphate-buffered saline to obtain intact microvesicles. The sample was loaded onto an SEC column. Fractions of 1 ml were collected. On the basis of the chromatogram, fractions were combined to obtain a HMW and a low molecular weight fraction. Both fractions were concentrated by centrifugation at 3000 *g* using Amicon Ultra-4 (Millipore, Billerica, MA, USA) (10 kDa molecular weight cut-off). The retentate was removed from the concentrator and 1% octyl β-D-glucopyranoside was added to strip the membrane of proteins adhered to the nanomembrane.[8] A micro bicinchoninic acid-protein assay was performed on all samples to determine the protein concentration.

One-dimensional gel electrophoresis and western blotting

Equal amounts of protein obtained using methods 1–3 were separated by one-dimensional SDS polyaclylamide gel electrophoresis.

For western blot analysis, proteins were electrophoretically transferred from one-dimensional SDS polyaclylamide gel electrophoresis to nitrocellulose membranes, which were blocked and probed with antigen-specific primary antibodies (as described in supporting methods). Horseradish peroxidase-conjugated secondary antibodies were used. Signals were developed using a luminol-based chemiluminescence reagent.

In-gel trypsin digestion and MALDI-TOF-TOF mass spectrometry

Samples were prepared using a modification of the technique described by Jensen *et al.*[22] The protein spots were excised from the stained gels. After destaining, proteins in the gel were reduced, alkylated, and trypsinized as described in the supporting methods. The trypsin digest was pipetted directly onto the stainless steel sample plate of the mass spectrometer. Mass spectral data were obtained using a MALDI-TOF-TOF instrument (AB4700 protein analyzer, Applied Biosystems, Foster City, CA, USA). Data were analyzed using Mascot 2.1 against the 20051115/20061212 Swiss Protein database. A Mascot score of $\geqslant 56$ was considered to be statistically significant ($P<0.05$).

Transmission electron microscopy

Isolated microvesicles were prepared as described in the Supplementary methods and mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and viewed in a Philips (Eindhoven, The Netherlands) CM12 transmission electron microscope operating at 60 KV.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

IMR was supported by a Kolff Student Grant Abroad from the Dutch Kidney Foundation. JKJD was supported by a Kolff Postdoc Grant Abroad from the Dutch Kidney Foundation. JBK and MLM were supported by 5U01DK085673-01 and DOE DE-FG02-05ER6406.

SUPPLEMENTARY MATERIAL

Table S1. List of proteins identified by MALDI-TOF-TOF in nephrotic urine prepared by nanomembrane ultrafiltration or ultracentrifugation.

Table S2. List of proteins identified by MALDI-TOF-TOF in urine prepared by ultracentrifugation followed by size-exclusion chromatography.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Thongboonkerd V, McLeish KR, Arthur JM *et al.* Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. *Kidney Int* 2002; **62**: 1461–1469.
2. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci USA* 2004; **101**: 13368–13373.
3. Simpson RJ, Jensen SS, Lim JW. Proteomic profiling of exosomes: current perspectives. *Proteomics* 2008; **8**: 4083–4099.
4. Gonzales P, Pisitkun T, Knepper MA. Urinary exosomes: is there a future? *Nephrol Dial Transplant* 2008; **23**: 1799–1801.
5. Pisitkun T, Johnstone R, Knepper MA. Discovery of urinary biomarkers. *Mol Cell Proteomics* 2006; **5**: 1760–1771.
6. Knepper MA, Pisitkun T. Exosomes in urine: who would have thought…? *Kidney Int* 2007; **72**: 1043–1045.
7. Horstman LL, Jy W, Jimenez JJ *et al.* Endothelial microparticles as markers of endothelial dysfunction. *Front Biosci* 2004; **9**: 1118–1135.
8. Cheruvanky A, Zhou H, Pisitkun T *et al.* Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *Am J Physiol Renal Physiol* 2007; **292**: F1657–F1661.
9. Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science* 2006; **312**: 212–217.
10. Gonzales PA, Pisitkun T, Hoffert JD *et al.* Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol* 2009; **20**: 363–379.
11. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 2002; **1**: 845–867.
12. Fernandez-Llama P, Khositseth S, Gonzales PA *et al.* Tamm–Horsfall protein and urinary exosome isolation. *Kidney Int* 2010; **77**: 736–742.
13. Zhou H, Cheruvanky A, Hu X *et al.* Urinary exosomal transcription factors, a new class of biomarkers for renal disease. *Kidney Int* 2008; **74**: 613–621.
14. Pap E, Pallinger E, Pasztoi M *et al.* Highlights of a new type of intercellular communication: microvesicle-based information transfer. *Inflamm Res* 2009; **58**: 1–8.
15. Redman CW, Sargent IL. Microparticles and immunomodulation in pregnancy and pre-eclampsia. *J Reprod Immunol* 2007; **76**: 61–67.
16. Nangaku M, Pippin J, Couser WG. C6 mediates chronic progression of tubulointerstitial damage in rats with remnant kidneys. *J Am Soc Nephrol* 2002; **13**: 928–936.
17. Camussi G, Stratta P, Mazzucco G *et al. In vivo* localization of C3 on the brush border of proximal tubules of kidneys from nephrotic patients. *Clin Nephrol* 1985; **23**: 134–141.
18. Gaarkeuken H, Siezenga MA, Zuidwijk K *et al.* Complement activation by tubular cells is mediated by properdin binding. *Am J Physiol Renal Physiol* 2008; **295**: F1397–F1403.
19. Huang Z, Wen Q, Zhou SF *et al.* Differential chemokine expression in tubular cells in response to urinary proteins from patients with nephrotic syndrome. *Cytokine* 2008; **42**: 222–233.
20. Perico N, Codreanu I, Schieppati A *et al.* Pathophysiology of disease progression in proteinuric nephropathies. *Kidney Int Suppl* 2005; **94**: S79–S82.
21. Zhou H, Yuen PS, Pisitkun T *et al.* Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. *Kidney Int* 2006; **69**: 1471–1476.
22. Jensen ON, Wilm M, Shevchenko A *et al.* Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. *Methods Mol Biol* 1999; **112**: 513–530.