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ORIGINAL PAPER

Isolation and characterization of human urine extracellular vesicles

Zhanguo Liu[1,2] · David M. Cauvi[1] · Erika M. A. Bernardino[1] · Bernardo Lara[1] · Radhames E. Lizardo[3] · Dennis Hawisher[1] · Stephen Bickler[1] · Antonio De Maio[1,4]

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Abstract

Extracellular vesicles (ECV) reflect physiological or pathological conditions, emerging as potential biomarkers for disease. They can be obtained from a variety of body fluids, particularly urine that is an ideal source because it can be obtained in great quantities, recurrently and with minimal intervention. However, the characterization of urine ECV is challenging because the preparation is usually contaminated with soluble proteins, such as uromodulin (UMOD) or Tamm-Horsfall glycoprotein that forms large extracellular filaments co-sedimenting with ECV. We developed a method to obtain human urine ECV free of UMOD by the addition of $ZnSO_4$ prior to vesicle isolation by differential centrifugation. Treatment with $ZnSO_4$ did not affect the size and concentration of the vesicle preparation and preserved the storage of the samples at low temperatures. We did not observe a variation in the number of vesicles isolated during different times of the day or different days between different donors. The glycoprotein pattern of urine ECV was characterized by binding to concanavalin A (Con A) and mass spectroscopy. Several markers were found, including dipeptidyl peptidase IV (CD26), vacuolar protein sorting factor 4A (VPS4A) and dipeptidase 1 (DPEP1), and galectin 3 binding protein (G3-BP). The levels of VPS4A and DPEP1 were similar in ECV preparations obtained from several donors of both sexes. Con A binding pattern and monosaccharide composition were also comparable between subjects. In summary, our method for the isolation of highly pure ECV derived from human urine is likely to help in the use of these vesicles as potential biomarkers.

Keywords Extracellular vesicles · Exosomes · Isolation · Glycoproteins · Biomarkers

Introduction

Cellular communication is a fundamental process to maintain tissue and systemic homeostasis. Cells communicate by a variety of mechanisms including soluble mediators, such as hormones that are secreted by one cell and captured by another involving a receptor-mediated process activating a respective signal transduction mechanism necessary for an orchestrated response. Adjacent cells exchange low molecular weight metabolites via the passage of the material through intracellular channels named gap junctions that modulate function (De Maio et al. 2002). Other forms of cellular communication involve surface contact mediated by adhesion proteins or receptors forming cellular synapses (Ahmed and Xiang 2011), or by the direct transfer of membrane portions, called trogocytosis (Davis 2007). An alternative form of cellular communication is mediated by vesicles derived from the plasma membrane that are released by one cell and captured by another. These vesicles, known as extracellular vesicles (ECV), exosomes or microparticles, contain typical elements of the plasma membrane, such as lipids, proteins and glyconjugates as well as elements from the cytosol, such as soluble proteins, nucleic acids, and other metabolites. ECV are exported by different mechanisms, including their assembly into multivesicular bodies or by direct protuberances from the plasma membrane (Thery et al. 2009; De Maio 2011). ECV are released during normal physiological conditions likely reflecting the physiological conditions of the cell. In addition,

✉ Antonio De Maio
ademaio@ucsd.edu

[1] Department of Surgery, School of Medicine University of California San Diego, 9500 Gilman Drive, #0739, La Jolla, CA 92093-0739, USA

[2] Department of Critical Care Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, China

[3] Naval Medical Center San Diego, San Diego, CA, USA

[4] Department of Neurosciences, School of Medicine University of California San Diego, La Jolla, CA, USA

ECV are exported after insults also revealing the stress condition. We have proposed that the recognition of normal or stress vesicles is mediated by a surveillance mechanism that we have coined the Stress Observation System (De Maio 2011). Therefore, it is not surprising that ECV released during pathological conditions, such as cancer, diabetes, etc., contain specific markers for the disease, emerging as potential biomarkers.

ECV can be isolated from a variety of body fluids, such as blood, saliva, and urine. However, their characterization has been very challenging. First, they are isolated from a great pool derived from different cell types and likely contain a variety of markers. Second, the methods for their characterization revealed a tremendous number of elements, including proteins and nucleic acids. Therefore, it has been very difficult to establish the identity of a specific marker within a unique vesicle population or a particular disease. Thus, it is important to develop systematic approaches to characterize ECV and correlate their markers to a pathological condition. In this study, we have performed a careful analysis of ECV derived from human urine as a prelude for their use as a soluble biopsy. Urine is an excellent source of ECV because samples can be obtained in great quantities, recurrently and with minimal intervention (Franzen et al. 2016; Harpole et al. 2016). However, isolation of urine ECV is challenging due to contamination with non-ECV associated proteins, such as uromodulin (UMOD) or Tamm Horsfall glycoprotein that forms large extracellular filaments (Bokhove 2016). Several different approaches have been used for the isolation of urine ECV, including immobilized antibodies against ECV markers (Duijvesz et al. 2015), hydrostatic dialysis (Musante et al. 2014), nanomembrane ultrafiltration, ultracentrifugation, and ultracentrifugation/size-exclusion chromatography (Rood et al. 2010). We described herewith the aspects of collection, isolation, and storage as well as the potential variability among individuals. Finally, the presence of glycoproteins on ECV was determined.

Methods

Urine sample collection, treatment, and ECV isolation

Urine samples were obtained from healthy adult male and female donors and transferred into sterile urine cups (Simport Scientific, Beloeil, Canada). As indicated, samples were supplemented with $ZnSO_4$ (1–4 mM final concentration) and maintained at 4 °C until processing. Urine samples were centrifuged at 2,000×*g* for 15 min at 4 °C (Allegra 25R centrifuge, Beckman Coulter, Indianapolis, IN, USA) to remove cells and debris. The supernatants were centrifuged at 10,000×*g* for 60 min at 4 °C and then centrifuged again at 100,000×*g* for 60 min at 4 °C (Optima L-90K Ultracentrifuge, Beckman Coulter) to obtain ECV pellets. The resulting pellets were resuspended in 1 ml of phosphate buffered saline (PBS), transferred to microcentrifuge tubes (Beckman Coulter), centrifuged at 100,000×*g* for 60 min at 4 °C (Optima TLX Ultracentrifuge, Beckman Coulter) and resuspended in PBS. Determination of ECV concentration and ECV mean and mode size was performed for each ECV preparation by nanoparticle tracking analysis using the NanoSight NS300 instrument (Malvern Instruments, Westborough, MA, USA). When indicated, ECV after trypsin treatment were analyzed by HPLC coupled with tandem mass spectrometry (LC-MS/MS) using nanospray ionization (TripleTOF 5600 hybrid mass spectrometer (AB SCIEX)). Data were analyzed using MASCOT® (Matrix Science) and Protein Pilot 4.0 (AB SCIEX) for peptide identifications.

Electrophoresis

An equivalent number of ECV (1×10^{11} ECV for Coomassie blue staining and lectin blots, and 5×10^{10} ECV for immunoblotting) were solubilized in NuPAGE LDS sample buffer (Life Technologies, Carlsbad, CA), and proteins were separated using NuPAGE 4–12% Bis-Tris gels (Life Technologies). Protein bands were visualized by Coomassie brilliant blue G-250 (Sigma-Aldrich, St. Louis, MO, USA) staining.

Western blotting

Electrophoretically separated proteins were transferred onto nitrocellulose membranes (Tris transfer buffer for 1 h at 30 V). Membranes were blocked with 5% BSA-Tris-buffered saline (TBS) for 1 h at 25 °C. Blots were probed with the following primary anti-human antibodies: mouse anti-human CD9 (1/500, clone ALB6, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-human CD81 (1/500, clone H-121, Santa Cruz), rabbit anti-human CD26 (1/1000, clone H-270, Santa Cruz), rabbit anti-human DPEP1 (1/2000, GeneTex, Irvine, CA, USA), mouse anti-human VPS4A (1/1000, clone VPS4A-110, Sigma-Aldrich) and mouse anti-human galectin-3 binding protein (1/1000, clone 2B1, Bio-Rad, Hercules, CA, USA). Blots were incubated with the respective primary antibodies in 5% BSA dissolved in TBS supplemented with 0.1% Tween 20 (TBST) at 4 °C for 16 h followed by three 15 min washes in TBST at 25 °C. Blots were then incubated with either HRP-conjugated goat anti-mouse IgG antibodies (1/1000, Thermo Fisher Scientific, Fremont, CA, USA) or with HRP-conjugated goat anti-rabbit IgG antibodies (1/1000, Thermo Fisher Scientific) in 5% BSA-TBST for 1 h at 25 °C. After three 15 min washes in TBST, bands were detected by chemiluminescence using SuperSignal reagents (Thermo Fisher Scientific).

Lectin overlays

Electrophoretically separated proteins were transferred onto nitrocellulose membranes (Tris transfer buffer for 1 h at 30 V). Membranes were blocked with 5% BSA-Tris-buffered saline (TBS) for 1 h at 25 °C. Blots were then incubated with 1 μg/mL horseradish peroxidase-conjugated concanavalin A (HRP-ConA, Sigma-Aldrich) for 16 h at 4 °C on a rocking platform. In some experiments, incubation of HRP-ConA was performed in the presence of α-Methyl D-mannoside (α MM, Sigma-Aldrich) to compete for ConA binding, therefore, ensuring binding specificity. At the end of the incubation period, blots were washed 3 times for 15 min each time in TBST. Bands were detected by chemiluminescence using SuperSignal reagents (Thermo Fisher Scientific).

Lectin affinity precipitation

Freshly isolated ECV were incubated with Sepharose 4B (Sigma-Aldrich) resuspended in TBS containing 0.25% Triton X-100 (TBSTX) for 1 h at 25 °C on a rocking platform to pre-clear samples. The mixture was centrifuged at 14,000×*g* for 5 min and the supernatants containing pre-cleared ECV were transferred into new microcentrifuge tubes and incubated with ConA-Sepharose beads 4B (Sigma-Aldrich) in TBSTX added for 16 h at 4 °C on a rocking platform. At the end of the incubation period, samples were washed 3 times with TBSTX, and finally once with TBS. The resulting pellet after trypsin treatment was analyzed HPLC coupled with tandem mass spectrometry (LC-MS/MS) using nanospray ionization (TripleTOF 5600 hybrid mass spectrometer (AB SCIEX). Data were analyzed using MASCOT® (Matrix Science) and Protein Pilot 4.0 (AB SCIEX) for peptide identifications.

Monosaccharide analysis

Isolated ECV were lyophilized in a screw cap glass hydrolysing tube. The dried samples were hydrolysed using 2 N trifluoroacetic acid at 100 °C for 4 h. Acid hydrolysed samples were cooled to room temperature and centrifuged at 2000 rpm for 2 min. Excess acid was removed by dry nitrogen flush followed by co-evaporation using 100 ul of 50% isopropyl alcohol twice. The dried samples were re-suspended in 200 ul of MilliQ water and 25% was injected onto Dionex ICS-3000, high-performance anion exchange chromatography attached with a pulsed amperometric detector (HPAEC-PAD). Monosaccharide profiling was done using a Dionex CarboPac™ PA1 column 4 mm × 250 mm with 4 mm × 50 mm guard column. An isocratic mixture of 19 mM sodium hydroxide with 0.95 mM sodium acetate was used at a flow rate of 1 mL/min with total run time of 25 min per sample. Monosaccharide data was collected using the standard Quad waveform for Carbohydrate as supplied by Chromeleon software version 6.8. All neutral and amino sugars were identified and quantified by comparison with known amount of authentic monosaccharide standard mixture consisting of L-fucose, D-galactosamine, D-glucosamine, D-galactose, D-glucose, and D-mannose. Naturally, the amino sugars galactosamine and glucosamine come as an N-acetylated form during hydrolysis the N-acetyl groups are converted to amino groups hence the reported galactosamine and glucosamine represents the N-acetylated form of the corresponding.

Results

Urine ECV could be isolated without cross-contamination of uromodulin

The most practical and cost-effective method to isolate ECV is differential centrifugation that requires standard laboratory equipment without the need for sophisticated reagents. The process requires three consecutive steps, a low-speed centrifugation to remove cells and large debris, a second centrifugation to eliminate large vesicles and apoptotic bodies and the last, at high speed, to concentrate the ECV population. Therefore, the isolation of ECV from urine samples may appear to be a straightforward approach. However, that is not the case. The most abundant protein in human urine is UMOD that is secreted after cleavage of the GPI anchor precursor protein. UMOD forms large extracellular filaments by the interaction of a cysteine-rich domain of the protein (Bokhove 2016). These large and abundant aggregates co-sediment with urine ECV as shown in Fig. 1a (lane 1), resulting in a false assumption that this glycoprotein could be a major component of urine ECV. Therefore, any intervention to remove UMOD is likely to improve the quality of ECV preparation. We found that the addition of $ZnSO_4$ from 1 to 4 mM to fresh urine samples prior to the isolation of ECV resulted in the reduction of UMOD cross-contamination of the vesicle preparation (Fig. 1a, lane 2–4). The addition of $ZnSO_4$ did not affect the electrophoretic pattern of isolated ECV as shown by staining of the gel with coomassie blue (Fig. 1a). Moreover, the size and number of ECV isolated from samples treated with $ZnSO_4$ are very similar to ECV isolated in absence of $ZnSO_4$ treatment (Fig. 1b, Table 1). Other divalent anions, such as $MgCl_2$ or $MnCl_2$, did not produce the same effect on reducing UMOD cross-contamination as $ZnSO_4$.

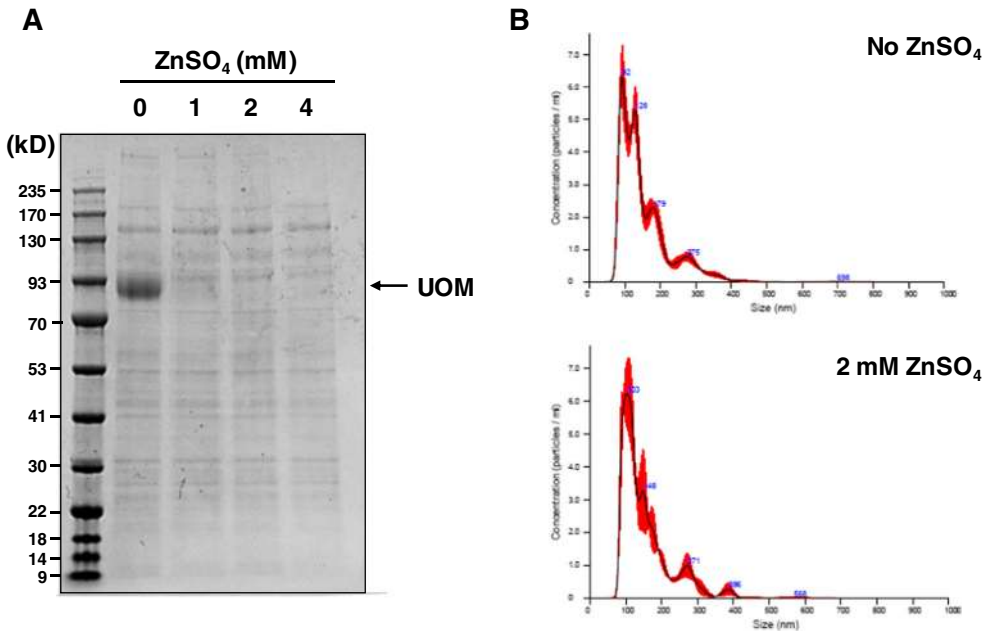

Fig. 1 The addition of $ZnSO_4$ depleted UMOD from the preparation of urine ECV. Urine samples were freshly obtained from a donor and maintained at 4 °C until processing. Aliquots of the urine sample (20 mL) were combined with PBS (lane 1) or $ZnSO_4$ at a final concentration of 1 (lane 2), 2 (lane 3) or 4 mM (lane 4). Then, urine ECV were isolated by differential centrifugation as described in Materials and Methods. ECV concentration was estimated using NanoSight NS300. Equal amounts of ECV for each condition (1×10^{11} per lane) were separated by SDS-PAGE (4–12% polyacrylamide gradient gel) and the protein bands detected by Coomassie brilliant blue staining (**a**). Notice that the band corresponding to URM of approximately 90 kD (see arrow) was only present in the sample without $ZnSO_4$. The difference in urine ECV distribution assessed by NanoSight NS300 between samples in presence or absence of $ZnSO_4$ is also presented (**b**)

The quality of urine ECV is not affected by the time of sample collection

An important consideration for the potential use of ECV as biomarkers is whether or not there is variation in samples obtained at different times of the day or during different days. We isolated ECV from fresh human samples of urine using the $ZnSO_4$ protocol from the same subject at two different times during the day, early morning and midmorning. In addition, we obtained samples from the same subject during four consecutive days. Analysis of ECV in these samples did not show major differences in the concentration of isolated vesicles whether they were collected on the same day or obtained on different days (Table 2). Finally, we analyzed whether there was a major variability in the number of isolated ECV from various donors. We obtained urine samples from five individuals and isolated ECV using the standard $ZnSO_4$ protocol. There was only a modest variation in the number of vesicles isolated from each donor (Table 3).

Table 1 Concentration and size of urine ECV isolated using $ZnSO_4$. The effect of $ZnSO_4$ on the size and concentration of urine ECV was analyzed by NanoSight NS300. The ECV mean corresponds to the average size of all particles whereas ECV mode corresponded to the size of the most abundant particle population

	$ZnSO_4$ (mM)			
	0	1	2	4
ECV ($\times 10^{10}$)/ml urine	1.4	1.1	1.4	1.1
ECV mean size (nm)	136.9 ± 2.6	126.5 ± 1.1	140.0 ± 2.2	128.1 ± 0.7
ECV mode size (nm)	97.8 ± 6.0	93.2 ± 4.9	95.8 ± 7.8	95.5 ± 1.4

Table 2 Concentration of urine ECV isolated from the same donor during four consecutive days. Urine samples were freshly obtained from a human donor at two different times during the day (early and midmorning) for four consecutive days and maintained at 4 °C until processing. $ZnSO_4$ (1 mM) was added to aliquots of the urine sample (20 mL) and ECV isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300 and expressed as 10^{10} ECV/ml of urine. Notice that the concentration of ECV was very similar between the two samples obtained during the same day as well as samples obtained during consecutive days

	Day 1	Day 2	Day 3	Day 4	Average ± SD
First sample	1.1	1.3	0.9	0.9	1.1 ± 0.2
Second sample	0.9	1	1.1	0.9	1.0 ± 0.1

Table 3 The concentration of urine ECV isolated from various donors was very similar. Urine samples were freshly obtained from five different human donors and maintained at 4 °C until processing. $ZnSO_4$ (1 mM) was added to aliquots of the urine sample (20 mL) and ECV isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300. Notice that the concentration of ECV was very similar between donors

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Average ± SD
ECV ($\times 10^{10}$)/ml of urine	0.6	0.4	0.6	0.4	1	0.6 ± 0.1

The quality of urine ECV is affected by the storage conditions

For clinical applications, the storage of biological samples is key for their potential use as biomarkers. We focused on the storage of the urine sample prior to ECV isolation as a practical approach for the collection and storage of urine samples. Urine samples were collected from the same donor and divided into two groups in which $ZnSO_4$ was added before or after storage. Urine samples were maintained at 4 °C, − 20 °C or − 80 °C for 7 days until ECV isolation. When urine samples were stored at 4 °C, there was not a major difference if $ZnSO_4$ was added before or after storage, but always before isolation by differential centrifugation (Fig. 2). Moreover, we found that urine samples could be stored at 4 °C for 7 days without significantly affecting the quantity and quality of isolated ECV, based on vesicle number and electrophoretic pattern, respectively. However, freezing samples at either −20 °C or − 80 °C in absence of $ZnSO_4$ resulted in substantial contamination with UMOD even if $ZnSO_4$ was added after thawing and before vesicle isolation, which was not observed if $ZnSO_4$ was added before storage (Fig. 2).

Urine ECV present typical and new exosome markers

In order to characterize ECV isolated from urine, we analyzed the presence of typical exosome markers, such as CD9 and CD81, by Western blotting, using the same number of vesicles for each determination. The signal for CD9 was very strong whereas CD81 was detectable but weak (Fig. 3). In order to

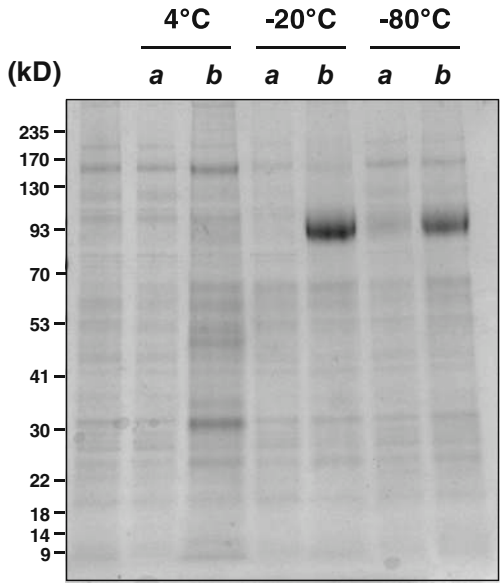

Fig. 2 Storage conditions affect the quality of urine ECV. Urine samples were freshly obtained from the same donor and stored at 4 °C, − 20 °C, or − 80 °C for 7 days in the presence of 1 mM $ZnSO_4$ added before (a) or after (b) storage. ECV were isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300. The same number of ECV (1×10^{11}) were added per lane and subjected to SDS-PAGE (4–12% polyacrylamide gradient gel) and visualized by Coomassie brilliant blue staining. The first lane corresponds to ECV isolated from freshly obtained (no storage) urine sample in the presence of 1 mM $ZnSO_4$

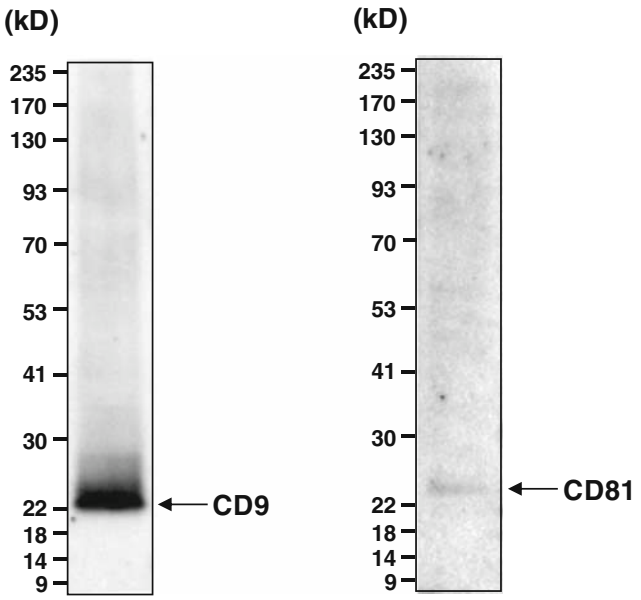

Fig. 3 Detection of CD9 and CD81 in urine ECV by Western blotting. Urine samples were freshly obtained from the same donor and stored at 4 °C in the presence of $ZnSO_4$ (1 mM). ECV were isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300. The same number of ECV (5×10^{10}) were added per lane and subjected to SDS-PAGE (4–12% polyacrylamide gradient gel), transfer onto nitrocellulose membranes and the presence of CD9 and CD81 detected by Western blotting

identify specific markers for human urine ECV, samples from three different donors were analyzed by mass spectroscopy. As expected, a large number of peptides corresponding to over 600 proteins were obtained. Some of the most common proteins identified in these samples are presented in Table 4. They include membrane proteins, actin, and some cytosolic proteins such as Hsc70 (HSPA8). The selection of specific markers for urine ECV among this large pool of proteins is very challenging. Thus, we decided to focus on a small group of proteins. We directed our attention to glycoproteins that are likely corresponding to membrane components of ECV. The use of lectins, specifically concanavalin A (Con A), is a great resource for the identification of glycoproteins (Sharon and Lis 2001). ECV were separated on SDS-PAGE, transferred to nitrocellulose membranes and probed with HRP-conjugated Con A, which is a lectin that recognizes high mannose and hybrid glycoconjugates. At least eight distinct bands were detected by Con A binding (Fig. 4). To demonstrate that Con A binding is specific, the Con A was co-incubated with α-methylmannoside (αMM) that is a specific competitor for the binding site of the lectin. Absolutely no signals were detected by competition with αMM (Fig. 4). Based on this result, we performed a pull-down experiment using Con A conjugated to sepharose beads and detergent solubilized ECV. After extensive washing, Con A immobilized glycoproteins were analyzed by mass spectroscopy. A reduced number of peptides were obtained, the majority corresponding to glycoconjugates (Table 5), with the exception of actin that was still present in the preparation, perhaps because this protein is associated with membrane glycoproteins. Among all the potential targets, we selected four glycoproteins, galectin 3 binding protein (G3-BP), dipeptidyl peptidase IV (CD26), vacuolar protein sorting factor 4A (VPS4A) and dipeptidase 1 (DPEP1), for further analysis. The presence of these

Table 4 Mass spectrometry analysis of urine ECV isolated from three donors. Urine samples were freshly obtained from three human donors and stored at 4 °C in the presence of $ZnSO_4$ (1 mM). ECV were isolated by differential centrifugation as described in Material and Methods and digested with trypsin. The resulting peptides were analyzed by HPLC coupled with tandem mass spectrometry. The list of the 25 most common proteins identified in all three samples is reported

Most common proteins identified by mass spect. analysis
Low density lipoprotein-related protein 2
Membrane alanine aminopeptidase precursor
Dipeptidylpeptidase IV / CD26
Membrane metallo-endopeptidase
Programmed cell death 6 interacting protein
Galectin 3 binding protein
Beta actin
IST1 homolog isoform a
Dipeptidase 1 (renal)
Solute carrier family 12 (sodium/chloride transporters), member 3
Uromodulin precursor
Albumin precursor
Heat shock 70kDa protein 8 isoform 1
X-prolyl aminopeptidase 2, membrane-bound
Maltase-glucomylase
Galactosidase, beta 1 isoform b
Galactosidase, beta 1 isoform a
Gamma-glutamyltransferase 1 precursor
Prominin 1
Annexin A11
Syntenin isoform 1
Ezrin
Calpain 7
ATP-binding cassette sub-family B member 1
Alpha-1-microglobulin/bikunin precursor

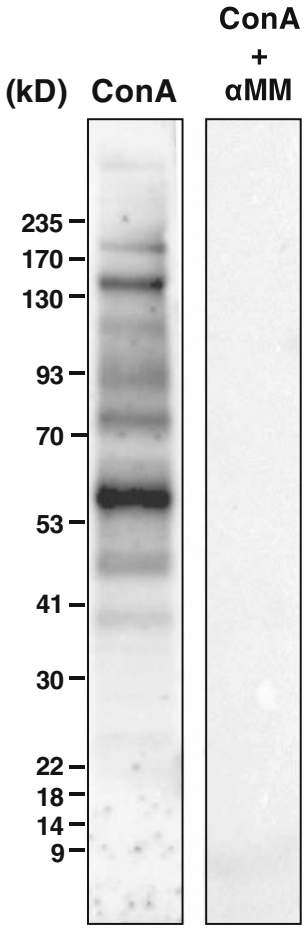

Fig. 4 Detection of glycoproteins from urine ECV by ConA staining. Urine samples were freshly obtained from the same donor and stored at 4 °C in the presence of $ZnSO_4$ (1 mM). ECV were isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300. The same number of ECV (5×10^{10}) were added per lane and subjected to SDS-PAGE (4–12% polyacrylamide gradient gel), transfer onto nitrocellulose membranes and the presence of glycoproteins were detected by ConA staining in the presence of absence of αMM used as a competitive inhibitor for ConA binding

glycoproteins in urine-derived ECV was evaluated by Western blotting. All of them gave a positive signal in the ECV preparation, although the signal for CD26 was relatively weak with respect to the others (Fig. 5).

Markers for urine ECV vary among different subjects

We were interested in evaluating the distribution of urine ECV markers in various subjects. Urine samples were obtained from four males and four female donors and freshly isolated using $ZnSO_4$ protocol. Equal amounts of isolated ECV were solubilized, separated by SDS-PAGE and analyzed by Western blotting. CD9 gave a very similar quantitative pattern among all subjects, suggesting that this glycoprotein could be used for the normalization of urine ECV samples (Fig. 6, top panel). DPEP1 and VPS4A were more variable among donor samples (Fig. 6). Finally, the electrophoretic pattern of G3-BP was highly variable among donors, in some a double band was very evident, whereas others only displayed a single band (Fig. 6).

Glucoconjugate pattern between different donors

Differences in the electrophoretic mobility among glycoproteins within urine ECV between different subjects could be due to variation in the pattern of glycosylation. To test this possibility, we analyzed the Con A binding electrophoretic profile of various subjects on isolated ECV. We found that the pattern of glycoproteins was very similar among donors with few bands that were different (Fig. 7). To further substantiate these observations, we analyzed the total sugar composition of urine ECV from three different donors. There were no major differences in the sugar composition of samples from these donors (Table 6A). Moreover, the sugar composition of the same donor obtained at different days was also very consistent (Table 6B). Thus, it is possible that the glycoprotein profile of urine ECV is not extremely variable.

Discussion

In recent years, ECV have emerged as an important mechanism for cellular communication during normal physiological conditions as well as stress and pathological situations (Thery et al. 2009; De Maio 2011). During these conditions, the composition of ECV is likely to change reflecting the physiological or pathological conditions of the cells from which they are derived. The recognition of normal or stress derived ECV is part of a surveillance mechanism for the detection of normal or stress ECV that has been named the Stress Observation System, and is likely involved in the activation of an appropriate cellular response directed at maintaining homeostasis (De Maio 2011). The fact that ECV could reflect a pathological condition has opened the possibility that they could be used as potential biomarkers of disease, also coined "liquid biopsies." The potential use of ECV as biomarkers has been enhanced for their relatively easy isolation and analysis from body fluids, such as blood, saliva, and urine. However, the characterization of ECV derived from bodily fluids has not been easy due to the natural heterogeneity of the ECV pool.

The isolation of ECV from urine samples appears to be an ideal system because samples are easily

Table 5 Mass spectrometry analysis of ConA affinity-precipitated ECV isolated from a donor. Urine samples were freshly obtained from a human donor and stored at 4 °C in the presence of $ZnSO_4$ (1 mM). ECV were isolated by differential centrifugation as described in Material and Methods, and affinity-precipitated by incubation with ConA-Sepharose beads for 16 h at 4 °C, followed by centrifugation. The resulting pellet after centrifugation was subjected to trypsin digestion. The digested peptides were analyzed by HPLC coupled with tandem mass spectrometry. A list of the most common glycoproteins identified is reported. Four candidates' glycoproteins were chosen for validation by Western blotting

List of proteins identified by mass spectrometry
uromodulin precursor
galectin 3 binding protein (G3-BP)
membrane alanine aminopeptidase precursor
actin, gamma 1 propeptide
beta actin
dipeptidylpeptidase IV/CD26
vacuolar protein sorting factor 4A (VPS4A)
dipeptidase 1 (renal) (DPEP1)
G protein-coupled receptor, family C, group 5, member B precursor
alpha-1-microglobulin/bikunin precursor
cathepsin A precursor
prominin 1
albumin precursor
retinoid X receptor, gamma isoform a

List of proteins selected for mass spec. validation	
Name	**Mol. Weight (kDa)**
galectin 3 binding protein (G3-BP)	90
dipeptidylpeptidase IV/CD26	110
vacuolar protein sorting factor 4A (VPS4A)	48.9
dipeptidase 1 (renal) (DPEP1)	45.6

obtained in large quantities, recurrently and with minimal intervention (Franzen et al. 2016). A potential problem with the analysis of ECV isolated from urine samples is the presence UMOD that cross-contaminates the vesicle preparation. UMOD makes large complexes that co-sediment with ECV when they are isolated by differential centrifugation. These large fibrotic complexes are part of the antibacterial function of UMOD directed at trapping microbes within these nets. UMOD contains a rich cysteine domain that apparently is responsible for the assembly of these complexes (Bokhove 2016). Indeed, the contamination of ECV with UMOD could wrongly add this glycoprotein as a component of urine ECV.

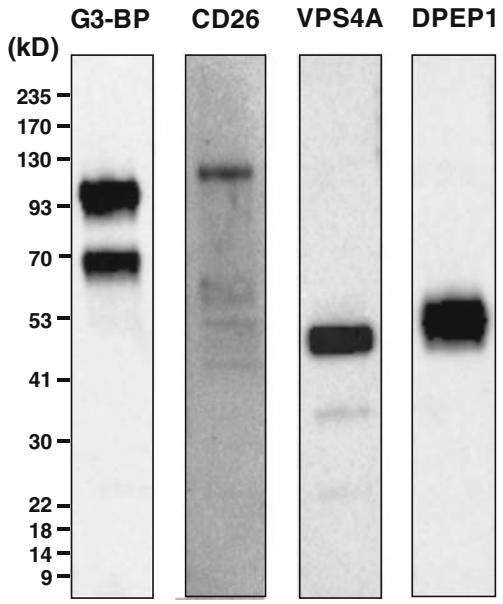

Fig. 5 Detection of G3-BP, CD26, VPS4A, and DPEP1 on urine ECV by Western blotting. Urine samples were freshly obtained from the same donor and stored at 4 °C in the presence of $ZnSO_4$ (1 mM). ECV were isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300. The same number of ECV (5×10^{10}) were added per lane and subjected to SDS-PAGE (4–12% polyacrylamide gradient gel), transfer onto nitrocellulose membranes and the presence of G3-BP, CD26, VPS4A and DPEP1 detected by Western blotting

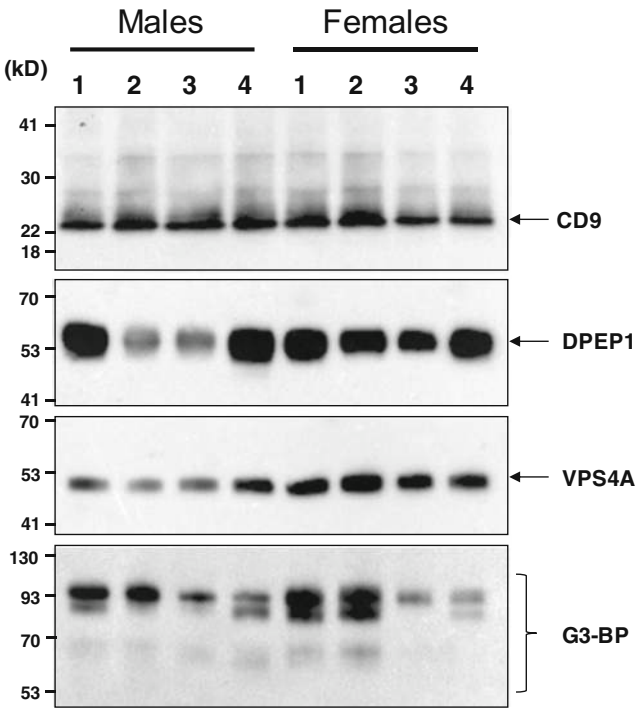

Fig. 6 Detection of CD9, DPEP1, VPS4A, and G3-BP on urine ECV isolated from various male and female donors by Western blotting. Urine samples were freshly obtained from the various donors of both sexes and stored at 4 °C in the presence of $ZnSO_4$ (1 mM). ECV were isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300. The same number of ECV (5×10^{10}) were added per lane and subjected to SDS-PAGE (4–12% polyacrylamide gradient gel), transfer onto nitrocellulose membranes and the presence of CD9, DPE1, VPS4A and G3-BP detected by Western blotting. Notice the consistency of CD9 content in urine ECV samples from various donors

Some investigators have approached the reduction of these complexes by the addition of DTT in great amounts to disrupt the cysteine-cysteine interactions, which is economically challenging and could potentially modify other proteins within ECV (Fernandez-Llama et al. 2010). Other studies have used a large dilution of the urine sample (Puhka et al. 2017), changes in the ionic strength of the urine solution, or the addition of calcium (Gamez-Valero et al. 2015). Although all of these approaches have produced positive results, there is still concern about the quality of the sample. We have developed a very simple approach for the isolation of UMOD-free ECV preparations by the addition of ZnSO4 in the urine sample prior to vesicle isolation. The $ZnSO_4$ protocol did not affect the size or number of isolated ECV. The cause of UMOD clearance after the addition of $ZnSO_4$ could be explained by changes in the oligomerization degree of the protein by either making bigger aggregates that sediment more rapidly after low-speed centrifugation or very small aggregates that remain in the supernatant after high-speed centrifugation. The effect of $ZnSO_4$ was not observed by the addition of other divalent anions. We have also shown that the addition of $ZnSO_4$ prior to storage of the sample improves the quality of the preparation, particularly if the urine sample will be stored at −20 °C or − 80 °C. Prior studies have established that storage at −80 °C preserved the sample better than −20 °C (Zhou et al. 2006). In addition, these authors recommended the use of protease inhibitors (Zhou et al. 2006), which we found unnecessary.

In spite of the tremendous potential for the use of urine ECV as liquid biopsies, they are likely derived from a variety of cell types under an array of pathophysiological conditions that are likely to complicate their characterization. Therefore, the standard isolation process does not differentiate the origin of the vesicles. This fact has affected the full characterization of the pool of ECV obtained from urine. Therefore, we have embarked on a careful characterization of human-derived ECV in spite of the fact that we are aware that it will be difficult to determine their cell of origin. Since the pool of proteins within ECV is very large, we focused on high

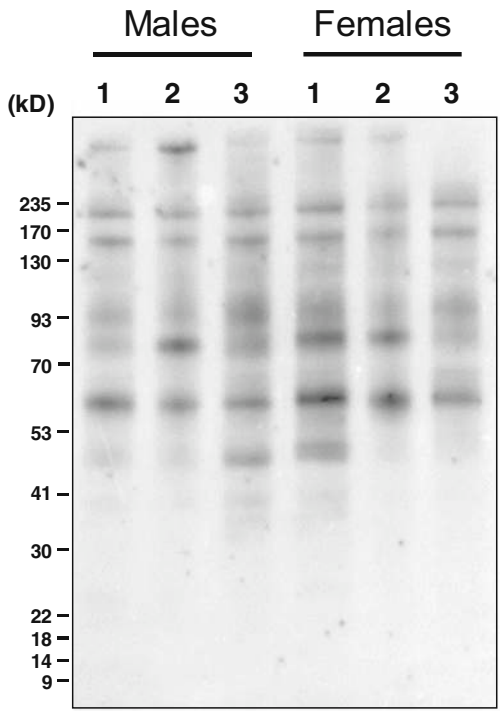

Fig. 7 Comparison of glycoprotein profiles by ConA staining from urine ECV samples isolated from male and female donors. Urine samples were freshly obtained from the same donor and stored at 4 °C in the presence of $ZnSO_4$ (1 mM). ECV were isolated by differential centrifugation as described in Material and Methods. 7ECV concentration was measured by NanoSight NS300. The same number of ECV (5×10^{10}) were added per lane and subjected to SDS-PAGE (4–12% polyacrylamide gradient gel), transfer onto nitrocellulose membranes and the presence of glycoproteins detected by ConA staining

mannose or hybrid glycoproteins aided by the use of Con A. Some of the new markers detected, DPEP1 and VPS4A, were quantitatively very similar among samples collected from healthy donors of both sexes. In addition, the traditional exosome marker, CD9 (Thery et al. 2009), was consistently quantitatively similar among donors suggesting that eventually they could be used as a control for the number of vesicles analyzed. Prior studies have identified an extensive number of proteins as part of urine ECV detected by LC-MS/MS (Pisitkun et al. 2004; Gonzales et al. 2009). Other markers that have been identified include CD24 (Keller et al. 2007), aquaporin (Oshikawa et al. 2016), α1-antitrypsin and H2B1K (Lin et al. 2016), and non-coding RNA (Khurana et al. 2017).

The protein that displayed major variability among donors was G3-BP. The level of this glycoprotein was different among subjects. In addition, the electrophoretic mobility of this glycoprotein was also different displaying single or double bands among samples. This difference in electrophoretic mobility could be due to proteolysis or differences in the glycosylation. However, human urine-derived ECV displays a complex pattern of glycosylation that was not very different among individuals. Indeed, the total sugar composition of human ECV was similar between donors. This observation opens the possibility of analyzing the glycoprotein pattern of human urine ECV for diagnostics.

Table 6 The sugar composition of urine ECV isolated from various donors was very similar. Urine samples were freshly obtained from five different human donors (A) or the same donor three different days (B). Samples were maintained at 4 °C until processing. $ZnSO_4$ (1 mM) was added to aliquots of the urine sample (20 mL) and ECV isolated by differential centrifugation as described in Material and Methods. ECV concentration was measured by NanoSight NS300. Sugar composition of each sample was obtained as described in Material and Methods and presented as a percentage of the total

A			
Name	Donor 1 (%)	Donor 2 (%)	Donor 3 (%)
Fucose	3.79	4.74	6.52
GalNH2	15.32	14.24	8.28
GlcNH2	32.84	32.76	34.98
Galactose	30.53	28.72	35.63
Glucose	13.9	14.83	11.74
Mannose	3.62	4.71	2.86
B			
Name	Day 1 (%)	Day 2 (%)	Day 3 (%)
Fucose	4.32	3.34	7.08
GalNH2	19.72	16.15	16.4
GlcNH2	26.41	28.82	29.53
Galactose	35.12	25.47	35.57
Glucose	12.88	24.51	9.93
Mannose	1.54	1.71	1.49

In summary, we have developed a very effective method for the isolation of highly pure human ECV derived from urine samples, which may be of help in further development of these vesicles as potential biomarkers. In addition, we have established conditions for sample collection and storage that may also contribute to their utility as liquid biopsies. Finally, we identified novel markers for these vesicles that we hope may serve in principle for the comparison between ECV isolated from healthy and disease conditions.

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Compliance with ethical standards

Competing interests The authors report no conflicts of interest.

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