see commentary on page 1043

CD24 is a marker of exosomes secreted into urine and amniotic fluid

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Exosomes are small membrane vesicles that are secreted from a variety of cell types into various body fluids including the blood and urine. These vesicles are thought to play a role in cell–cell interactions. CD24 is a small but extensively glycosylated protein linked to the cell surface by means of a glycosyl-phosphatidylinositol anchor. In this study we found that CD24 is present in membrane vesicles characterized as exosomes that were isolated from the urine of normal individuals. CD24 was expressed by both tubule cells and podocytes and treatment of the latter with a cholesterol-extracting agent, but not with a calcium ionophore, caused the release of CD24-containing exosomes. Using CD24 as a marker, we found exosomes in the urine of newborn infants and in the amniotic fluid of pregnant women with similar findings made in mice. Interestingly, studies with CD24 knockout mice showed that the exosomes are released from the fetus but not from the mother; however, exosome release was similar from both the knockout and the wild-type mice. This indicates that CD24 is not essential for exosome formation or release but may be a convenient exosome marker. Our studies suggest that exosomal secretion from the embryonic kidney could play a biological role at the fetal–maternal interphase.

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Exosomes are membrane vesicles with a size of 40–100 nm that are released from a variety of different cell types including tumor cells, red blood cells, platelets, lymphocytes, and dendritic cells.[1,2] Exosomes are formed by invagination and budding from the limiting membrane of late endosomes.[3] They accumulate in cytosolic multivesicular bodies from where they are released by fusion with the plasma membrane.[3] The process of vesicle shedding is particularly active in proliferating cells, such as cancer cells, where the release can occur continuously.[4] Depending on the cellular origin, exosomes recruit various cellular proteins that can be different from the plasma membrane including major histocompatibility complex molecules, tetraspanins, adhesion molecules, and metalloproteinases.[1,2,5] Exosomes have been found in various body fluids such as blood plasma,[6] malignant ascites,[7] and urine.[8] It has been proposed that under physiological conditions exosomes could play a role in cell–cell interactions.[9,10] When released from tumor cells, exosomes can promote invasion and migration.[4,11]

CD24 is a membrane glycoprotein with unusual lipid-like, organic-solvent soluble and heat-stable features.[12] These characteristics are due to a small protein (mouse CD24 has 27 amino acids, human CD24 consists of 31 amino acids), extensive core N- or O-linked glycosylation, and the linkage to the cell membrane via a glycosyl-phosphatidylinositol (GPI) anchor.[12] In humans, CD24 is expressed by hematopoietic subpopulations of B- and T lymphocytes but also on granulocytes and many carcinomas (for review see Kristiansen *et al.*[13]). Due to a GPI anchor, CD24 is exclusively localized in lipid rafts.[14] GPI-anchored proteins are highly enriched in exosomes.[2,3,15]

Exosomes in human urine have been studied as a novel diagnostic means for kidney-related diseases.[8] By marker analysis it was shown that exosomes are derived from different kidney regions.[8] Although exosomal analysis in human urine may offer novel diagnostic insights, a central question remains unaddressed: why are exosomes present in urine and what is the biological role of the exosomal secretion mechanism? Several possibilities could be envisaged: (i) exosome secretion from the kidney is important for

the detoxification of the organism; (ii) the exosome secretion may be part of a protein and lipid degradation pathway in the kidney; and (iii) exosome secretion is a primordial mechanism in adults, but plays a role during fetal development when the kidney of the infant releases exosomes into the amniotic fluid. The latter hypothesis leads to explicit predictions that can be tested directly. To do so, we have analyzed exosomes in the urine using the GPI-linked protein CD24 as a marker. We find that CD24 is indeed a marker for urinary exosomes and is present in the urine of men and mice. The exosomes are likely derived from cells that face the urinary space including those that express CD24. Importantly, we observed CD24-positive exosomes already in the urine of newborn and in the amniotic fluid during pregnancy. These findings suggest that exosomal secretion from the kidney is indeed an early event during embryogenesis, continues for lifetime and is preserved over species barriers.

RESULTS

CD24-containing exosomes in urine of adults

We observed that the urine of healthy individuals contained significant amounts of CD24 as detected by Western blot analysis (Figure 1a, left panel). To find out whether CD24 was in a soluble or membrane-bound form, we subjected urine to ultracentrifugation. The analysis of depleted supernatant and pelleted vesicles indicated that the CD24 activity was present in the 100 000 g pellet (Figure 1a, middle panel) but was not detected in the cleared supernatant (Figure 1a, right panel). The analyses were confirmed for $n > 30$ individuals and representative examples are shown in Figure 1b. Some individuals donated urine samples during the course of the day but only little variation in the amount of CD24-positive vesicles were noted. An average of 7.21 μg/ml (range 1.6–13 μg/ml, $n = 11$) of protein was recovered per urine sample. Of approximately 120 samples analyzed, only $< 5\%$ were negative or low for CD24-vesicles content for reasons presently unknown (data not shown).

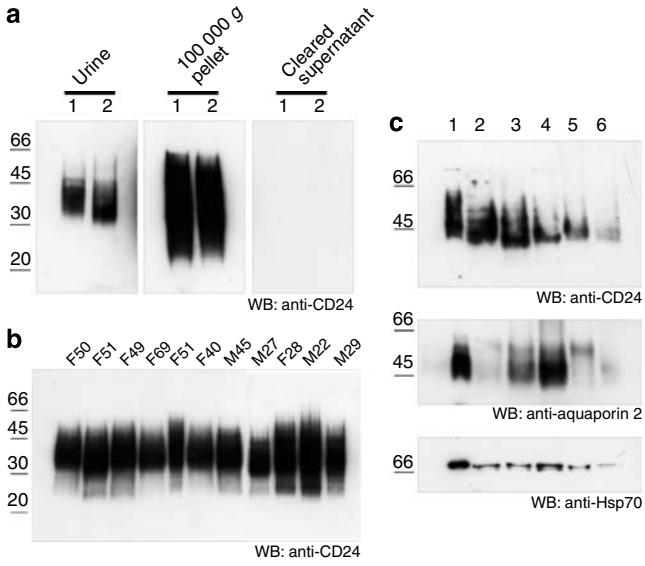

Figure 1 | Identification of CD24-containing urinary vesicles. (**a**) Two representative urine samples were either analyzed after trichloroacetic acid precipitation (left panel) or after ultracentrifugation at 100 000 g for 3 h. Pelleted vesicles (middle panel) or cleared supernatants (right panel) were analyzed by SDS-polyacrylamide gel electrophoresis and Western blot analysis using mAb SWA11 to CD24 and enhanced chemiluminescence detection. (**b**) A representative panel of CD24 containing urinary vesicles is shown. Membrane vesicles were obtained after ultracentrifugation of urine and analyzed by Western blot analysis using mAb SWA11 to CD24 and enhanced chemiluminescence detection. F50: female 50 years old; M27: male 27 years old. (**c**) Membrane vesicles were analyzed for CD24, aquaporin-2, and HSP70 by Western blot analysis using respective antibodies.

We suspected that the pelleted material might represent membrane vesicles in the form of exosomes. Indeed, vesicles showed positive reactivity with antibodies to HSP70 (Figure 1c), CD9, and annexin-I (see below). These antigens are enriched in exosomes.[1,2] All samples were positive for the kidney marker aquaporin-2 that is known to occur in urinary exosomes (Figure 1c).

Exosomes are released from cells by fusion of multivesicular bodies with the plasma membrane and have an orientation of cell surface antigens similar to the cell.[1–3] To determine the orientation of antigens, we immobilized urinary vesicles onto latex beads and carried out fluorescence-activated cell sorter (FACS) analysis. Vesicles were readily stained with antibodies to CD24 (Figure 2a). As detected by electronmicroscopy, the vesicles were of variable size ranging predominantly from 20 to 100 nm (Figure 2c).

We carried out sucrose density centrifugation, in combination with Western blot analysis to further characterize the released membrane vesicles. Fractions in the middle part of the gradient (density 1.05–1.15 mg/ml) representing exosomes were positive for CD24 and annexin-I (Figure 2b). We concluded that the urinary vesicles containing CD24 had all characteristics of being exosomes.

Origin of CD24-positive exosomes

Marker analysis in adults has shown earlier that urinary vesicles are derived from distinct regions of the kidney.[8] Using immunohistochemistry on serial sections, we investigated the expression of CD24 in human kidney in combination with defined cell subset markers. CD24 expression was observed in the aquaporin-2-positive collecting duct (Figure 3a), in calbinin-positive distal tubular cells (Figure 3b), and the Tamm–Horsfall-positive thick ascending limp of the Henle loop (Figure 3c). To examine whether podocytes were CD24-positive, we employed two color staining using monoclonal antibody (mAb) WT1 against a podocyte-specific nuclear antigen and SWA11 for CD24. As shown in Figure 3d, double-positive cells, but also CD24-single-positive cells, were clearly detected in the glomeruli.

We also examined the expression of CD24 in cultured cell lines derived from the human kidney. We observed CD24 expression by FACS analysis in immortalized human podocytes (HPCs), the HK-2 human proximal tubular epithelial cell line, and in human mesangial cells (Figure 3c).

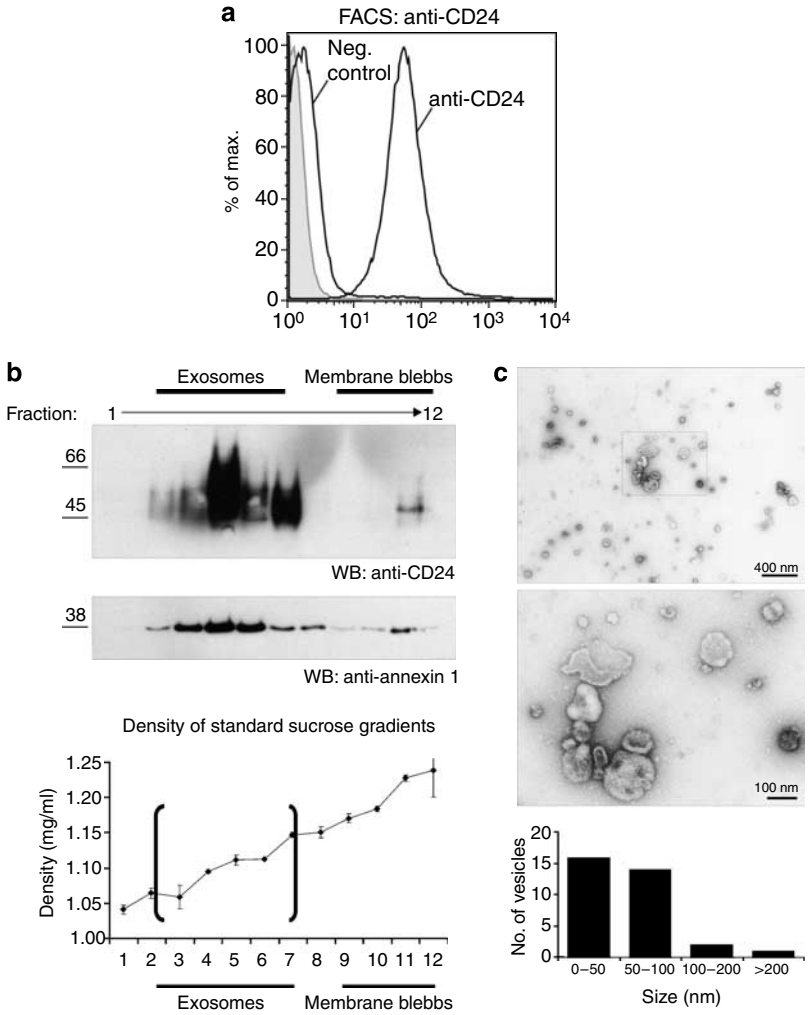

Figure 2 | Characterization of urinary vesicles. (**a**) Exosomes isolated from urine were adsorbed to latex beads and stained for CD24 followed by phycoerythrin-conjugated goat anti-mouse immunoglobulin G and FACS analysis. The negative control represent beads stained with the secondary antibody only. The gray curve represents the autofluorescence of unstained beads. (**b**) Exosomes were subjected to sucrose density centrifugation and the collected fractions from the gradient were analyzed by Western blot analysis with the indicated antibodies to CD24 and the exosomal marker protein annexin-I followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The density of the gradient fractions is depicted in the graph below. (**c**) Electronmicroscopy pictures and size distribution analysis of urinary vesicles.

These findings suggested that CD24-containing exosomes in the urine were likely derived from the kidney.

Regulated exosomal secretion in HPCs

We previously reported that the secretion of exosomes is constitutive but can be significantly enhanced in human tumor cells by exposure to substances such as the Ca-ionophore ionomycin, 4-aminophenylmercuric acetate, methyl-β-cyclodextrin (MCD), or cisplatin.[16,17] We treated differentiated HPC with ionomycin (10 mg/ml), MCD (10 μg/ml), or cisplatin (1 μg/ml) and analyzed the amount of exosomes released. Only MCD significantly augmented the release of CD24-containing exosomes (Figure 4). The vesicles contained the exosomal marker HSP70 but not annexin-I. These results suggest that the exosomal release can be regulated in HPC.

CD24 exosomes are present in the urine of newborn and amniotic fluid

We observed that the presence of CD24-containing exosomes in urine samples was not restricted to adults but could also be found in infants. As shown in Figure 5a, the urine of newborns contained significant amounts of CD24-positive exosomes that were both positive for aquaporin-2 and HSP70.

The renal system of the fetus contributes to the production of amniotic fluid. To analyze whether exosomes from the fetus were already released early in the pregnancy, we examined amniotic fluid collected at approximately week 16 of gestation for routine amniocentesis. Indeed, in all ($n = 4$) amniotic fluids tested, exosomes containing CD24 and CD9 were detected (Figure 5b). To analyze whether the CD24-positive exosomes were derived from the fetus, we

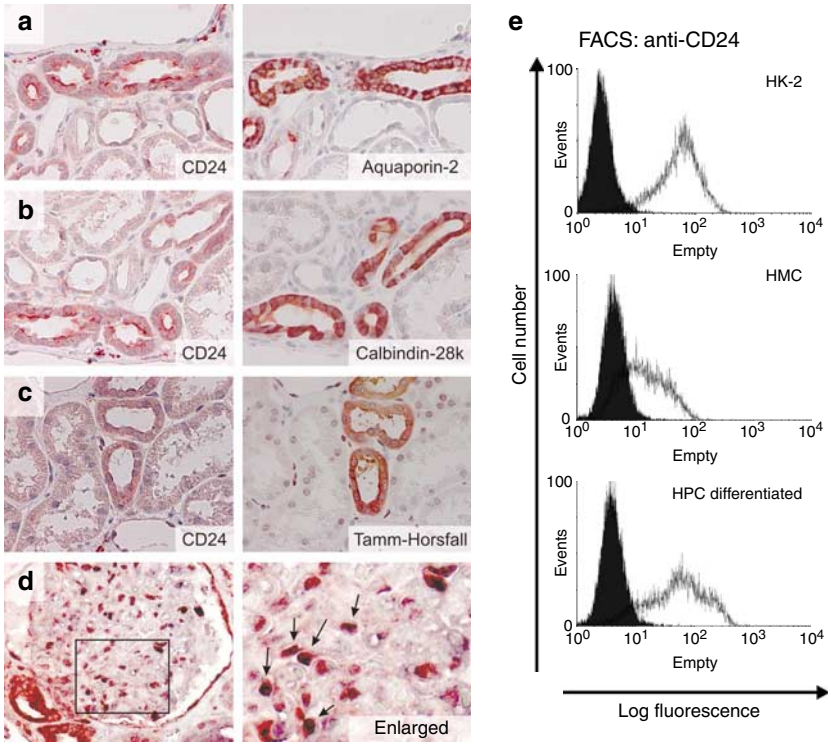

Figure 3 | Expression of CD24 in adult kidney. Staining was carried out in combination with cell type-specific markers in serial sections. (**a**) Analysis of CD24 and aquaporin-2 (collecting duct); (**b**) CD24 and calbindin (distal tubules); (**c**) CD24 and Tamm–Horsfall (thick ascending limp of Henle loop). (**d**) Analysis of CD24 staining in glomeruli using the podocyte marker WT1 (blue) and SWA11 (red). Note the enlarged picture (right) marking double-stained cells with arrows. (**e**) Cultured human tubular cells (HK-2), human mesangial cells, or differentiated HPC were stained with mAb to CD24 followed by phycoerythrin-conjugated goat anti-mouse immunoglobulin G and FACS analysis.

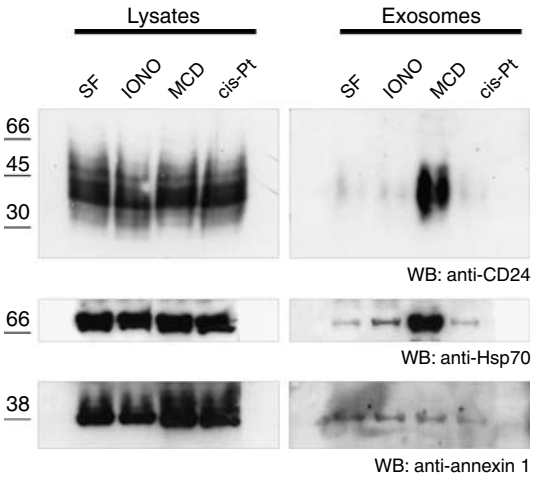

Figure 4 | Regulation of exosome secretion. Cultured HPC (differentiated) were treated with ionomycin (1 μM), MCD (10 mM), or cisplatin (15 μM) for 2 h. Secreted membrane vesicles were harvested and cells were lysed in lysis buffer. Lysates and isolated vesicles were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with the indicated antibodies to CD24 and the exosomal marker proteins, HSP70 and annexin-I. Note the low level of constitutive vesicle secretion that is augmented by MCD.

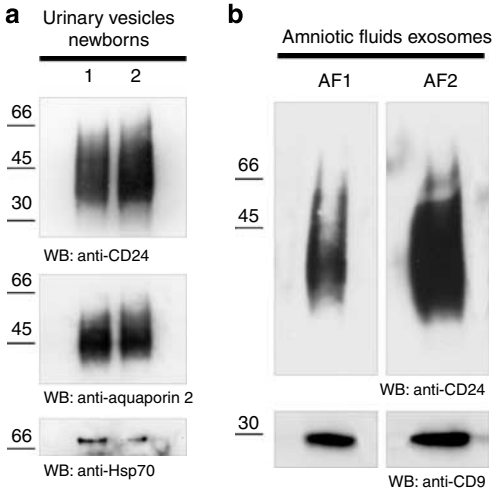

Figure 5 | CD24-containing exosomes in the urine from newborn and amniotic fluid. (**a**) Exosomes isolated from the urine of newborn were analyzed for CD24, aquaporin-2, and HSP70 by Western blot analysis. Two examples from $n = 5$ samples with similar results are shown. (**b**) Exosomes isolated from the amniotic fluid of women undergoing amnioncentesis were analyzed for CD24 and CD9 by Western blot analysis. Two examples from $n = 4$ with similar results are shown.

used a refined sucrose gradient capable of separating distinct exosome subpopulations on the basis of buoyant density (see Figure 6a). The analysis revealed two populations of exosomes: a light fraction floating on top of the gradient (1.08–1.11 mg/ml) contained CD24, the kidney marker aquaporin-2; and annexin-I, a more dense fraction (1.11–1.14 mg/ml)

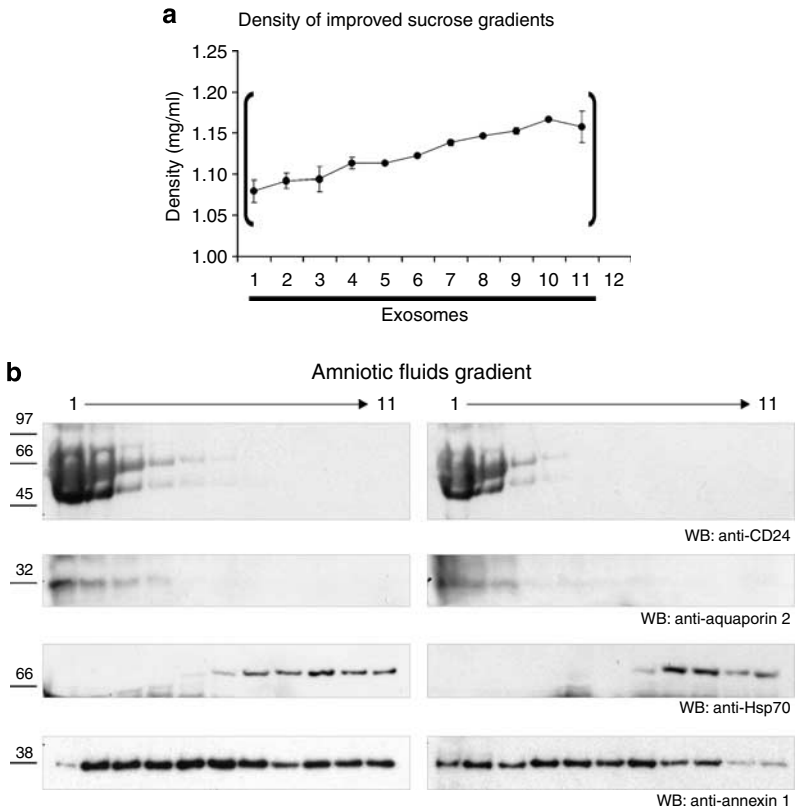

Figure 6 | Sucrose gradient analysis of exosomes from amniotic fluid. (**a**) Density profile of an expanded sucrose gradient used for the analysis of human amniotic fluid. Note the expansion of exosomal fractions compared to the gradient shown in Figure 2b. (**b**) Exosomes isolated from the amniotic fluid of women undergoing amniocentesis were analyzed for the indicated marker proteins by Western blot analysis.

containing only HSP70 and annexin-I (Figure 6b). These results suggested that the CD24-positive exosomes were likely derived from the kidney of the fetus.

CD24 exosomes in the urine and amniotic fluid of mice

We wished to examine whether or not CD24 was an essential component for vesicle formation. The human CD24 ortholog in the mouse is heat-stable antigen (HSA/mouse CD24).[12] We compared CD24+/+ mice and CD24−/− mice for vesicle release into the urine. Urinary vesicles were collected and analyzed by Western blot. As shown in Figure 7a, in CD24+/+ animals, exosomes were present in urine containing CD24 and CD9, whereas in CD24−/− animals the CD24 signal was missing. Given the identical band intensity of CD9 (Figure 7a) and the identical protein content of exosomes (data not shown), we concluded that CD24 was not essential for the process of exosome secretion.

We also investigated whether CD24-positive exosomes were present in mouse amniotic fluid. As shown in Figure 7b and similar to the results in humans, CD24-positive exosomes were clearly detected in amniotic fluid of CD24+/+ mice but were absent in CD24−/− mice. To unequivocally prove that the exosomes were derived from the fetus and not from the mother, we crossed CD24−/− female mice with wild-type male. This will give CD24+/− offspring in a CD24−/− mother. As shown in Figure 7b, the amniotic fluid contained CD24-positive exosomes, suggesting their origin from the fetus.

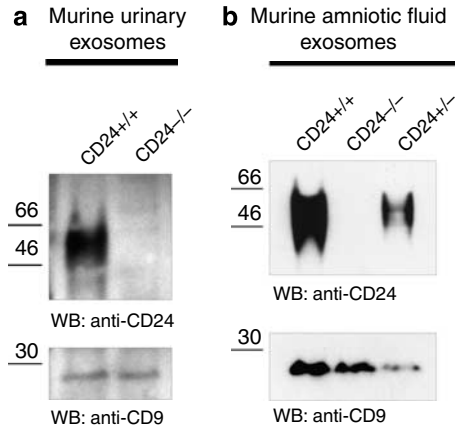

Figure 7 | CD24-containing exosomes in the urine and amniotic fluid of mice. (**a**) Urine from C57B6 mice (CD24+/+) and CD24−/− mice were analyzed for CD24 and CD9 by Western blot analysis. Note that the CD9 signals are similar in both CD24+/+ and CD24−/− samples. (**b**) Amniotic fluid from pregnant CD24+/+, CD24−/−, or CD24−/− female mice mated with CD24+/+ males (CD24+/−) was analyzed for CD24 and CD9 by Western blot analysis.

DISCUSSION

Membrane vesicles in the form of exosomes are released from normal and cancer cells. In this study we have analyzed vesicles present in human urine containing the GPI-anchored protein CD24. We find that (i) CD24 vesicles are exosomes and are most likely derived from the kidney; (ii) CD24 exosomes are present in the urine of newborn and in the amniotic fluid of pregnant women and these vesicles are derived from the kidney of the infant; (iii) CD24 exosomes are also found in the urine and amniotic fluid of mice; (iv) in CD24−/− mice the secretion of vesicles into the urine is not affected suggesting that CD24 is not essential in the vesicle-release process. Our results suggest that exosomal secretion into the urine and amniotic fluid is a biological process that is conserved over species barriers and could play a role in embryogenesis as well as in later life.

Clinical proteomics are extensively used to explore urinary biomarkers.[18] A surprisingly high abundance of membrane-derived proteins was noticed in a recent study on total urinary proteins.[19] This publication did not address the fact that urine contains both soluble proteins and urinary exosomes that contain plasma membrane proteins derived from the kidney and the urinary tract. Pisitkun *et al.*[8] analyzed urinary exosomes using immunoelectron microscopy and proteomic analysis. They noticed that released exosomes were small (<100 nm) with variable size and were oriented 'cytoplasmic-side inward'. The authors also identified by liquid chromatography-tandem mass spectrometry 295 proteins including proteins known to be involved in endosomal trafficking and exosome formation. In addition, there were cytoskeletal and motor proteins, many integral membrane proteins as well as eight GPI-anchored proteins.[8] The cytosolic proteins identified were presumably trapped in the lumina of forming exosomes. In our study the density and orientation of urinary exosomes were similar to that reported by Pisitkun *et al.*[8] For unknown reasons the study by Pisitkun *et al.*[8] did not identify CD24 as a component of urinary exosomes. This may be due to the unique feature of human CD24 being a highly glycosylated mucin with a protein core of only 31 amino acids. These characteristics may have hampered the detection by proteomic analysis. Nevertheless, the recruitment of CD24 from the plasma membrane into exosomes is not surprising as GPI-anchored proteins are known to be recruited into exosomes.[15]

Although the proteomic analysis of urinary exosomes can provide important information about the pathophysiology of the kidney, the biological relevance of the exosome secretion process remains unaddressed. Using CD24 as a marker, we found that the presence of exosomes in the urine of adults is a constant feature with very little variation between individuals and during the course of the day. Importantly, we found that exosomes are present in the urine of newborns and can be detected in the amniotic fluid of pregnant women undergoing amniocentesis, that is, as early as 14–18 weeks after gestation. We used a separation technique on the basis of buoyant density to further dissect the exosomes present in amniotic fluid. We observed a fraction of exosomes that were lighter in density and contained the kidney marker aquaporin-2, CD24, and annexin-I. These exosomes were most likely derived from the fetus. A second fraction of exosomes contained annexin-I and HSP70 but not CD24. It is possible that this subfraction originated from the mother. The fetal origin of CD24 exosomes was confirmed by genetic experiments in CD24 knockout mice. The presence of fetal exosomes in the amniotic fluid is perhaps not so surprising in the light of the know role of the fetal kidney in the production of amniotic fluid. Nevertheless, these observations raise questions about the biological function of urinary exosomes.

One possibility is that exosome secretion from the kidney is important for the detoxification of the organism. Indeed, recent data in tumors have shown that exosomes or microvesicles with similar characteristics may cargo lipid-solving drugs from tumors. An enhanced exosomal export of cisplatin and abnormal lysosomal trafficking of the cisplatin transporter was observed in drug-resistant human ovarian carcinoma cells.[20] Experiments with doxorubicin and other small molecules confirmed drug accumulation and expulsion in shed vesicles.[21] Moreover, there was an accumulation of drugs in membrane domains from which vesicles originated.[21] It was proposed that by virtue of their hydrophobic character, these molecules can be shuttled to the plasma membrane via vesicle-mediated traffic, for final elimination in complex with shed vesicles.[21] If this was the case also in an organism, then exosomes could cargo lipophilic substances via the blood stream that are meant for secretion via the urine, such as steroids or other substances that are not water-soluble. In fact, exosomes are present in blood plasma[8] and this has been confirmed in our own studies (C Rupp, unpublished data).

Exosomes were also implicated in the regulation of immune responses.[5,22] A role for exosomes in the modulation of T-cell signaling during pregnancy has been suggested.[23] Exosomes obtained from the serum of pregnant women could suppress the expression of important T-cell signaling components including CD3-ζ and JAK3. This suppression was correlated with exosome-associated Fas ligand, and a striking difference was noted between women delivering at term and those delivering pre-term.[23] This study assumed a maternal origin of the isolated exosomes and did not take a possible fetal origin into account. Our study shows for the first time that fetal exosomes are present in amniotic fluid. As fetal cells are known to occur in the maternal blood circulation, we cannot rule out the possibility that exosomes from the fetus are present in the bloodstream of the mother. Interestingly, exosomes display both immunostimulatory but also immunosuppressive properties.[22] For example, exosomes produced by mouse dendritic cells pulsed with tumor peptide are able to mediate the rejection of established tumors.[24] These antitumor effects were antigen-specific and were associated with the activity of T cells. Direct stimulation of T cells by membrane vesicles from antigen-presenting cells

has also been reported.[25] Conversely, it has been suggested that intestinal epithelial cells, T-cell tumors, and melanoma cells can secrete exosomes capable of inducing antigen-specific tolerance and Fas ligand-mediated T-cell apoptosis.[26] Exosomes derived from tumor cells were also shown to inhibit the cytolytic activity of natural killer (NK) cells by reducing the levels of perforin in NK cells, a molecule that is essential for target cell lysis.[27] It is of interest to note that a major fraction of cells in the amniotic fluid are decidual NK cells that play an important role in trophoblast invasion and the vascularization of the uterus.[28] In contrast to peripheral NK cells, decidual NK cells secrete a variety of cytokines but are non-cytolytic.[28] It is tempting to speculate that exosomes may be involved in the inhibition of cytolytic activity of decidual NK cells similar to the tumor situation. Finally, a recent study has shown that exosomes can transport mRNA and microRNAs and represent a novel mechanism of genetic exchange between cell.[29] For urinary exosomes future work will be required to analyze closely a possible role for nephron function. Also the immunological properties of fetal exosomes at the maternal–fetal interphase need to be investigated further.

In summary, our results describe the phenomenon of exosome secretion in the human renal system. The data provided on mouse CD24 exosomes in the urine and amniotic fluid highlight that the secretion process is conserved over species borders. This similarity now allows experimental studies in animals to further unravel the biological role of exosome secretion.

MATERIALS AND METHODS

Chemicals and antibodies

Antibodies to human CD24 (SWA11) and CD9 (TS9) were described.[7,14] The mAbs to HSP70, annexin-I, and mouse CD9 were from BD-Transduction (Heidelberg, Germany). The antibodies to aquaporin-2 and calbindin 28K were from Sigma (Taufkirchen, Germany), and the antibody to WT1 (sc-846) was obtained from Santa Cruz (Heidelberg, Germany). The Tamm–Horsfall antibody was from Biotrend (Köln, Germany). The antibodies to mouse CD24 are described before.[30]

Cell culture and animals

Human conditionally immortalized podocytes (HPC) were isolated and cultivated as described previously.[31] Cells were grown in flasks either at the permissive temperature of 32°C (in 5% CO_2) to promote cell propagation as a cobblestone phenotype (undifferentiated) or at the nonpermissive temperature of 37°C (in 5% CO_2) to inactivate the SV40 T antigen and to allow the cells to differentiate. Before stimulation, cells were incubated for 16 h in RPMI-1640 medium, supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin. HK-2 human proximal tubular epithelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human mesangial cells were kindly provided by Professor Dr Radeke (Department of Pharmacology, University of Frankfurt). Briefly cells were grown in RPMI-1640 medium supplemented with nonessential amino acids (1 ml/dl), L-glutamine (2 mM), sodium pyruvate (1 mM), transferrin (5 mg/ml), insulin (125 U/ml), and fetal calf serum (10%). Cells were grown at 37°C in 5% CO_2. For passaging, human mesangial cell were detached by trypsin-EDTA and split into 1:3. CD24−/− mice (C57BL6) were originally obtained from Dr Peter Nielsen (Max-Planck Institute for Immunobiology, Freiburg, Germany) and maintained at the animal house of the DKFZ. Normal age-matched C57BL6 mice served as control.

Isolation of vesicles from urine

Urinary samples were obtained from healthy volunteers (male and female age range 22–57 years) and were collected during the course of a working day. Urine samples (50 ml from adults; 5 ml from newborn) were centrifuged for 10 min at 300 *g* and 20 min at 10 000 *g* to remove cellular debris. Membrane vesicles were collected by centrifugation at 100 000 *g* for 2 or 18 h at 4°C using a Beckman SW 40 rotor. Vesicles were directly dissolved in sodium dodecyl sulfate (SDS) sample buffer in relation to the original volume or processed further for gradient centrifugation (see below). Mouse urine was collected in metabolic cages. Drinking water contained 16% sucrose and urine (approximately 5 ml/16 h) was collected on ice. Mouse urine was processed as described above.

Isolation of vesicles from amniotic fluid

Human amniotic fluid ($n = 4$) was collected for routine amniocentesis and analyzed after removal of cells. Membrane vesicles were obtained after differential centrifugation as described above. Amniotic fluids from mice (approximately 100–500 μl) were collected on day 18 of gestation and were spun in an Eppendorf centrifuge at maximum speed for 20 min. The cell debris-free supernatant was diluted into 4 ml of phosphate-buffered saline, and vesicles were pelleted by overnight centrifugation using a Beckman SW60 rotor at 120 000 g. The vesicle pellet was taken up in SDS sample buffer.

Sucrose density gradient fractionation

Vesicles isolated from ascites were loaded onto the top of a step gradient comprising layers of 2, 1.3, 1.16, 0.8, 0.5, and 0.25 M sucrose as described.[18] The gradients were centrifuged for 2.5 h at 100 000 g in a Beckman SW40 rotor. Twelve 1 ml fractions were collected from the top of the gradient and precipitated by chloroform/methanol or acetone as described.[18] A modified sucrose gradient was designed to allow a more refined analysis of exosomes according to differences in the density.

FACS analysis

FACS analysis of isolated vesicles was carried out after adsorbing of isolated vesicles to 4 nm (Surfactant-free) aldehyde-sulfate latex beads (Interfacial Dynamics Corp, Portland, OR, USA) as described.[32] The staining of beads or cultivated cells with mAbs and phycoerythrin-conjugated secondary antibodies has been described.[14,18] Stained beads or cells were analyzed with a FACScan using Cellquest software (Becton Dickinson, Heidelberg, Germany).

Immunohistochemistry

This was described previously.[33] Briefly, paraffin tissue sections were deparaffinized and antigens were retrieved for 20 min by incubating the tissue sections in 0.01 M sodium citrate buffer, pH 6.0, in a microwave (500 W). The intrinsic peroxidase activity was abolished by pretreatment in 3% H_2O_2 in methanol for 30 min at RT. After blocking, the sections were incubated with an Avidin/Biotin Blocking kit (Linaris, Wertheim, Germany) following the manufacturer's protocol. The sections were incubated overnight with

CD24 antibody (SWA11 hybridoma supernatant diluted 1:5 in blocking reagent). After washing the slides, the Universal Quick kit (Linaris) was used for staining. As substrate, the AEC Substrate kit from BioGenex (San Ramon, CA, USA) was used to detect immune complexes. To counterstain the sections, slides were incubated with hematoxylin (Roth, Karlsruhe, Germany). For double-staining CD24 with WT1, a specific podocyte marker, sections were not counterstained with hematoxylin. WT1 antibody was diluted 1:250 in blocking reagent (1% bovine serum albumin in phosphate-buffered saline) and incubated overnight. After washing the sections, the alkaline phosphatase-conjugated goat-anti rabbit antibody (1:250 diluted in blocking reagent) was added for 1 h at 37°C. Endogenous alkaline phosphatase was blocked with levamisole (Sigma) before adding the substrate. The immune complex was detected adding the Sigma fast™ BCIP/NBT (Sigma) substrate tablet until a blue color developed.

Biochemical analysis

SDS-polyacrylamide gel electrophoresis under reducing conditions and transfer of proteins to an immobilon membrane using semi-dry blotting has been described.[14,18] After blocking with 5% skim milk in Tris-buffered saline, the blots were developed with the respective primary antibody followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection.

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