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Exosomal transfer from human renal proximal tubule cells to distal tubule and collecting duct cells

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ABSTRACT

Objectives: Exosomes are 50–90 nm extracellular membrane particles that may mediate trans-cellular communication between cells and tissues. We have reported that human urinary exosomes contain miRNA that are biomarkers for salt sensitivity and inverse salt sensitivity of blood pressure. This study examines exosomal transfer between cultured human renal proximal tubule cells (RPTCs) and from RPTCs to human distal tubule and collecting duct cells.

Design and methods: For RPTC-to-RPTC exosomal transfer, we utilized 5 RPTC lines producing exosomes that were fluorescently labeled with exosomal-specific markers CD63-EGFP or CD9-RFP. Transfer between RPTCs was demonstrated by co-culturing CD63-EGFP and CD9-RFP stable clones and performing live confocal microscopy. For RPTC-to-distal segment exosomal transfer, we utilized 5 distal tubule and 3 collecting duct immortalized cell lines.

Results: Time-lapse videos revealed unique proximal tubule cellular uptake patterns for exosomes and eventual accumulation into the multivesicular body. Using culture supernatant containing exosomes from 3 CD9-RFP and 2 CD63-EGFP RPTC cell lines, all 5 distal tubule cell lines and all 3 collecting duct cell lines showed exosomal uptake as measured by microplate fluorometry. Furthermore, we found that RPTCs stimulated with fenoldopam (dopamine receptor agonist) had increased production of exosomes, which upon transfer to distal tubule and collecting duct cells, reduced the basal reactive oxygen species (ROS) production rates in those recipient cells.

Conclusion: Due to the complex diversity of exosomal contents, this proximal-to-distal vesicular inter-nephron transfer may represent a previously unrecognized trans-renal communication system.

Introduction

During normal cellular metabolism, exosomes (50–90 nm plasma membrane particles) are secreted from specific membrane areas and contain selectively packaged mRNA, proteins, miRNA and other cellular contents [1]. Apparently, exosomal signaling represents a higher order of inter-cellular communication as compared to autocrine or paracrine signaling since exosomes can change according to alterations in cellular metabolism and influence specific cellular structures and processes in the recipient cells. For example, hypoxia can increase the concentration of caveolin-1 in plasma exosomes in patients with glioblastoma [2].

Exosomal signaling in the lumen of the renal nephron is unique in that plasma exosomes cannot cross the glomerular filtration apparatus and thus intra-nephron exosomes originate exclusively from the luminal epithelial cells [3]. The regulation of renal function may be influenced by exosomal contents. Protein epitopes exposed on the surface of exosomes may interact with extracellular targets on cells in contact with the tubular lumen [4]. Some of the targeting molecules on the exosomes and docking molecules on the recipient cells are known in certain immune cell interactions [5]; however, in the context of the human nephron, neither the targeting molecules nor the docking molecules have yet been identified. In addition, exosomes isolated from human urine contain mRNA from every segment of the renal tubule [6]. Selective knockout of a collecting duct-selective marker (V-VATPase-B1) in mice eliminated this marker from exosomes in their urine, demonstrating that exosomes may contain nephron-specific contents and accurately represent the expression levels of the donor cells. One report of exosomal stimulation resulting in a change of phenotype in the kidney involved a single tubule segment and the use of desmopressin to stimulate an increase in aquaporin 2 content of secreted exosomes from cortical collecting duct cells, which then increased both aquaporin 2 expression and water transport in another batch of cortical collecting duct cells [7].

Since inter-nephron segment exosomal signaling has not been demonstrated in human kidney, we examined both intra- and inter-nephron segment exosomal signaling in cells derived from proximal and distal tubules as well as collecting duct. Renal tubular reactive

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oxygen species (ROS) have been linked to hypertension in experimental animals [8,9]. Furthermore, dopamine and angiotensin II (Ang II) oppose each other in regulating renal sodium excretion, which is a mechanism by which sodium homeostasis and hence blood pressure is maintained [8,10]. Since it is known that dopamine decreases ROS [10] while Ang II increases ROS in the renal proximal tubule [11], we investigated whether exosomes produced by cultured renal proximal tubule cells (RPTCs), after dopaminergic or Ang II stimulation, would modulate the local ROS production in RPTCs, distal tubule (DT) cells, or collecting duct (CD) cells.

Materials and methods

Renal proximal tubule cell culture

RPTCs were obtained from normal tissue from nephrectomies in human subjects, under an institutional review board-approved protocol according to the Declaration of Helsinki, Title 45, Part 46, and U.S. Code of Federal Regulations. Cell lines were isolated and immortalized as previously described [12–14]. RPTCs were extensively characterized and demonstrated only proximal tubule-specific characteristics [15]. We utilized i16, i22 and i25 RPTC cell lines in the current studies.

Distal tubule and collecting duct cell isolation, culture and characterization

DT and CD cell lines were selectively isolated (Invitrogen Cellection). Human kidney tissue was chopped into 1 mm sections (McIlwain) and then digested with collagenase A (Roche Diagnostics, 2 mg/mL, 37 °C, 25 min). Single cell suspensions were isolated (40 micron filter, Becton Dickinson) followed by lectin or immuno-magnetic affinity separation.

Three DT cell lines were isolated using biotinylated *dolichos biflorus agglutinin* [16] (DBA, Vector Labs, 2.5 mg/500 mL) incubated with the cell suspension for 30 min at room temperature, followed by anti-biotin magnetic nanoparticle conjugated secondary antibody (Invitrogen). Alternatively 2 cell lines were established using anti-Tamm-Horsfall protein [17] (THP, Santa Cruz 20631,1:200 dilution) antibody and an anti-rabbit magnetic secondary antibody (Invitrogen). Three immortalized human CD cell lines were isolated using antibody to L1CAM (1:100) and an anti-mouse magnetic secondary antibody (Invitrogen).

Both the DT and CD were cultured in DMEM-F12 medium with10% FBS and the same supplements used for growing RPTC cells [13]. Cells were immortalized using human terminal transferase (hTERT)-containing lentiviral infection and selected with G418 [13]. The DBA and THP-isolated DT cell lines and CD cell lines were characterized (Online Supplement).

Exosome purification

Exosomes were isolated from cultured cells grown in serum free media using our previously published method [18], (modified method of Gonzales et al.) [19]. Serum free media were found to be necessary because of the high concentration of exosomes in serum competing during transfer. Details of this ultracentrifugation method and characterization of exosomes are in the Online Supplement.

Western blotting for TSG101

Purified exosome pellets were re-suspended in MPER lysis buffer (Thermo Scientific) and protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Scientific). 10 μg protein per lane was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and western blotting using an anti-TSG101 antibody (Sigma HPA006161, 1:1000 dilution) and Licor anti-rabbit secondary antibody (IRDye 800CW, 1:15,000 dilution) and scanning on an Odyssey infrared imaging system (Licor).

Proximal tubule-derived fluorescently labeled exosomes

Proximal tubule cell lines were created which stably express two different GFP fusion proteins known to fluorescently label exosomes. Two different lentiviral constructs with green or red fluorescent proteins (GFP, RFP) were purchased from System Biosciences: CYTO-tracer pCT-CD63-GFP and pCT-CD9-RFP. The plasmids were packaged into virus and three cell lines were transduced with CD63-GFP containing lentiviral particles, and two cell lines were created with CD9-RFP containing lentiviral particles. The exosomal origin of the fluorescence found in cell culture supernatant was verified by microplate fluorometry (PHERAstar FS, BMG Labtech) using purified exosomes.

An additional method, using transient transfection of RPTCs with plasmids with yellow fluorescent proteins (YFP), was utilized (LAMP1-YFP or CD82-YFP fusion protein, Addgene), and was carried out under conditions previously described [15,20]. The transfected RPTCs were grown on magnetic GEM™ microcarriers [13], and then were co-incubated with non-transfected RPTCs on non-magnetic GEMs. YFP fluorescence transfer was measured on the non-magnetic GEMS by fluorometry after removal of the donor fluorescent magnetic GEM™ microcarriers.

Exosomal transfer

Proximal tubule-derived exosomes were isolated by ultracentrifugation of serum-free RPTC 24 h culture supernatants and re-suspended back into the same volume of serum-free medium. The purified exosomes were incubated with RPTCs, DT cells, and CD cells for 24 h. The recipient cells were then washed three times with HBSS and analyzed by microplate fluorometry. The amount of exosomal transfer was calculated as the fluorescence at the appropriate emission wavelength minus the background fluorescence obtained from a well containing the same number of recipient cells that received exosomes isolated from non-fluorescing RPTC cells.

Measurement of ROS production

RPTC, DT and CD cells were labeled with a fluorescent total ROS detection agent (Enzo), which was detected using a PHERAstar FS microplate reader. A basal ROS production rate was measured for each well for 30 min using identical gain and timing for all experiments, after which fenoldopam (FEN, D_1-like receptor agonist, 1 μmol/L) or angiotensin II (Ang II, 10 nmol/L) were added to each well using automated injectors. The change in ROS production rate was measured for 30 min, after which a ROS inhibitor (N-acetyl-L-cysteine, NAC, 5 mmol/L) was added and the change in ROS production rate (relative fluorescence units (RFU)/min) for each treatment was measured and normalized to the negative vehicle control (VEH).

For RPTC exosome-mediated effects on the change in ROS production rate in recipient distal segment cells, fenoldopam-stimulated RPTCs were incubated for 16 h and the exosomes were purified by ultracentrifugation. The purified exosomes were then placed onto DT and CD cells for 24 h before measuring basal NAC-inhibitable ROS production in those recipient cells. The ROS production was compared to that in cells that had received exosome-free media or exosomes from vehicle-treated donor cells.

Confocal microscopy of transferred exosomes

Purified exosomes from CD63-GFP transfected RPTCs were added to non-transfected RPTCs in culture and incubated overnight. Time lapse confocal microscopy was performed in an automated spinning disk confocal microscope using a 60× water immersion objective (Olympus IX81 with Disk Scanning Unit) in a temperature, CO_2 and humidity controlled incubator. Static images of transferred CD9-RFP containing