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Human aqueous humor exosomes

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ABSTRACT

Aqueous humor (AH) is a dynamic intraocular fluid that supports the vitality of tissues that regulate intraocular pressure. We recently discovered that extracellular nanovesicles called exosomes are a major constituent of AH. Exosomes function in extracellular communication and contain proteins and small RNA. Our goal was to characterize the physical properties of AH exosomes and their exosomal RNA (esRNA) content. We isolated exosomes from human AH collected during cataract surgery from five patients using serial ultracentrifugation. We measured the size and concentration of AH exosomes in solution using nanoparticle tracking analysis. We found a single population of vesicles having a mean size of 121 ± 11 nm in the unprocessed AH. Data show that centrifugation does not significantly affect the mean particle size (121 ± 11 nm versus 124 ± 21 nm), but does impact the final number of exosomes in solution (87% loss from the unprocessed AH; n = 5). We extracted esRNA from the pooled human AH samples using miRCURY RNA isolation kit from Exiqon. The quality of extracted esRNA was evaluated using Agilent Bioanalyzer 2100 and was used to generate a sequencing library for small RNA sequencing with Illumina MiSeq sequencer. More than 10 different miRNAs were identified; abundant species included miR-486-5p, miR-204, and miR-184. We found that the majority of extracellular vesicles in the AH were in the exosome size range, suggesting that miRNAs housed within exosomes may function in communication between AH inflow and outflow tissues.

Glaucoma is a leading cause of irreversible blindness worldwide (Quigley and Broman, 2006). It is a heterogeneous group of disorders and is defined as the progressive loss of retinal ganglion cells leading to characteristic optic neuropathy and associated visual field loss (Quigley, 2011). One of the major risk factors for glaucoma is elevated intraocular pressure (IOP). IOP reflects the balance between inflow and outflow of aqueous humor (AH). Currently, lowering IOP is the only therapeutic approach that effectively slows down the progression of glaucoma (AGIS Investigators, 2000). Lowering IOP is accomplished by decreasing the rate of AH production by the ciliary epithelium or by increasing AH outflow facility. Previous studies suggested a cross-talk communication may exist between inflow tissue and outflow tissues through potential modulators in aqueous humor (Coca-Prados and Escribano, 2007; Escribano and Coca-Prados, 2002; Lee et al., 2011; Zhang et al., 2009). These modulators, including neuropeptides, hormones and growth factors, are likely soluble agents present in AH. Our previous research indicates that a major component of AH is an extracellular vesicle called an exosome (Perkumas et al., 2007). There appear to be only three other AH exosome studies conducted previously: One found immunosuppressive activity of exosomes from rabbit AH on T lymphocytes (Liao et al., 2012). The second attempted to identify protein biomarkers from exosomes in AH of patients with age-related macular degeneration (Kang et al., 2014). The third study examined total miRNAs from human AH but not miRNAs within exosomes (Tanaka et al., 2014). However, none of these studies reported the physical properties of exosomes in AH, nor their RNA payload.

Exosomes are nanovesicles (50–175 nm in diameter) made of a lipid bilayer with common exosomal marker proteins plus cell-type-specific proteins that can be used as markers (Bang and Thum, 2012; Gercel-Taylor et al., 2012; Henderson and Azorsa, 2012; Sokolova et al., 2011; Stamer et al., 2011). Exosomes contain proteins and RNAs such as messenger RNA (mRNA) and small RNAs (miRNA) that are transported to surrounding target cells

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(Henderson and Azorsa, 2012; Rani et al., 2011). Exosomes are released into the extracellular environment by many cell types, including epithelial cells, bone marrow-derived cells and trabecular meshwork cells (Dowling and Clynes, 2011; Keller et al., 2011; Kesimer et al., 2009; Stamer et al., 2011; Wang et al., 2009). They are detected in a number of body fluids, such as blood plasma, urine, saliva, breast milk and AH (Admyre et al., 2007; Caby et al., 2005; Keller et al., 2011; Michael et al., 2010; Perkumas et al., 2007; Vlassov et al., 2012). For a long time exosomes were considered organelles used to remove cell debris and obsolete surface molecules from the cell (Henderson and Azorsa, 2012; Staals and Pruijn, 2010). Recently, exosomes have been implicated in cell-to-cell communication partly by transferring exosomal RNA between cells (Camussi et al., 2010; Schneider and Simons, 2012; Vlassov et al., 2012) and they are emerging as important biomarkers of human disease (Bang and Thum, 2012; Conde-Vancells and Falcon-Perez, 2012; Henderson and Azorsa, 2012; Simpson et al., 2009; Staals and Pruijn, 2010). For example, melanoma-derived exosomes contain a unique RNA signature for prognostic and therapeutic potential (Gajos-Michniewicz et al., 2014). Different types of cancer cells produce distinct exosomal RNA profiles that are used to modify their environment and evade immune surveillance (Clayton and Mason, 2009; Corcoran et al., 2011). There is growing interest in serum biomarker tests for ovarian cancer, melanoma, and prostate cancer including the potential role for exosome screening (Li et al., 2009; Logozzi et al., 2009; Lu et al., 2009; Poliakov et al., 2009; Smith et al., 1997; Tavoosidana et al., 2011; Taylor and Gercel-Taylor, 2008). A number of miRNAs, such as miR-34a, miR-130, miR-98, miR-124, miR-204, miR-142, miR-184 and miR-130a, have been shown to contribute to autophagy and phagocytosis (Alexandrov et al., 2013; Murad et al., 2014; Zhai et al., 2013). These are two cellular processes thought to play important roles in the pathogenesis of glaucoma, particularly at the level of the trabecular meshwork (Gasiorowski and Russell, 2009). In this study, our objectives were 1) to characterize the size distribution and concentration of all extracellular vesicles in human AH using Nanoparticle Tracking Analysis (NTA), and 2) to identify the esRNAs in these exosomes using small RNA sequencing.

Experiments conducted in the present study adhered to the tenets of the Declaration of Helsinki. The research was reviewed and approved by the Institutional Review Board from Duke University Medical Center. Written informed consent was obtained for all participating individuals. We collected human AH from patients undergoing cataract surgery at Duke University Eye Center prior to phacoemulsification to eliminate possible contamination of the AH. Exosomes were isolated using our established methods of serial ultracentrifugation (Hardy et al., 2005; Hoffman et al., 2009; Perkumas et al., 2007). Concentration and size distribution of extracellular vesicles in the AH samples was determined by NTA (NanoSight NS500, Malvern Inc., United Kingdom). Specifically, each AH sample was split into three equal portions and subjected to incremental serial ultracentrifugation steps to isolate extracellular vesicles (Fig. 1A). In the unprocessed AH, we found a single vesicle population with a mean diameter of 121 ± 11 nm (Fig. 1B), while the vesicle concentration ranged from 8.6 to 62.3 × 10^8 particles/ml between patient samples. We also examined how the isolation process affected the size and concentration of exosomes in AH. The data shows that neither centrifugation steps significantly affect the mean particle size (113 ± 11 nm with low speed spin; 124 ± 21 nm with both spins, mean ± SD). However, each purification step impacts the final yield of exosomes in solution. For example, up to 60% loss was observed with low speed spin and up to 87% loss from combined full spins, when compared to the total amount of exosomes in the original AH samples (n = 5) (Fig. 1B).

We extracted esRNA from the pooled human AH samples using miRCURY RNA isolation kit from Exiqon according to the recommended procedure. Briefly, the isolated exosomes were lysed using the supplied miRCURY lysis buffer and the RNA was isolated using resin spin column after three spin washes. Quality of extracted esRNA was evaluated using Agilent Bioanalyzer 2100 with Agilent RNA 6000 Pico Kit. All the extracted esRNAs had a single peak with the size less than 200 nucleotides, at around 25 nucleotides (Fig. 2A and B). As expected, no 18S or 28S RNA bands were detected due to the absence of mRNA. For small RNA sequencing, all the extracted esRNA from each sample was used to generate libraries with Illumina TruSeq Small RNA Sample Prep kit with 12 unique indexes (Illumina, San Diego, CA, USA) according to the manufacturer's recommendations. Briefly, esRNA was ligated with specific adapters at the 3′- and 5′-end separately. The ligated esRNAs were amplified with universal primers and a sample-specific index sequence was integrated. The amplified esRNAs were gel-purified based on their sizes and validated with High Sensitivity DNA chips on an Agilent 2100 Bioanalyzer. Denatured DNA templates from different samples

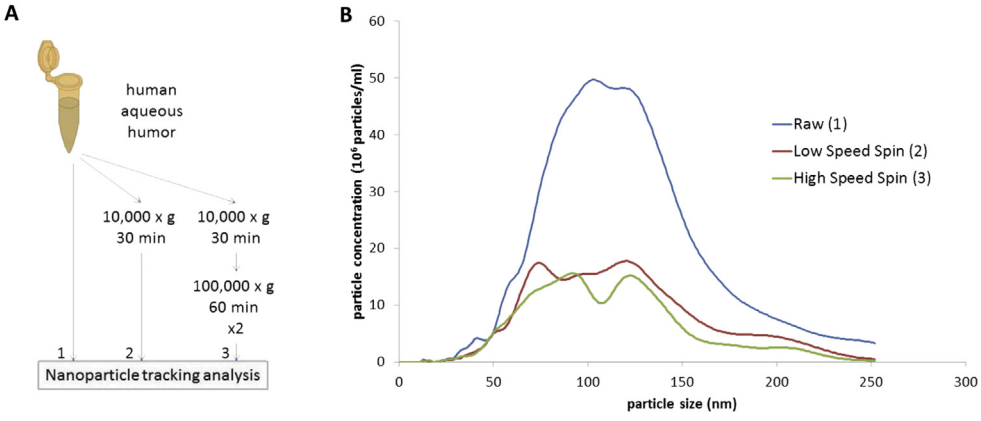

Fig. 1. Human aqueous humor contains a single nanovesicle population with a size characteristic of exosomes. A) Flow chart detailing our experimental design. Each aqueous humor sample from a single patient was equally split 3 ways and processed as shown. Specifically, AH samples were centrifuged at low speed (10,000 × *g* for 30 min at 4 °C in a Beckman TLS-55 rotor) to pellet cell debris and other large contaminants. This cleared supernatant was then centrifuged at high speed (100,000 × *g* for 60 min at 4 °C in the same rotor) to pellet extracellular vesicles. This vesicle pellet was re-suspended in PBS and centrifuged again at high speed to remove any contaminants. Size and concentration of the vesicle populations in these three portions were measured by nanoparticle tracking analysis. B) A histogram showing the vesicle size versus concentration resulting from the vesicle isolation steps (mean of 5 individual samples).