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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.

Keywords

aqueous humor; glaucoma; exosome; exosomal RNA; miRNA

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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 crosstalk communication may exist between inflow tissue and outflow tissues through potential modulators in aqueous humor (Coca-Prados and Escibano, 2007; Escibano 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 (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 (Figure 1A). In the unprocessed AH, we found a single vesicle population with a mean diameter of 121 ± 11 nm (Figure 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 $\pm$ 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) (Figure 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 (Figure 2A and 2B). 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 and PhiX controls from Illumina were pooled together and

sequenced using MiSeq Reagent Kit v2 (50 cycles) with an Illumina MiSeq Personal Sequencing System. Generated sequence data in fastq format was trimmed to remove adapter sequences, and aligned against human reference database using Bowtie software. Only exact matches to known mature miRNA sequences were counted. These matches must align to the start of the reference sequence on the same strand, but not contaminants provided by Illumina.

From the isolated human AH exosomes, Figure 2C showed that we identified more than 10 mature miRNAs, including miR-486-5p, miR-184, and miR-204. Other esRNAs in human AH included miR-181a, miR-191, miR-148a, miR-26a, miR-125a-5p, let-7a, and let-7b. By comparison, the exosomes isolated from conditioned media of primary human trabecular meshwork (HTM) cells contained a different set of miRNAs including miR-143, miR-22, miR-21, let-7f, miR-27b, and let-7i. Both miR-191 and miR-26a were among the most abundant miRNAs in esRNAs derived from human AH and HTM. Our most abundant miRNAs in AH differed from two previous studies (Dunmire et al., 2013; Tanaka et al., 2014). Several factors could contribute to this inconsistency. Our study was the first based on small RNA sequencing while the other two were based on either microarray (Tanaka et al., 2014) or non-probe based realtime PCR array (Dunmire et al., 2013). Our identification was extremely stringent, requiring 100% sequence match to the mature miRNA while the array-based approaches were not. Also, our esRNAs were derived exclusively from the *isolated exosomes* in AH while the other two studies isolated miRNAs from the whole AH, which may include free floating miRNAs, cellular debris, and exosomes. Finally, our bioinformatics analysis removed all precursor miRNAs while the array-based approaches might not have removed them.

Here we show that exosomes are a component of human aqueous humor and contain miRNAs, however, the origin of these exosomes and their miRNAs are unknown. Currently, little is known about exosome generation in anterior chamber tissues outside of the trabecular meshwork (Hardy et al., 2005; Hoffman et al., 2009; Resch et al., 2010). While any cell in contact with aqueous humor may contribute to aqueous humor exosomes/esRNAs, there is insufficient data in the literature to support a single tissue/cell type. However, there are reports identifying miRNAs within anterior chamber tissues. For example, miR-184 is expressed in lens and cornea while miR-204 is expressed in lens, ciliary body and trabecular meshwork cells (Ryan et al., 2006). We detected these two miRNAs in the aqueous humor exosomes but the question remains as to whether the lens, cornea and trabecular meshwork cells are releasing these miRNAs into the AH via exosomes or accumulating them as the AH exosomes are internalized. Future studies will have to answer this question.

We hypothesize that the primary source of exosomes found in the aqueous humor is from the non-pigmented ciliary epithelium, where aqueous humor is actively secreted. Further, we postulate that the release of exosomes, and their esRNA cargo, is a means for the aqueous “inflow” tissues to communicate with the outflow tissues in order to maintain optimum IOP homeostasis. In fact, some of the miRNAs we identified in exosomes from the human AH are reported to affect cellular processes known to be important for proper outflow facility regulation. For example, we identified miR-204 and miR-148a, which are

negative regulators of the pathways involved in the transdifferentiation of normal cells into pro-fibrotic, mesenchymal-like cells (Li et al., 2013; Qiu et al., 2013; Wang et al., 2013; Zhang et al., 2014). Conversely, we identified miR-181a which is thought to promote the transdifferentiation toward mesenchymal-like cells (Parikh et al., 2014). Activation of these pathways is thought to contribute to the dysfunction in conventional outflow facility (Takahashi et al., 2014). Additionally, another miRNA we identified, miR-191, has been shown to reduce expression of the matrix metalloproteinase (MMP) inhibitor TIMP3 (Qin et al., 2014). Inhibition of MMPs in the trabecular meshwork results in decreased outflow facility (Bradley et al., 1998), so conversely, miR-191 reducing TIMP levels in the trabecular meshwork may result in increased outflow facility. Finally, trabecular meshwork cells are highly phagocytic (Shirato et al., 1989) and the miR-184 we identified in the aqueous humor has been shown to regulate phagocytosis (Murad et al., 2014).

Despite the intriguing possibility of our identified miRNAs affecting the specific cellular processes mentioned above, we need to approach these ideas with caution. First, miRNAs are not specific to a single gene. The individual miRNAs reduce expression of a number of genes, though these genes may be involved in a single cellular process like extracellular matrix remodeling (Ibrahim et al., 2014) or transdifferentiation into mesenchymal-like cells (Yan et al., 2013). Second, the effect of a single miRNA will most likely be cell type dependent. Third, we need to determine the efficacy of the exosomes/miRNAs on outflow tissues including the trabecular meshwork. It is possible that while present in the aqueous humor, the miRNA copy number may not be high enough to affect cellular processes (Chevillet et al., 2014). Fourth, and most importantly, we need to identify whether ocular diseases correlate with changes in either the specific miRNAs or copy numbers of the miRNAs. We are currently addressing these issues experimentally. Fifth, despite our best effort, the human aqueous humor samples collected from cataract surgeries are not really “normal” aqueous humor and that some of the exosomes could possibly be derived from the cataractous lens.

The results we report here are the initial findings of a broader project to understand the role of aqueous humor derived miRNAs and exosomes in eye health. A tantalizing possibility is that changes in the aqueous humor exosomes/miRNAs could be used as a biomarker for prognosis and diagnosis of ocular disease. Furthermore, it is hypothesized that exosomes from diverse origins eventually wind up in blood (Properzi et al., 2013). Since it is difficult to obtain aqueous humor samples from patients and aqueous humor eventually rejoins the venous system, a simple blood draw could provide information about anterior eye tissue health. However unlikely this possibility may be, obtaining prognostic or diagnostic information pertaining to ocular disease from a simple blood sample remains a tantalizing goal. In summary, for the first time we demonstrate that exosomes are the major extracellular vesicle population in AH and that these exosomes contain characteristic esRNAs. Thus, AH exosomes appear to serve as transport vehicles, delivering esRNAs, possibly from the ciliary body, to other anterior chamber tissues including the outflow tissues; the trabecular meshwork, Schlemm’s canal and episcleral vessels. This esRNA transfer may synchronize AH production and drainage, and ultimately IOP homeostasis. Further analysis of these exosomes and their esRNA may provide significant information for

potential disease biomarkers for glaucoma diagnosis, clinical treatment, and potential prognosis.

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- Human aqueous humor contains a single vesicle population with a size characteristic of exosomes, measured by nanoparticle tracking analysis
- Isolated human aqueous humor exosomes contain small RNAs
- More than 10 different miRNAs were identified from isolated aqueous humor exosomes using small RNA sequencing
- miRNAs housed within exosomes may function in communication between AH inflow and outflow tissues

Figure 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 30min 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 60min 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).

Figure 2.

Characterization of exosomal RNA (esRNA) extracted from purified nanovesicles from fresh human aqueous humor. The esRNA was evaluated using Agilent Bioanalyzer 2100 with Agilent RNA 6000 Pico Kit. A) Shows the RNA ladder and B) displays extracted esRNA from one of the human aqueous humor samples. The size of extracted esRNA was less than 200 nucleotides (nt), mostly around 25 nt. C) shows the miRNAs that were identified via small RNA sequencing using Illumina MiSeq sequencing system. The height of each bar indicated the number of sequence reads with perfect match to mature miRNA.