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Research article

Variations in active outflow along the trabecular outflow pathway

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ABSTRACT

Previous tracer studies have shown segmental outflow in the trabecular meshwork (TM) and along the inner wall (IW) of Schlemm's canal (SC). Whether segmental outflow is conserved distal to SC has not yet been investigated. This study aims to investigate whether the segmented pattern of outflow is conserved in distal outflow pathways by using a newly developed global imaging method and to evaluate variations of active outflow in three distinct regions along trabecular outflow pathway.

Six normal whole globe human eyes were first perfused at 15 mmHg to establish a stable baseline outflow facility. The anterior chamber was then exchanged (5 mL) and perfused with fluorescent microspheres (0.002% v/v, 200 μL) to label areas of active outflow. All eyes were perfusion fixed and dissected into anterior segments. The TM and scleral surface were en face imaged globally. Effective filtration area (EFA) and fluorescent tracer distribution and intensity were analyzed in global images for both the TM and episcleral veins (EPVs). Anterior segments were further dissected into a minimum of 16 radial wedges, from which frontal sections were cut, stained, and imaged, using confocal microscopy. EFA from all three locations along the trabecular outflow pathway were measured and compared. Additionally, TM thickness, SC height, and total number of collector channels (CC) were analyzed and compared between active and inactive areas of outflow. Statistical analysis was performed using Student's t-tests and Wilcoxon signed-rank test with a required significance of $p \leq 0.05$.

All three locations showed a segmental outflow pattern. The TM had a significantly higher mean EFA (86.3 ± 3.5%) compared to both the IW (34.7 ± 2.9%; $p \leq 0.01$) and EPVs (41.1 ± 3.8%; $p \leq 0.01$). No significant difference in mean EFA was found between IW and EPVs. Preferential active outflow was observed in the nasal and inferior quadrants. TM thickness was significantly larger in areas of active outflow (103.3 ± 4.0 μm; $p \leq 0.01$) compared to areas of inactive outflow (78.5 ± 6.5 μm), but there was no significant difference in SC height between active and inactive outflow areas. Among all eyes, a total of 80 CCs were counted with 63 associated with active outflow and 17 associated with inactive outflow. A higher number of CCs associated with areas of active outflow were found in the nasal (26 of 63) and inferior (20 of 63) quadrants compared to the temporal (9 of 63) and superior (8 of 63) quadrants.

A segmental nature of outflow is conserved along the trabecular outflow pathway with variations in three distinct locations (TM, IW, and EPVs). IW and EPVs showed a similar mean EFA. Preferential active outflow was observed in the nasal and inferior quadrants of the eye, which are associated with more expanded TM and higher number of CCs. Normal outflow patterns and its variations along the outflow pathway reported in this study will provide the basis for future studies of the outflow changes in eyes with glaucoma.

1. Introduction

Normal intraocular pressure (IOP) is maintained through a dynamic balance between the amount of aqueous humor production and drainage. Dysfunction or impairment of the aqueous outflow drainage pathway due to increased outflow resistance results in elevated IOP, which is a primary risk factor for primary open angle glaucoma (POAG). The majority of aqueous humor (50–80%) is removed through the trabecular outflow pathway (Bill, 1965; Bill and Hellsing, 1965; Toris et al., 1999) and several previous studies

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have localized the primary site of outflow resistance to a thin region of tissue within the trabecular meshwork (TM), that includes the inner wall (IW) endothelium of Schlemm's canal (SC) and its basement membrane, as well as the underlying juxtacanalicular connective tissue (JCT) (Grant, 1958, 1963; Maepea and Bill, 1989; Maepea and Bill, 1992). However, following complete trabeculotomy at normal IOP (7 mmHg) in enucleated human eyes, only 49% of outflow resistance was eliminated and at a higher IOP (25 mmHg) 71% of outflow resistance was eliminated (Rosenquist et al., 1989). Additionally, Schuman et al. (1999) reported that following a 1-clock hour ablation of the tissue distal to the outer wall of SC, using an excimer laser at a perfusion pressure of 10 mmHg, 35% of outflow resistance was eliminated. Collectively, these studies suggest that one-third to one-half of the outflow resistance lies distal to the IW of SC at various pressures. However, the mechanism by which outflow resistance is generated and regulated in each location remains unclear and is even further complicated by segmental flow.

Earlier tracer studies using electron microscopy in monkey (MacRae and Sears, 1970; Epstein and Rohen, 1991; Sabanay et al., 2000) and human eyes (MacRae and Sears, 1970; de Kater et al., 1989; Ethier and Chan, 2001; Hann et al., 2005) found variable distribution of outflow tracers within the TM and along the abluminal and luminal side of the IW of SC. While these observations were the first to describe the non-uniform or segmental outflow, as labeled by various tracers, it also suggested that outflow resistance might vary in different locations along the limbus. A limitation of these studies was that only a small fraction of the TM along the circumference of the limbus was examined by electron microscopy. More recent studies took on a more macroscopic approach to visualizing outflow patterns in larger areas within the TM and along the IW by combining the use of fluorescent microspheres and confocal microscopy. It was found that even on a larger scale, outflow patterns remained segmental in both non-human (Lu et al., 2007; Battista et al., 2008; Lu et al., 2008; Zhu et al., 2010) and human eyes (Keller et al., 2011; Yang et al., 2013; Chang et al., 2014). In order to understand how segmental outflow is regulated, more recent studies have begun to explore the potential mechanisms that contribute to this pattern and its association with outflow resistance. In particular, differences in both morphology and extracellular matrix composition have been associated with high and low flow regions of outflow in both human (Keller et al., 2011; Yang et al., 2013; Vranka et al., 2015) and mouse eyes (Swaminathan et al., 2013). Additionally, an inverse relationship between the percent of the effective filtration area (EFA) and outflow resistance was also found in non-human (Lu et al., 2007; Battista et al., 2008; Lu et al., 2008; Scott et al., 2009; Zhu et al., 2010) and human eyes (Yang et al., 2013).

Despite numerous studies looking at the segmental outflow patterns within the TM and along the IW, whether this same pattern is conserved distal to SC on a whole eye scale has not yet been investigated. Therefore, the aims of this study are: 1) To evaluate whether the segmented nature of outflow is conserved in distal outflow pathways by using our newly developed global imaging method; 2) To compare variations of areas of active outflow along the entire trabecular outflow pathway in three distinct locations (TM, IW, episcleral veins); and 3) Investigate which structures may contribute to the segmental pattern of outflow.

2. Materials and methods

A total of 3 pairs of human eyes (N = 6) were used in this study with an age range of 74–78 years old (76 ± 2; Mean ± SD). Whole globe human eyes were received from the National Disease Research Interchange (Philadelphia, PA) within 24-h post-mortem and inspected for any physical damage under a dissecting microscope. Criteria for acceptable globes included a clear history of no diabetes, no chemotherapy within 1 year, and no history of known ocular diseases or surgeries.

2.1. Ocular perfusion

Whole globe ocular perfusion was carried out using a similar protocol previously established (Scott et al., 2009). Briefly, all eyes were first perfused with a perfusion fluid containing 5.5 mM D-glucose + Dulbecco's phosphate buffered saline (DPBS) for 30 min at constant pressure (15 mmHg) to establish a stable baseline outflow facility. Following establishment of baseline outflow facility, the anterior chamber fluid was exchanged with 5 mL of carboxylated polystyrene fluorescent microspheres (0.002% v/v, 500 nm, Life Technologies, Grand Island, NY) and additionally perfused with a fixed volume (200 μL) of the same solution to label areas of active outflow. Once perfusion of microspheres was completed, the anterior chamber fluid was exchanged with 5 mL of modified Karnovsky's fixative and perfusion-fixed for 30 min. A small equatorial cut was then made and eyes were additionally immersion-fixed overnight and stored in DPBS at 4 °C until further processed.

2.2. Global imaging

To image active outflow patterns in the entire trabecular meshwork (TM) and episcleral veins (EPV) from anterior segments of each eye, fixed whole globes (N = 6) were first oriented into four quadrants (nasal, inferior, temporal, superior) based on the location of extra ocular muscle tissue, optic nerve head and macula lutea. Eyes were then dissected along the equator into an anterior and posterior segment. Anterior segments were retained and further processed by removing the iris, ciliary body, cornea (10 mm trephine), vitreous, and excess conjunctiva. Processed anterior segments were then imaged *en face* on both their TM and scleral surfaces with a fixed exposure time of 5 s (Fig. 1A) through a 300 mm lens on a 4000 MP VersaDock imaging system (Bio-Rad Laboratories, Hercules, CA) using Quantity One (Bio-Rad Laboratories, Hercules, CA).

2.3. Analysis of areas of active outflow from global images

Fluorescent tracer distribution and intensity in the TM and EPVs of each global image was analyzed for areas of active outflow. To avoid identifying the auto-fluorescent sclera as a false positive, each global image underwent background subtraction using a fixed value (60 pixels) in Fiji (NIH, Bethesda, MD). Images were then digitally separated into a minimum of 16 different “wedges” (Fig. 1B) and analyzed for areas of active outflow using two different approaches.

The first approach was to quantify the percent of the scleral and TM surfaces that showed fluorescing tracers. We termed this measurement the effective filtration area (EFA). To calculate whole eye EFA, global images of the TM and scleral surfaces were measured for the total scleral length (TSL) and total filtration (tracer-labeled) length (TFL) along the circumference of the eye. Only one measurement for TSL and TFL was taken from global images of the TM surface (Fig. 2A), while three different measurements (inner, middle, outer) for TSL and TFL were taken from global images of the scleral surface (Fig. 2B). The average of three measurements was used to account for a larger scleral area compared to one measurement for the TM. EFA was calculated as $EFA = 100 \times (\Sigma TFL/\Sigma TSL)$.

The second approach was to evaluate both the distribution and