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The three-dimensional organisation of the post-trabecular aqueous outflow pathway and limbal vasculature in the mouse

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ABSTRACT

The mouse eye has been used as a model for studies on the microanatomy of the outflow pathways but most of what is known comes from histological sections. These studies have focused mainly on the morphological features of the trabecular meshwork, Schlemm's canal and aqueous channels that link to the superficial episcleral vasculature. However, the anatomical architecture of the aqueous outflow vessels and their relationship to each other and to the general vascular circulation is not well understood. The aim of this study was to provide a detailed description of the microarchitecture of the aqueous outflow vessels and their relationship to the superficial limbal/episcleral vasculature throughout the entire limbus. The aqueous outflow vessels and blood and lymphatic vessels were imaged in PECAM-1 and LYVE-1 immunostained whole anterior segments of adult mice and three-dimensional (3-D) reconstructions of the optical sections were generated to reveal the aqueous, blood and lymphatic architecture. The arterial supply, venous drainage, organisation of perilimbal vasculature, collector channels/aqueous veins and the morphology of Schlemm's canal were revealed in their entirety and the relationships between these structures is described. Schlemm's canal was PECAM-1 positive but there was no affinity for the lymphatic marker LYVE-1. We show that Schlemm's canal is a continuous circular structure and more often seen as a single, broad, varicose vessel with short regions appearing as a plexus. Aqueous veins link Schlemm's canal to the superficial vasculature and there were no direct links seen between the canal and the lymphatic vessels.

1. Introduction

The outflow of aqueous humour from the anterior chamber follows two distinct routes, the conventional and unconventional pathways (Aihara et al., 2003; Goel et al., 2010 (review); Lindsey and Weinreb, 2002; Llobet et al., 2003). The conventional pathway is located in the corneoscleral limbus and is of clinical importance because it plays a pivotal role in regulating intraocular pressure (Llobet et al., 2003). The posterior surface of the corneoscleral limbus at the iridocorneal angle houses the trabecular meshwork through which the aqueous humour percolates and is filtered en route to entering the canal of Schlemm (Schlemm's canal) that encircles the entire periphery of the cornea. The aqueous fluid exits Schlemm's canal through collector channels and aqueous veins. These feed into the circulatory system which is located midway and at the outermost regions (anterior surface) of the corneoscleral limbus. The major function of the conventional aqueous outflow pathway is to maintain the intraocular pressure within the critical range by modulating the outflow resistance of the aqueous humour. Structural aberrations in this pathway can lead to raised intraocular pressure, greatly increasing the risk for developing glaucoma.

Studies on the microanatomy of the murine aqueous outflow structures of the limbus and/or limbal vasculature have been carried out by three different means; examination of histological cross sections (Chang et al., 2001; Smith et al., 2000), wholemount immunostaining (Chan et al., 2004) and vascular corrosion casts (Ninomiya and Inomata, 2006). Studies investigating effect of the genes *Foxc1* and *Bmp4, using the former approach,* revealed considerable variation between individuals of the same genetic background and between individuals from different genetic backgrounds (Chang et al., 2001; Smith et al., 2000). However, since the eye is a spherical globe-like structure, true cross-sections of the limbus can deviate substantially depending on the plane of sectioning, thus limiting the regions/sections of the limbus from

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which measurable data can be collected. This points to a need to study the vasculature throughout the entire limbus. The vascular corrosion cast explorations of Ninomiya and Inomata (2006) have contributed partially to such understanding. However details of the aqueous vessels and their relationships to the superficial limbal vasculature (episcleral vessels) throughout the limbus have not been uncovered in much detail. By contrast, the three dimensional anatomical relationships of the limbal aqueous and blood vessels are well described in humans (Ashton, 1951, 1952; Ashton and Smith, 1953), primates (Selbach et al., 2005; Ujiie and Bill, 1984), dogs (Van Buskirk, 1979), rabbits (Selbach et al., 1998), hamsters (Ninomiya and Inomata, 2005) and rats (Bhutto and Amemiya, 2001; Morrison et al., 1995; Ninomiya and Kuno, 2001; Selbach et al., 1998).

The aim of this study was to develop a protocol and analytical method for viewing the entire outflow pathway in the mouse eye. By utilising wholemount immunostaining and three-dimensional imaging, we reveal here the microanatomy of the entire aqueous outflow pathway and limbal architecture of the mouse eye. The detail provided in this report will allow investigators to more accurately explore the developmental genetics of the eye and the role that the vascular architectural variations may play on the functioning of the aqueous outflow pathway.

2. Material and methods

2.1. Animals

Studies on ocular tissues obtained from euthanized mice were approved by the Health Science Faculty Animal Ethics Committee (HSFAEC), University of Cape Town. Care, handling and euthanasia of mice were in accordance with the guidelines of the HSFAEC, and are compliant with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and EU Directive 2010/63/EU for animal experiments at http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm. Animals were fed *ad libitum* and had free access to fresh water. Ambient temperature was maintained at 21 °C, and mice were exposed to 12 h day and night cycles. Mice were euthanized according to standard practice (either by CO_2 inhalation followed by cervical dislocation, or by cervical dislocation performed by an experienced animal technologist of the Health Sciences Animal Unit at the University of Cape Town. Both eyes were harvested from mice aged 6–8 months, (C57BL/6Jx129 WT ($n = 10$), and ICR WT ($n = 12$)), and mice aged 10–12 months (C57BL/6Jx129 WT ($n = 16$)).

2.2. Wholemount immunostaining

Mouse eyes were enucleated immediately after euthanasia, briefly immersed in ice cold phosphate buffered saline (PBS) and fixed in 100% ice cold methanol by intracameral injection followed by immersion in 100% methanol for a further 15 min. Release of excessive intraocular pressure from the injected fixative was achieved by puncturing the posterior segment prior to the intracameral injection. Anterior segments with intact cornea, limbus, iris and ciliary body were removed and fixed for a further 30 min in methanol. In most cases the iris and ciliary body were gently dissected away as these structures are heavily vascularized since they contribute to the out-of-focus glare when viewing by widefield epifluorescence microscopy. In order to correlate known structures in sectioned material to those observed in the corneoscleral wholemounts, histological analysis and immunofluorescence was performed on sections of epoxy-embedded and frozen mouse eyes respectively.

Fixed corneoscleral wholemounts (hereafter referred to as wholemounts) were washed in phosphate buffered saline (PBS), incubated in 50 mM ammonium chloride for 2 h to reduce background fluorescence, blocked in 1% bovine serum albumin (BSA) containing 0.01% dimethylsulphoxide in PBS for 2–3 h and incubated in rat anti-mouse PECAM-1 (PharMingen International) (1:250 dilution/4 μg/ml) to detect vascular endothelium. To determine whether Schlemm's canal had any anatomical relationship to lymphatic vessels or may have expressed a lymphatic phenotype, a number of eyes were double immunostained with PECAM-1 antibody together with rabbit anti-mouse LYVE-1 (Abcam Inc.) (1:500 dilution/2 μg/ml). Wholemounts were incubated overnight at 4 °C, rinsed in PBS and incubated in anti-rat Alexa Fluor 488 (Molecular Probes, Invitrogen, USA) and anti-rabbit Cy3 IgG (Jackson Laboratories) both at 1:2000 dilution. Wholemounts and frozen sections that served as negative controls were immunostained as above but not exposed to primary antibodies. For viewing, flat mounts were prepared by making radial incisions around the wholemount and mounted in PBS/glycerol containing antifade (n-propylgallate or 1,4 diazobizlyclo[2.2.2]octane – DABCO).

2.3. Imaging

Viewing and image capture was done using a Zeiss Axiovert 200W fluorescence microscope (fitted with HR Axiocam and Axiovision software) or a Zeiss 510 Meta confocal microscope using the LSM imaging software. The entire limbal vessel network including Schlemm's canal was captured by taking consecutive images around the limbus. The superficial limbal plexus and anatomical details of Schlemm's canal were photographed using the 10× and 20× objective lenses respectively. Complete coverage of Schlemm's canal (from inner to outer walls) required Z-stacks comprised of optical sections each separated by 3 microns. For confocal microscopy, the pinhole was set to 180 μm (approximately 1.8 Airy units). This allowed better visual continuity of the limbal vessels in the Z-plane due to the “thicker” optical sections in the Z stacks. Extended focus/flattened projection images were generated from Z-stacks using NIH ImageJ (Rasband W at http://rsb.info.nih.gov.info//ij/) or the proprietary LSM software for images captured by widefield or confocal microscopy respectively in order to produce a clear outline of Schlemm's canal. For the purpose of publication, images have resized and subjected to digital contract enhancement. Comparisons relating to staining intensity would thus only apply to immunostained structures within the same image and not between images.

2.4. Morphological analysis

The morphological features of arteries, veins, capillaries and aqueous vessels in our immunostained wholemounts were identified in this study based on features for these vessels as shown by wholemount immunostaining and vascular corrosion casts reported in other studies (Baluk et al., 2004a, 2004b; Cursiefen et al., 2005; Ezaki et al., 2001; Gale et al., 2002; Hamrah et al., 2004; Murfee et al., 2007). The anatomical identification of specific PECAM-1 immunostained ocular vessels in the murine eye was based on comparing the organisation of vessels, their points of anastomosis and precise location as revealed by vascular corrosion casts of ocular limbal vessels from humans (Ashton, 1951, 1952; Ashton and Smith, 1953), primates (Selbach et al., 2005; Ujiie and Bill, 1984), dogs (Van Buskirk, 1979), rabbits (Selbach et al., 1998) and rats (Bhutto and Amemiya, 2001; Morrison et al., 1995; Ninomiya and Kuno, 2001; Selbach et al., 1998) and mice (Ninomiya and Inomata, 2006). In addition, blood vessels leading to and draining from the anterior segment from the mouse eye were