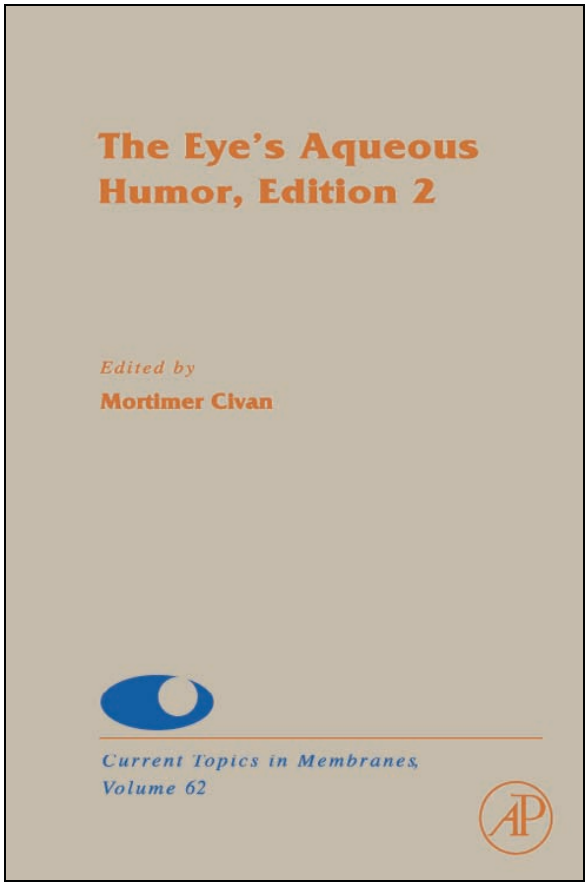

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CHAPTER 6

Aqueous Humor Outflow Resistance

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I. OVERVIEW

This chapter provides a summary of the work that has led to our current day understanding of conventional aqueous outflow. The anatomy and basic physiology of aqueous humor outflow through the trabecular meshwork, via Schlemm's canal, to the episcleral venous system is reviewed. Various postulates concerning the manner in which aqueous outflow resistance is generated are reviewed as well.

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II. INTRODUCTION

It was already recognized in the seventeenth century by Rikchard Banister that glaucoma was associated with an elevated intraocular pressure (IOP) (Sorsby, 1963), although this was not widely accepted until the nineteenth century. Recognition that this elevated pressure was related to a blockage of the aqueous humor outflow pathway began with the studies of Leber, Schwalbe, Knies, and Smith (Schwalbe, 1870; Leber, 1873; Knies, 1875; Smith, 1888). The flow resistance of this pathway, in both normal and glaucomatous eyes, has been the focus of study for the past 130 years. Despite this effort medical treatments for glaucoma, which are directed at the principal outflow pathway, remain few. And none of these are among the current first line of therapy.

Aqueous humor is secreted by the ciliary body into the posterior chamber of the eye. Aqueous humor cannot traverse the intact iris and thus it passes through the pupil to reach the anterior chamber of the eye. Since the pupillary margin rests on the crystalline lens of the eye, the pupil acts as a one-way valve, preventing reflux of aqueous into the posterior chamber. Changes in the relationship between the pupil and the lens can alter aqueous flow. In some eyes, a mid-size pupil diameter can produce a relative blockage of aqueous movement into the anterior chamber. In the face of intraocular inflammation, the pupillary margin may become bound to the lens (aka posterior synechia). The result may be an elevation of IOP.

Aqueous humor that has reached the anterior chamber circulates in a convective current driven by the temperature difference between the warm iris and the cooler cornea. Aqueous humor rises in the back of the anterior chamber and falls along the inner surface of the cornea, while at the same time, flowing toward the ''angle,'' where the iris and ciliary body insert into the sclera. At the angle, the bulk of this flow enters a pathway known as the conventional aqueous outflow pathway composed of the trabecular meshwork, the juxtacanalicular connective tissue (JCT), the endothelial lining of the inner wall of Schlemm's canal, Schlemm's canal itself, and the collecting channels that lead to the episcleral veins (Fig. 1).

Since this is a bulk flow, driven by a passive pressure gradient, it is clinically important to remember that anything leading to elevation of episcleral venous pressure will require IOP to rise to whatever level is required to surpass episcleral venous pressure in order for outflow to resume.

A small fraction of the aqueous humor flows out of a second pathway know as the ''unconventional'' pathway. The fluid traveling along this pathway also enters at the angle of the eye, but then travels posteriorly through the ciliary body and ciliary muscle, to the supraciliary and suprachoroidal spaces (Fig. 2; Bill, 1964a, b, 1965; Bill and Hellsing, 1965). The route by

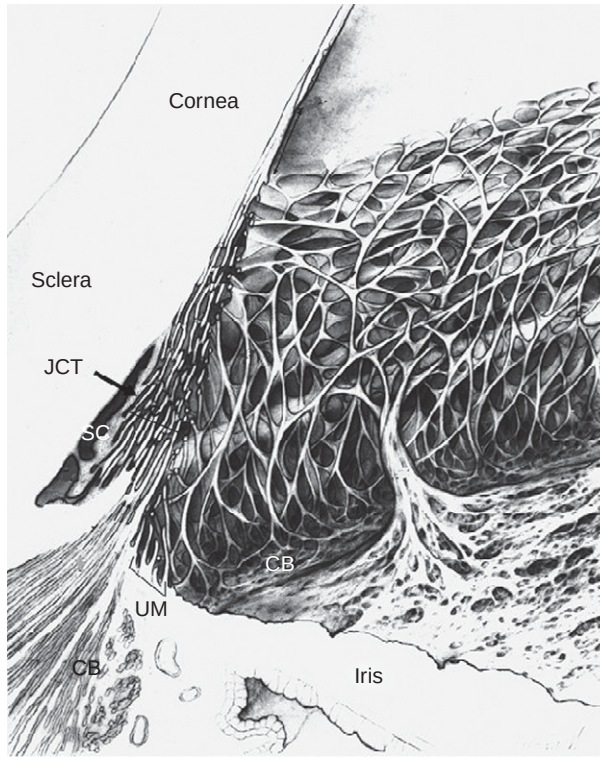

FIGURE 1 Sketch of the angle region of the human eye showing the relationship between the trabecular meshwork and surrounding structures. CB, ciliary body; UM, uveal meshwork; SC, Schlemm's canal; JCT, juxtacanalicular meshwork. [Adapted from Hogan, M., *et al.*, (1971). "Histology of the Human Eye" (W.B. Saunders), Figs. 4–16.]

which the fluid exits the eye from here is still debated. Some studies suggest that this fluid passes through the sclera into the episcleral tissue (Bill, 1966, 1975; Bill and Phillips, 1971), while other studies suggest that this fluid is absorbed osmotically into the choroidal vessels and vortex veins (Pederson *et al.*, 1977; Sherman *et al.*, 1978; Ethier *et al.*, 2004). This question is complicated by the difficulty of measuring flow through this pathway (Johnson and Erickson, 2000), and by the need to make this measurement *in vivo* (Wagner *et al.*, 2004). This pathway carries less than 10% of the total flow in the older adult human eye, but is important in the treatment of glaucoma as the mechanism of action of $PGF_{2\alpha}$ and commercially available prostanoids is on this pathway (Crawford and Kaufman, 1987; Gabelt and Kaufman, 1989).

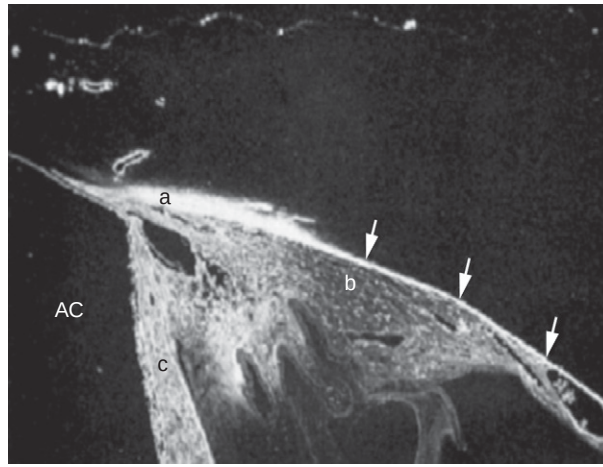

FIGURE 2 Sagittal section of a rabbit eye intracamerally injected with fluorescent tracer that has highlighted both the conventional outflow pathway (a) and the unconventional outflow pathway through the ciliary muscle (b) to the supraciliary space (arrows). AC, anterior chamber; c, iris. (Tripathi, 1977a; Fig. 2).

Our focus in this chapter is on the conventional outflow pathway. We begin with an examination of the aqueous humor itself. Then, we briefly review the flow resistance of those aspects of the outflow pathway that are thought to have minor roles in the generation of aqueous outflow resistance. Finally, we turn our focus to the region near the inner wall of Schlemm's canal where the bulk of outflow resistance is thought to be generated. However, we first begin by defining what we mean by flow resistance.

Fluid flow through a tissue, in the absence of active pumping, is driven by a gradient in hydrostatic and osmotic pressures. As there is no significant osmotic pressure difference between the fluid in the anterior chamber and the blood into which it empties (Bárány, 1963), it is simply the pressure difference ($\triangle P$, typically 5 mm Hg) between the IOP and the episcleral venous pressure that drives flow through the aqueous outflow network. The ratio of this pressure difference to the flow of aqueous humor passing through this system (Q, typically 2 μl/min) is the flow resistance:

$$R = \frac{\Delta P}{Q} \tag{1}$$

The inverse of the outflow resistance is known as the outflow facility.

III. THE AQUEOUS HUMOR

The question first arises as to what are the flow properties of the fluid passing through the aqueous outflow network. Aqueous humor is secreted by the ciliary epithelium from an ultrafiltrate of blood. It has a protein concentration roughly 0.5–1% of that in serum, depending on the specie (Gaasterland *et al.*, 1979; Dernouchamps, 1982; Pavao *et al.*, 1989). The most important hydrodynamic property of aqueous humor is its viscosity, and this has been measured to be essentially the same as that of saline (Beswick and McCulloch, 1956; Balazs *et al.*, 1959).

Johnson *et al.* (1986) reported that ultracentrifuged aqueous humor, when passed through microporous filters with pore sizes similar to the smallest openings found in the outflow pathway, can obstruct flow through these filters. Of interest, serum diluted to the same protein concentration as aqueous humor did not obstruct the filters. This obstruction with aqueous humor appeared to be due to a hydrophobic interaction between the filter surface and the proteins in the aqueous humor and could be relieved with a protease but not a GAGase (Johnson *et al.*, 1986; Ethier *et al.*, 1989; Pavao *et al.*, 1989). More recent work indicates that myocilin, a protein associated with juvenile and primary open-angle glaucoma (POAG) (Polansky *et al.*, 1997; Stone *et al.*, 1997; Fingert *et al.*, 2002), may be involved in this process of filter obstruction by aqueous humor (Russell *et al.*, 2001). It is not known whether this filter-blocking behavior of aqueous humor is of physiological significance.

IV. REGIONS OF LOW OUTFLOW RESISTANCE

Upon entering the conventional aqueous outflow pathway, the aqueous humor enters the trabecular meshwork, an avascular tissue composed of the uveal meshwork, the deeper corneoscleral meshwork, and the still deeper JCT (Fig. 1). The uveal meshwork consists of a set of beams organized into an irregular netlike structure (Fig. 3). This is a very open and porous network and negligible flow resistance is expected in the region, an observation confirmed experimentally by Grant (1963).

The corneoscleral meshwork extends from the uveal meshwork $\sim$100 μm in the direction of flow toward Schlemm's canal. It consists of a number of interconnected sheets or trabeculae that extend from the peripheral cornea to the scleral spur. These sheets, like the cores of the uveal meshwork beams, have an avascular core of collagen and elastin covered with a basal lamina and finally a single layer of endothelial cells (Fig. 3; Tripathi, 1974). The number of trabecular endothelial cells decreases with age and is further decreased in glaucoma (Alvarado *et al.*, 1981).

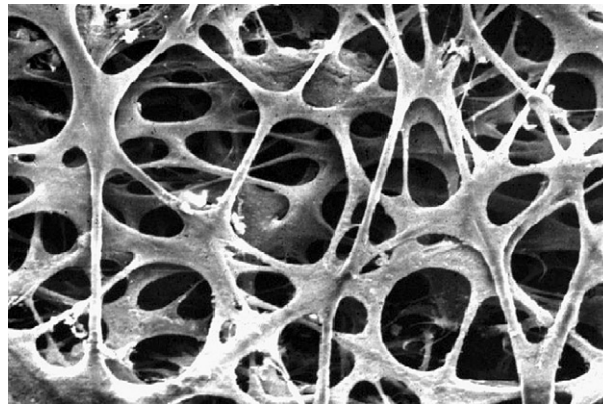

FIGURE 3 Scanning electron micrograph shows the uveal face of the trabecular meshwork showing branching and anastomosing trabecular beams covered in a thin layer of endothelial cells. Note how the open spaces get smaller in layers beneath the surface. [Freddo, T. F., *et al.* (1984)].

The trabecular meshwork exhibits openings that get progressively smaller as the deeper layers of the meshwork are reached (Fig. 3). Its design is like that of a filter. The trabecular endothelial cells on the surface of this filter are phagocytic and can thus ingest materials trapped by this filter (Rohen and van der Zypen, 1968). McEwen (1958) used Poiseuille's law to show that there is negligible flow resistance in the region.

The aqueous humor next passes through the JCT. The JCT and the endothelial lining of the inner wall of Schlemm's canal (and its basement membrane) are the regions where the bulk of outflow resistance is thought to be generated in the normal eye (Fig. 4). We defer our discussion of this region until the following section.

Upon passing through the inner wall of Schlemm's canal, the aqueous humor enters the canal itself. When cut in cross section, the canal has the appearance of a highly elongated ellipse [Freddo, 1993 (and revised 1999)], with its major axis having a diameter varying between 150 and 350 μm (Ten Hulzen and Johnson, 1996); while the minor axis (the distance between the inner and outer wall of the canal) can vary between roughly 1 and 30 μm, depending on the IOP (Fig. 5). As IOP increases, the canal progressively collapses (Johnstone and Grant, 1973; Johnson and Kamm, 1983). As it collapses, the outflow resistance it generates is increased (Moses, 1979; Van Buskirk, 1982). Preventing collapse of Schlemm's canal is likely the mechanism by which muscarinic agents (e.g., pilocarpine) act to decrease outflow resistance (Johnson and Erickson, 2000). These agents cause the longitudinal fibers of the ciliary muscle to contract, thus pulling on a system of elastic

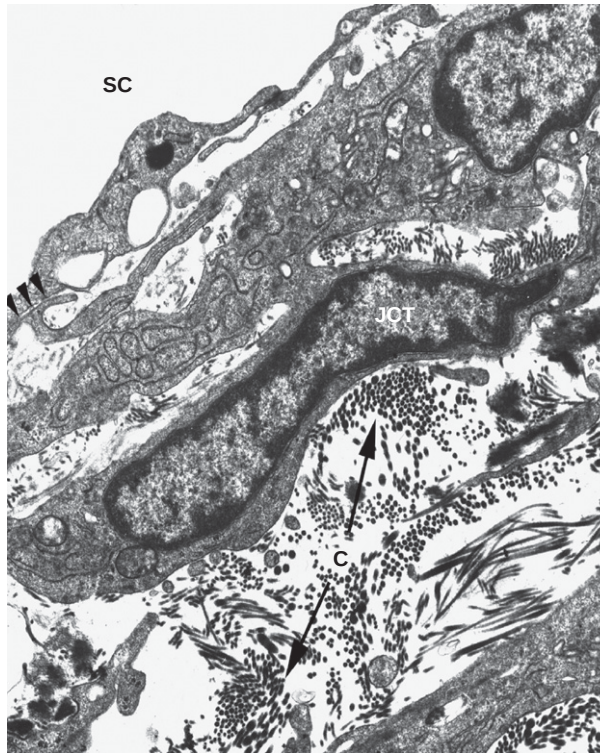

FIGURE 4 Transmission electron micrograph shows juxtacanalicular region (JCT) of the trabecular meshwork and both the endothelial lining and lumen of Schlemm's canal (SC). The JCT region contains an open extracellular matrix including collagen (C) and elastin. The fibroblast-like cells of the region extend filipodial connections to the endothelial cells lining Schlemm's canal (arrows) (Freddo, 1993).

fibers termed the cribriform plexus, which makes connections into the JCT region and the endothelial lining of inner wall of Schlemm's canal (Rohen, 1983; Gong *et al.*, 1989; Figs. 6 and 7).

A similar effect on outflow facility occurs experimentally when the lens is pushed posteriorly in the eye, thus pulling on the zonules that in turn pull the ciliary muscle in the posterior direction. This opens the canal in a fashion analogous to the action of muscarinic agents. Lens depression only decreases outflow resistance at elevated IOP when Schlemm's canal is collapsed (Fig. 8), confirming that the mechanism of action of muscarinic agents is largely one of preventing collapse of Schlemm's canal, despite earlier assumptions that the principal action of miotics was to pull the scleral spur posteriorly, thus "opening up" the trabecular meshwork (Van Buskirk, 1976; Rosenquist *et al.*, 1988).

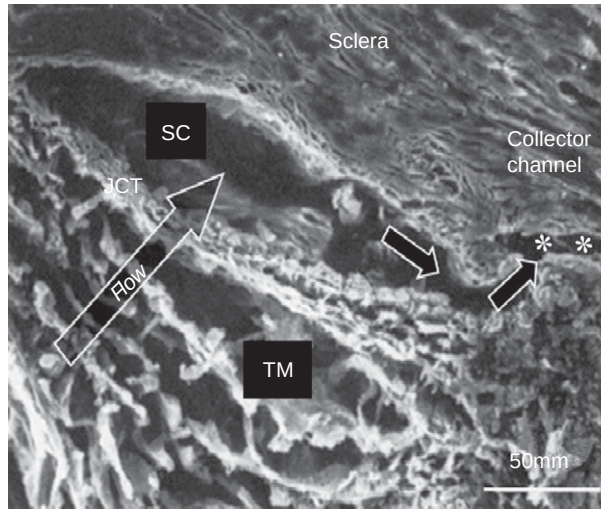

FIGURE 5 Scanning electron micrograph showing a sagittal section through the trabecular meshwork (TM), the juxtacanalicular region (JCT), Schlemm's canal (SC), and one of the external collector channels that leads from Schlemm's canal to the episcleral venous system (Freddo, 1993).

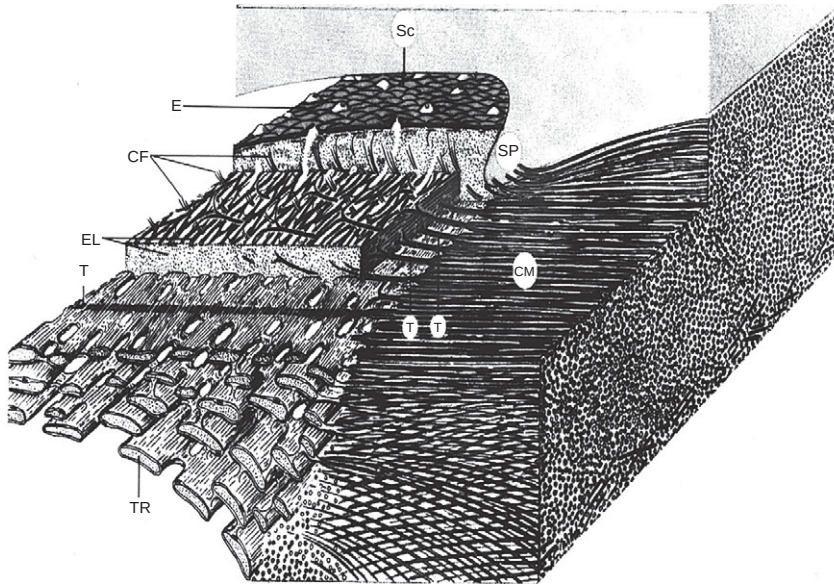

FIGURE 6 Tendons (T) extending from the longitudinal bundle of the ciliary muscle (CM) attach to the scleral spur (SP) but also extend elastic (EL) connecting fibrils (CF) to attach to the endothelial cells (E) lining Schlemm's canal (SC) (Rohen, 1983).

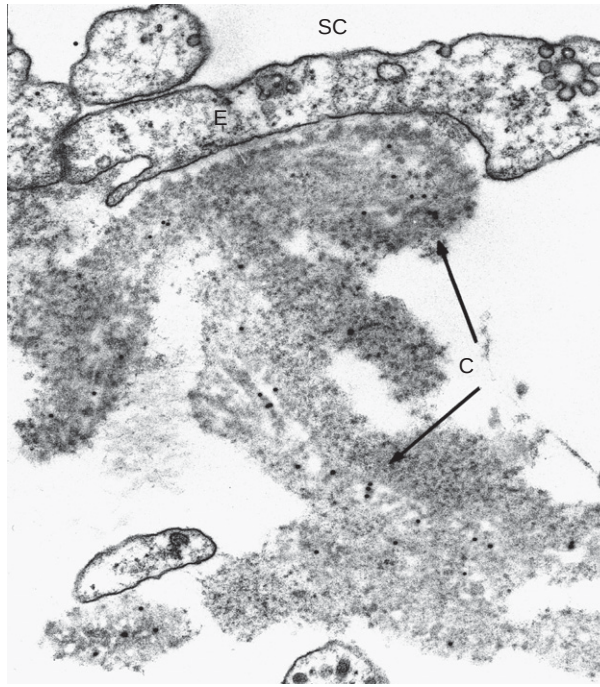

FIGURE 7 High magnification electron micrograph demonstrating detail of an elastic connecting fibril (C) of the cribriform plexus making connection with an endothelial cell (E) of the inner wall of Schlemm's canal (SC) (Gong *et al.*, 1989; Fig. 3).

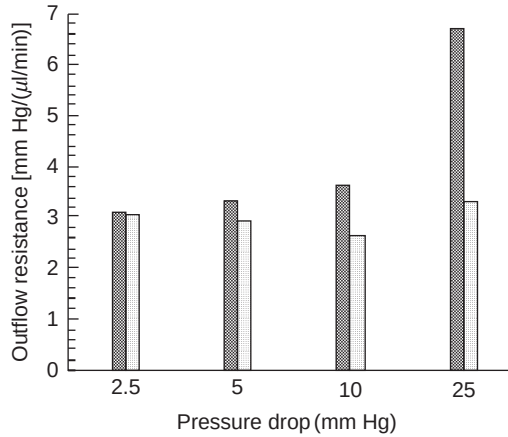

FIGURE 8 Effect of pressure drop (IOP, episcleral venous pressure) on outflow resistance in enucleated eyes without (▨) or with lens depression (▭); data from (Van Buskirk 1976).

The potential for collapse of Schlemm's canal as IOP increases has led some to speculate that this might be a cause of POAG (Nesterov, 1970). However, the outflow resistances of normal enucleated eyes, perfused at pressures that lead to extensive collapse of Schlemm's canal (Johnstone and Grant, 1973), were not as high as those of typical glaucomatous eyes that range from 10 to 100 mm Hg/(μl/min) in untreated glaucoma (Grant, 1951). Taken together, these findings suggest that while collapse of Schlemm's canal might make a glaucomatous condition worse, it is not likely to be a primary cause of the disease (Johnson and Kamm, 1983).

Leading from the outer wall of Schlemm's canal are approximately 30 collector channels spaced around the circumference of Schlemm's canal. These collector channels connect to the deep scleral plexus, then the intrascleral venous plexus, and finally, the episcleral veins where aqueous humor mixes with the venous blood. In some eyes, a smaller number of vessels lead directly from Schlemm's canal to the episcleral veins, bypassing the scleral plexi. These are termed aqueous veins and are identified clinically by the fact that aqueous humor and blood are seen to run within them in a laminar flow (Fig. 9). Both the collector channels and the channels to which they lead,

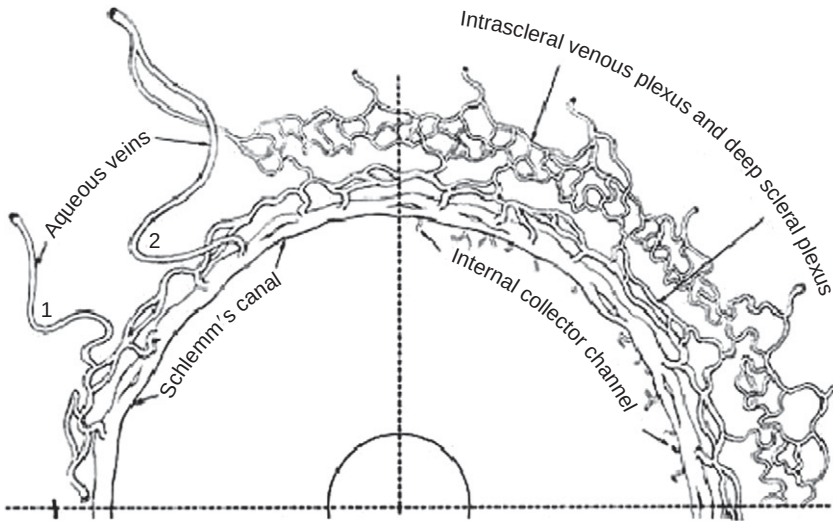

FIGURE 9 Diagram showing the aqueous outflow pathways from Schlemm's canal to the episcleral vessels. External collector channels emerging from the outer wall of Schlemm's canal (upper right) lead to deep and intrascleral plexuses and then to the episcleral vessels. Aqueous veins (upper left) arising from either external collector channels or the outer wall of Schlemm's canal, bypass this more convoluted pathway to the episcleral veins. [Modified from: Hogan, M., *et al.*, (1971). Figs. 4–19.]

enroute to the venous blood, have diameters that are tens of micrometers in size, and calculations indicate that these vessels should have negligible flow resistance (Dvorak-Theobald, 1934; Batmanov, 1968; Rohen and Rentsch, 1968; Rosenquist *et al.*, 1988).

The experimental evidence regarding this question is less clear. Mäepea and Bill (1992) using micropipettes measured the pressure in Schlemm's canal in living monkeys and received results in agreement with the theoretical calculations, namely, that the collector channels and vessels leading to the episcleral veins generated less than 10% of the total outflow resistance (Mäepea and Bill, 1989). However, a number of investigators have perfused enucleated primate and human eyes following a 360° trabeculotomy that should remove all flow resistance proximal to the collector channels and aqueous veins. These studies show that at high IOP, 25% of the flow resistance resides in collector channels and the vessels leading to the episcleral veins, while at low IOP (7 mm Hg in an enucleated eye), ~50% of the flow resistance resides in these vessels.

This discrepancy has not yet been resolved, perhaps partially because other findings indicate that the increased outflow resistance characteristic of POAG is not caused by an increased flow resistance of the collector channels or the vessels leading to the episcleral veins. Grant (1963) found that, in eight enucleated eyes from patients with POAG, a 360° trabeculotomy eliminated all of the elevated outflow resistance of these eyes, indicating that the enhanced flow resistance in POAG is proximal to the collector channels. This conclusion is further supported by the success of laser trabeculoplasty (LTP) in reducing the outflow resistance of such glaucomatous eyes (Wise and Witter, 1979). While it is not clear exactly how LTP works to lower IOP, the site of action appears to be in the trabecular meshwork rather than acting upon the collector channels or the vessels leading to the episcleral veins (Van Buskirk *et al.*, 1984; Bradley *et al.*, 2000; Johnson and Erickson, 2000).

The considerations above suggest that both in the normal eye and in the glaucomatous eye, the only tissues capable of generating a significant fraction of outflow resistance are those tissues in the immediate and vicinity of the JCT, the basement membrane of the inner wall endothelium of Schlemm's canal, and the endothelium itself. Experimental measurements attempting to localize the pressure drop in the outflow pathway lead to this same conclusion (at least in normal eyes) (Mäepea and Bill, 1992; Johnson, 2006).

V. REGIONS OF POTENTIAL SIGNIFICANT OUTFLOW RESISTANCE

Seidel (1921), examining the outflow pathway in 1921, stated that "the inner wall of Schlemm's canal stands in open communication with the anterior chamber, and that the aqueous humor directly washes around

the inner wall endothelium of Schlemm's canal and is only separated from the lumen by a thin, outer membrane''. It is either in or around this location that the bulk of outflow resistance likely resides in both the normal and the glaucomatous eye. We today tend to refer to this inner wall region as including the JCT, the basal lamina of the inner wall endothelium of Schlemm's canal, and the endothelium itself.

A. Extracellular Matrix and the JCT

The JCT, also called the endothelial meshwork or cribriform region, is the portion of the meshwork positioned between the beams of the corneoscleral meshwork and the basal lamina of the inner wall of Schlemm's canal. It varies in thickness between a few micrometers in some locations to perhaps 10 μm in others. It is not nearly as well ordered as is the corneoscleral meshwork. There is nothing resembling a beamlike structure. It is, instead, composed of a loose connective matrix that is very porous (30–40% open space) under typical flow conditions (Figs. 4, 10, and 11; Ten Hulzen and Johnson, 1996).

The cells in this region, whose type has not been definitively determined, are fibroblastic in appearance and lack a basal lamina (Gong *et al.*, 1996). They are connected to one another and also to the collagen and elastic fibrils in this tissue (Tervo *et al.*, 1995). These cells exhibit thin processes that make connections with the endothelium of the inner wall of Schlemm's canal (Fig. 4).

The extracellular matrix in the JCT region includes collagen types I, III, IV, V, and VI (but not type II) (Lütjen-Drecoll *et al.*, 1989; Marshall *et al.*, 1990, 1991) elastin, (Gong *et al.*, 1989); laminin (Marshall *et al.*, 1990); fibronectin (Gong *et al.*, 1996); and glycosaminoglycans (GAGs),

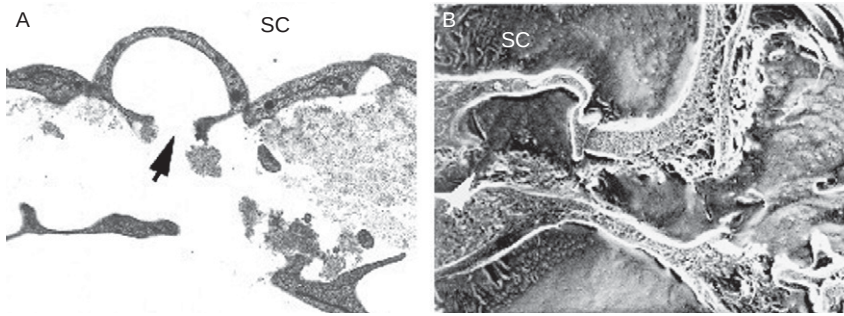

FIGURE 10 Micrographs of inner wall of Schlemm's canal (SC) and JCT from an 81-year-old eye. (A) Conventional TEM and (B) QFDE image preparation. Both show vacuoles with discontinuities in their basal lamina at the basal opening into the vacuole (arrows) (×11,100) (Gong *et al.* 2002).

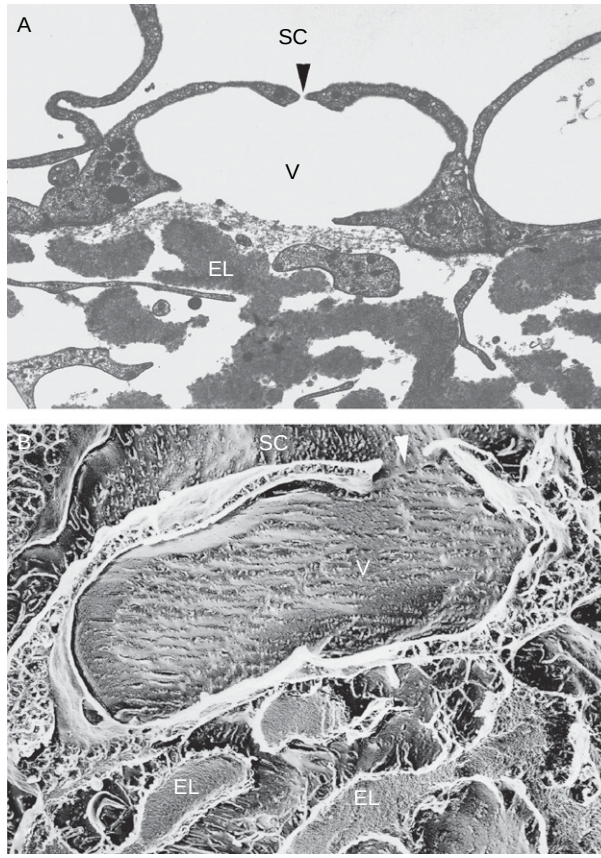

FIGURE 11 Transmission electron micrograph and quick-freeze, deep-etch micrograph of giant vacuoles exhibiting "pores" connecting the lumen of the giant vacuole (V) with the lumen of Schlemm's canal (SC). EL, elastic fibers in JCT region. (Courtesy of Haiyan Gong.).

particularly chondroitin sulfate, dermatan sulfate, and hyaluronic acid (Gong *et al.*, 1996). In glaucoma, there is a loss of hyaluronic acid from this region (Knepper *et al.*, 1996b). There is also an accumulation of a material called plaque (Lütjen-Drecoll *et al.*, 1981), although it does not appear to have any hydrodynamic consequences (Alvarado *et al.*, 1986; Murphy *et al.*, 1992).

The tortuous and relatively small flow pathways through the JCT were once attractive candidates for the generation of significant outflow resistance. Surprisingly, these expectations were not supported by hydrodynamic

considerations (Kamm *et al.*, 1983; Seiler and Wollensak 1985; Ethier *et al.*, 1986). Fluid flow through complicated structures such as soils, polymer networks, and connective tissues are frequently modeled by using porous media theory. In such an approach, the specific hydraulic conductivity (K) of the medium is the property that characterizes its intrinsic capacity to carry flow. It is closely related to the flow resistance of that tissue:

$$R = \frac{\mu L}{KA} \tag{2}$$

where μ is the viscosity of the fluid passing through the tissue, L is the flow-wise length, and A is the cross-sectional area facing flow. For most connective tissue, K ranges in value from 10^{-13} to 10^{-15} cm^2 (Johnson, 2006), although the very loose vitreous humor has a specific hydraulic conductivity of 10^{-11} cm^2.

If we assume that the entire pressure drop in the aqueous outflow pathway (roughly 5 mm Hg) occurs across the JCT, then we can use Eqs. (1) and (2) to estimate the K of the JCT. As noted above, aqueous humor has a viscosity similar to that of saline (0.007 g/cm s) and flows through the outflow pathway at a rate of $\sim$2 μl/min. The approximate cross-sectional area facing flow can be determined by multiplying the width of Schlemm's canal (150–350 μm) (Ten Hulzen and Johnson, 1996) by it length around the eye of $\sim$3.6 cm.

The only parameter that is not well known is the length over which the pressure drop occurs. Mäepea and Bill (1992) used micropressure measurements in the outflow pathway to find that this pressure drop occurs within 14 μm of the inner wall of Schlemm's canal. We can then use Eqs. (1) and (2) to conclude that if all or most of the pressure drop in the outflow pathway occurs across the JCT, then the K of this tissue must be less than 9×10^{-13} cm^2 (Johnson and Erickson, 2000; Johnson, 2006).

K for a tissue can also be estimated from micrographs showing the ultrastructure of that tissue (Johnson, 2006). By morphometrically characterizing the open spaces in that tissue, K can be determined. Carmen-Kozeny theory relates the structure of a porous medium to K as:

$$K = \frac{\varepsilon D_h^2}{80} \tag{3}$$

where D_h is the hydraulic diameter characterizing the open spaces in the porous medium and ϵ is the porosity or fraction of open space in the medium. D_h can be found by determining both the porosity of a tissue and the surface area per unit volume of its open spaces (α) as $D_h = 4\varepsilon^3/\alpha^2$.

Several groups have used such an approach to estimate K of the JCT based on its morphological appearance as seen by conventional electron microscopy (EM) (Kamm *et al.*, 1983; Seiler and Wollensak, 1985; Ethier *et al.*, 1986; Murphy *et al.*, 1992; Ten Hulzen and Johnson, 1996). In these studies, D_h was typically 1–1.5 μm, and more importantly, K of the JCT was calculated to be $\sim 200–1000 \times 10^{-13}$ cm^2. This is at least 20 times greater than the maximum value of K of this tissue based on experimental measurements.

Based on these findings, Ethier *et al.* (1986) concluded that the JCT region, as visualized using conventional EM techniques, could not generate an appreciable fraction of aqueous outflow resistance. They further concluded that this region must either be filled with an extracellular matrix gel that is poorly visualized using conventional EM techniques, or that this region is not the primary site of outflow resistance.

To evaluate the first possibility, Gong *et al.* (2002) used the quick-freeze/deep-etch (QFDE) method to examine the apparent open spaces in the JCT region in detail. QFDE is a technique that allows exquisite preservation of tissue ultrastructure at nanometer length scales. Using this technique, a much more elaborate and extensive extracellular matrix was seen in the JCT than seen using conventional techniques; however, openings nearly a micrometer in size were still seen in this region casting doubts as to whether a significant fraction of outflow resistance could be generated by this tissue (Figs. 10 and 11).

An important caveat pointed out by Gong *et al.* (2002) was that it was not clear whether and to what extent GAGs would be well preserved using their methods, and this reservation leaves the question of generation of significant outflow resistance in the JCT region in doubt. There is conflicting evidence in the literature as to whether GAGs and other extracellular moieties contribute to aqueous humor outflow resistance.

B. Possible Role of Glycosaminoglycans

Proteoglycans consist of a protein core to which negatively charged GAGs side chains are attached. The resulting structure is space filling as a consequence of the highly charged GAGs, and this gives rise to the potential to generate significant flow resistance. This characteristic also leads to these structures being difficult to preserve during morphological examination since the counterions used as stains for conventional TEM and the salt that remains after the sublimation step in QFDE would each be expected to collapse the GAG structures.

Along with GAGs, other extracellular moieties such as small nonfibrillar collagens and fibronectin have also been shown in other connective tissues to be associated with the generation of flow resistance. However, unlike GAGs,

these other extracellular macromolecules do not collapse when tissues are prepared for EM examination. Thus, as the appearance of the JCT as seen by both conventional EM and by QFDE preparation techniques appears not to be consistent with the generation of appreciable flow resistance, only GAGs are candidates as extracellular molecules in the JCT that might generate significant flow resistance.

Early studies by Bárány (1953, 1956) showed that testicular hyaluronidase dramatically decreased outflow resistance in enucleated bovine eyes. Pedler (1956) confirmed these findings. This was consistent with the role of GAGs in other tissues (Meyer, 1953; Comper and Laurent, 1978). Testicular hyaluronidase has been reported to increase outflow facility in a guinea pigs (Melton and DeVille, 1960), dogs (Van Buskirk and Brett, 1978), and rabbits (Knepper *et al.*, 1984). Knepper (1980) found that chondroitinase AC, chondroitinase ABC, and *Streptomyces* hyaluronidase increased outflow facility in the enucleated rabbit eye in a dose-dependent manner.

While the outflow pathways of nonprimates appear to be sensitive to these agents that degrade GAGs, the evidence is far less clear in primate and humans. Peterson and Jocson (1974) found a significant effect of testicular hyaluronidase on enucleated primates eye and Sawaguchi *et al.* (1992) reported that chondroitinase ABC decreased IOP in living cynomolgus monkeys as compared with control eyes receiving heat-inactivated enzymes. Hubbard *et al.* (1997) found no effect of chondroitinase ABC or *Streptomyces* hyaluronidase on IOP or outflow facility, either chronically or acutely, in living monkeys. Furthermore, studies on human eyes have shown no effects of GAGase on outflow resistance (Pedler, 1956; Grant, 1963).

Indeed, biochemical studies show a decrease in hyaluronan in the meshwork in glaucoma (Knepper *et al.*, 1996a), and additional studies also have shown a decrease in sulfated proteoglycans with age in normal human trabecular meshwork (Gong *et al.*, 1992). A decrease in these extracellular matrix components with age would be inconsistent with attribution of an increase in outflow resistance to excess accumulation of GAGs in an age-related disease such as glaucoma.

More recently, the role of the other extracellular matrix components in contributing to outflow resistance in human eyes has been supported by work of Acott's group showing that matrix metalloproteinases (MMPs) reversibly increase outflow facility in perfused human anterior segment organ culture (Bradley *et al.*, 1998). However, MMPs are relatively nonspecific in their action, and the locus of their activity was not determined in this study. It is possible that the MMPs were acting not on extracellular matrix in the JCT, but instead on the basement membrane of the cells of the inner wall endothelium of Schlemm's canal, the topic we address in the following section.

C. *Possible Role of the Basement Membrane of the Endothelial Lining of Schlemm's Canal*

The fundamental role of the basement membrane is as a structural support of the epithelial tissue it supports. Vascular endothelium requires a strong substrate to support it against the load of the vascular transmural pressure. In the case of the aqueous outflow pathway, attachments of the Schlemm's canal endothelial cells to this underlying substrate may assist the cell to "hold on" against the flow passing through this endothelium and entering Schlemm's canal.

The basement membrane can be a source of significant flow resistance in some tissues. Typically, the flow resistances of physiological membranes are described in term of their hydraulic conductivity (L_p) which is defined as the flow per unit area per pressure drop. This can be related to K as follows:

$$L_p \equiv \frac{Q/A}{\Delta P} = \frac{K}{\mu L} \tag{4}$$

Since the flow per unit area (Q/A) and the pressure drop of the outflow pathways are properties that are well known, it is straightforward to determine that L_p is between 4000×10^{-11} and 9000×10^{-11} cm^2 s/g (Johnson, 2006).

Table I shows measured value of L_p for several basement membranes. L_p for the aqueous outflow pathway is comparable to that of other basement membranes also involved in water transport, namely, Bruch's membrane through which the retinal pigment epithelium pumps fluid, and of course, the kidneys.

This supports the possibility that the basement membrane of the inner wall endothelium of Schlemm's canal might generate a significant flow resistance. Further supporting this possibility is the composition of basement membranes. The type IV collagen, heparan sulfate, fibronectin, and laminin that make up basement membrane would be expected to be degraded by the MMPs of the types shown to affect outflow resistance by Acott's group (Bradley *et al.*, 1998).

Morphological examination of the inner wall basement membrane using conventional methods of tissue preparation for EM do not preserve the basement membrane in sufficient detail to allow a morphometric analysis of the flow resistance such as has been done on the JCT. However, it has been reported (Gong *et al.*, 1996) that unlike vascular basement membranes, the basement membrane of the inner wall endothelium is discontinuous. Studies using QFDE appear to confirm this conclusion (Fig. 10; Gong *et al.*, 2002).

TABLE I
Hydraulic Conductivity of Basement Membranes.

Tissue	$L_p \times 10^{11}$ (cm^2 s/g)
Descement's membrane (Fatt, 1969)	15–37
Lens capsule (Fisher, 1982)	17–50
Bruch's membrane (eyes under 40 years old) (Starita *et al.*, 1996)	2000–12,500
Kidney tubule basement membrane (Bentzel and Reczek, 1978; Welling and Welling, 1978)	6300–13,700
Renal glomerulus basement membrane (Daniels *et al.*, 1992)	7600–25,000

It is hard to see how the basement membrane can be a significant source of flow resistance in the aqueous outflow pathway if it is a discontinuous layer, as the fluid would flow through the breaks rather than passing through the membrane itself. While the flow resistance of these breaks have not been explicitly calculated, these breaks are ubiquitous and typically are a fraction of a micrometer or larger in size. These breaks would not be expected to generate significant outflow resistance.

It is important to mention that Hann *et al.* (2001) found no difference in ultrastructural labeling for fibronectin, laminin, or type IV collagen comparing normal to glaucomatous eyes in the basal lamina of Schlemm's canal. This suggests that even though the basement membrane was found to be a significant source of outflow resistance in the normal eye, it is not likely to be responsible for the elevated flow resistance characteristic of the glaucomatous eye.

Changes have been found in glaucomatous eyes in the cells of their inner wall endothelium as compared to normal eyes. We now turn our examination to that tissue.

VI. ENDOTHELIAL LINING OF SCHLEMM'S CANAL

A. How Does Aqueous Humor Cross the Continuous Endothelial Barrier Presented by the Endothelial Lining of Schlemm's Canal?

The debate on how or even whether aqueous humor crosses the inner wall of Schlemm's canal was engaged early in the study of glaucoma. No less prominent luminaries than Schwalbe and Leber were diametrically opposed on this issue, the former contending that open communications across

Schlemm's canal must exist and the latter insisting that the inner wall of Schlemm's canal was a continuous membrane requiring either passive filtration or active transport (Leber, 1895). More than 100 years later the debate has been refined but certainly not resolved.

The inner wall of Schlemm's canal is composed of endothelial cells that appear to be vascular in their embryological origin (Krohn, 1999). These cells encounter a direction of flow that is inward, toward the lumen of the canal. As such, they are presumed to share more characteristics with postcapillary venules and lymphatics rather than with arterioles. A recent comprehensive comparison of known characteristics of blood capillaries, lymphatics, and the wall of Schlemm's canal concluded that the inner wall of Schlemm's canal is unique, sharing some but not all of the features of either vascular or lymphatic endothelia (Ramos *et al.*, 2007).

One of the clearly distinguishing features that makes the inner wall of Schlemm's canal unique is the way in which this endothelium responds to changes in pressure. When human eyes are placed into fixative fluid at zero (i.e., atmospheric) pressure, rather than being fixed under conditions of flow, the endothelium of Schlemm's canal is generally flat and featureless. When fixative is introduced under conditions of flow, however, remarkable endothelial blebs are found to bulge into the lumen of Schlemm's canal. These have been termed "giant vacuoles" (Figs. 10 and 11).

Giant vacuoles are discernable at the light microscopic level. Initially there was debate as to whether these structures were truly vacuoles, meaning transcytoplasmic channels that enveloped an aliquot of aqueous humor on the abluminal side of Schlemm's canal and conveyed it to the lumen (Tripathi, 1968, 1971, 1974). Electron microscopic studies using serial sections have since shown that the vacuoles all have an opening on their basal aspect and thus these structures appear to be outpouchings, bullous separations, or invaginations of the inner wall cells caused by the pressure drop across the inner wall, rather than intracellular structures (Inomata *et al.*, 1972; Johnson and Erickson, 2000).

Given the remarkable appearance of these structures, initial skepticism arose as to whether they were physiological or artifactual (Shabo *et al.*, 1973; Grierson and Johnson, 1981). In this regard, it is reassuring to appreciate that virtually identical "vacuoles" are found in the arachnoid villi, which reabsorb cerebrospinal fluid (Tripathi, 1977b).

A careful analysis of the baboon outflow pathway clearly distinguished giant vacuoles from postmortem changes in this tissue (Grierson and Johnson, 1981). Adding more credibility to the existence of giant vacuoles as a physiological phenomenon are findings that the number and size of giant vacuoles increases with increasing IOP. The number of vacuoles increases

linearly with pressure while the increase in size with increasing pressure (and therefore the volume of fluid contained within them) is nonlinear (Grierson and Lee, 1977, 1978a).

Current evidence points to the process of giant vacuole formation as being entirely passive and not requiring either the expenditure of cellular energy resources or protein synthesis. These vacuoles are found in greater number near the ostia of collector channels (Parc *et al.*, 2000). As an indication of their longevity, within 3 min of discontinuing perfusion, Schlemm's canal returns to its featureless state, exhibiting no giant vacuoles (Brilakis and Johnson, 2001).

One line of investigation explored the possibility that a loss of passive deformability of the endothelium could create a resistive element in the eye with glaucoma, possibly making it more difficult to passively form giant vacuoles. It has been shown that inner wall cells from eyes with glaucoma exhibit fewer sialic acid moieties, inferring that this led to the membranes being less deformable, making the process of vacuole formation more difficult (Tripathi*et al.*, 1987).

B. How Does Aqueous Humor Cross the Inner Wall of Schlemm's canal?: Pores or Paracellular Flow or Both?

The fact that the number of giant vacuoles increases with increasing pressure suggests a relationship between their formation and outflow. But how does aqueous humor actually cross the endothelial barrier presented by Schlemm's canal? Years ago, it was assumed that the expanding giant vacuole compressed the cytoplasm of the distended endothelial cells, ultimately resulting in development of a pore through which its aliquot of aqueous humor was discharged into Schlemm's canal (Tripathi, 1977a). This implied that giant vacuoles form, burst, and collapse, a process referred to as its "life cycle." Although nonvacuolar openings were identified in the inner wall in these studies, they were found to be occupied by wandering cells (Tripathi, 1974), implying that any pores found in the inner wall should be associated with giant vacuoles as illustrated in Fig. 11. By scanning electron microscopy (SEM), however, numerous pores are found when viewing the luminal surface of the inner wall of Schlemm's canal (Fig. 12) and not all of these are associated with giant vacuoles. Some of these have ragged edges, leading investigators to suspect this population as preparation artifacts (Ethier and Chan, 2001). But pores with smooth edges found both within the walls of giant vacuoles and in areas of the inner wall show no evidence of the typical bulging associated with giant vacuoles. The apparent lack of a one-to-one correspondence between pores and vacuoles, as seen by SEM, merits further

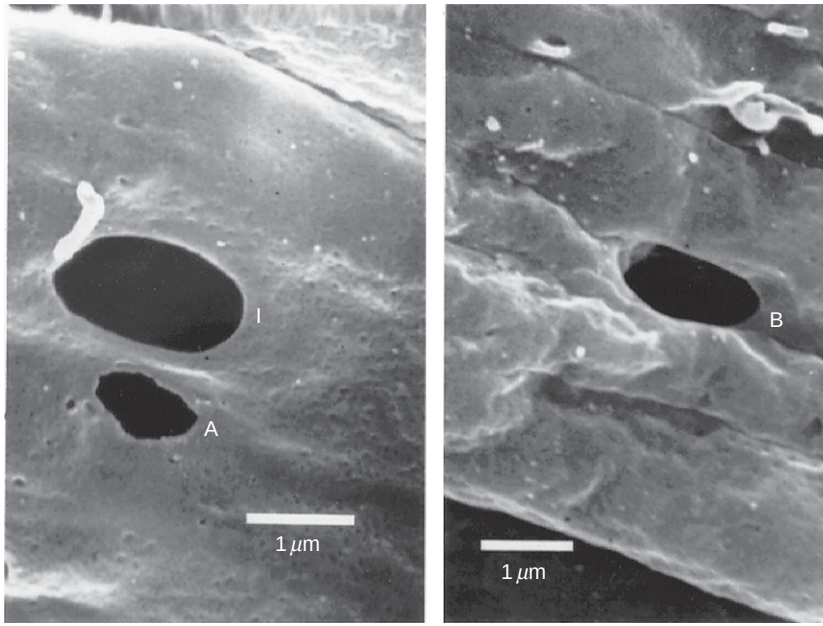

FIGURE 12 Scanning electron micrograph of pores in the inner wall endothelium. Left, an intracellular or I-pore (I) and an artifactual pore (A); and right, an intercellular or B-pore (B) (Ethier *et al.*, 1998).

study to better understand the role of the vacuole, if its purpose is not to give rise to a pore that releases the aliquot of aqueous contained within it. Equally important is the issue of whether pores associated with vacuoles and those not associated with vacuoles might form in different ways.

Allingham *et al.* (1992) reported that the density of pores in the inner wall endothelium was inversely correlated with outflow resistance and that fewer were found in eyes with POAG. This raised the question as to whether a reduced capacity to form pores might contribute to the added resistance in the outflow pathway of the glaucomatous eye. In that study, however, eyes were fixed at a constant pressure, resulting in much lower flow rates in glaucomatous eyes than in normal eyes.

In more recent studies (Sit *et al.*, 1997; Ethier *et al.*, 1998; Johnson *et al.*, 2002), in which fixation was completed under conditions of constant flow rather than constant pressure, pore density was not correlated with outflow facility but did increase with increasing volume of fixative passed through the system. Importantly, the fundamental notion that glaucomatous eyes exhibit fewer pores than normal eyes was confirmed.

Two subtypes of pores have more recently been described. Some of these occur at the border between adjacent endothelial cells and are termed "B" pores. Still others occur away from areas of cell borders. These are termed "I" (intracellular pores) (Ethier *et al.*, 1998). Studies attempting to better define these two types of pores have demonstrated that I-pores decrease with postmortem time suggesting that they are not artifactual (Johnson *et al.*, 2002). Both B-pore and I-pore density correlated with volume of fixative perfused, but only the I-pore result was statistically significant. Complicating this analysis was the fact that the density of B-pores correlated strongly ($p < 0.01$) with the number of I-pores, suggesting that either both were artifacts or neither were artifacts.

C. *Paracellular Flow?*

Whether either or both types of pores proves to be artifactual or real, other investigators favor paracellular flow, between inner wall endothelial cells, as the principal pathway for entry of aqueous humor into the lumen of Schlemm's canal. Epstein and Rohen (1991) perfused monkey eyes with cationized ferritin at normal and elevated IOPs. Remarkably, little tracer was found within or lining the giant vacuoles. Instead, the tracer was found to decorate the interendothelial clefts between inner wall cells and accumulate in paracellular channels that became more distended under conditions of elevated pressure. Such distentions would presumably have an impact on the permeability properties of intercellular junctions between adjacent endothelial cells. Subsequently, (Ethier *et al.* (2001) showed that cationized ferritin dramatically reduced outflow facility compared with its anionic counterpart, even with tenfold lower concentrations. Unlike anionic ferritin, cationized ferritin was shown to cluster and to distribute itself along the interendothelial clefts but especially around the openings of pores. These studies did not, however, reconcile the relative importance of the paracellular pathway versus pores.

In thin-sectioned material, several investigators reported various forms of intercellular junctions between cells forming the inner wall of Schlemm's canal (Vegge, 1967; Grierson *et al.*, 1978b). In one such study, horseradish peroxidase was perfused into the anterior chambers of normal human and monkey eyes. The junctions of the inner wall of Schlemm's canal blocked the passage of this material, suggesting the presence of tight junctions (MacRae and Sears, 1970).

Where tight junctions (zonulae occludentes) exist, they are invariably accompanied by zonular junctions of the adherens type. These latter junctions serve to provide adhesion, a prerequisite for junction formation and for

repair following major disruptions of tight junction integrity. But the adherens junction itself does not represent the occluding element of the junctional complex. Screens of cDNAs from inner wall cells have been shown to exhibit PECAM-1 and VE-cadherin (Heimark *et al.*, 2002). Their presence suggests the presence of adherens junctions, and this expression is also consistent with Schlemm's canal being embryologically of vascular origin (Heimark *et al.*, 2002). The protein ZO-1 has also been documented in inner wall cells (Alvarado *et al.*, 2004), but this protein is also intracellular and does not directly influence flow in the paracellular space (McNeil *et al.*, 2006).

Definitive demonstration of tight junctions (zonulae occludentes) between the endothelial cells of the inner wall of Schlemm's canal came with publication of freeze-fracture replicas showing a simple zonulae occludentes represented by as many as four discontinuous tight junctional strands in monkey eyes, with corridors through the junctional matrix that were termed "slit-pores" (Raviola and Raviola, 1981). It is possible that these "slit-pores" could be the freeze-fracture correlate of the distentions in the paracellular channels observed by Epstein and Rohen (1991) in sectioned material. Using their freeze-fracture replicas, the Raviolas completed exhaustive morphological studies to calculate potential flow through the "slit-pores" they documented, concluding that paracellular flow would be negligible. Unfortunately, these studies were performed on eyes fixed by immersion and not under physiological conditions of flow, a point raised by Epstein and Rohen to suggest that the Raviola calculations could have underestimated the potential for flow.

When freeze-fracture studies were first completed on the inner wall of Schlemm's canal in human eyes, immersion fixed material was again used. In these studies, the interendothelial junctions of Schlemm's canal inner wall cells were even more robust than those seen in monkey eyes, but pathways across the junctional matrix, similar to those described by Raviola and Raviola (1981) as "slit-pores" were still found (Bhatt *et al.*, 1995).

Following on from these studies, the same group repeated these freeze-fracture studies but on human eyes, now fixed at increasing pressures (0, 15, 45 mm Hg). In these eyes, increasing pressure was found to result in simplification of tight junctional structure but not wholesale disruption or distortion of the junctional matrix (Fig. 13). In these same studies, in sectioned material, a reduction in overlap of the adjoined endothelial cells was observed (Ye *et al.*, 1997). Equally important, it was demonstrated that where an interendothelial cleft existed within the wall of a giant vacuole, a *focal* reduction in junctional complexity resulted (Fig. 14). This finding raised the prospect that if "B" pores are real, they might be the result of focal simplification of tight junctions from a normal strand number of 3 to 0. Importantly, small disruptions of tight junctions have been shown to repair very rapidly, and without

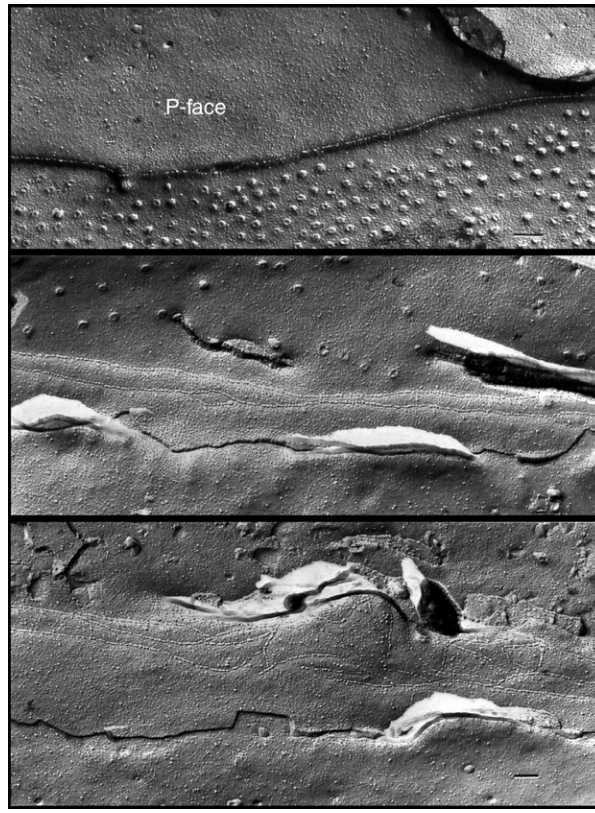

FIGURE 13 Freeze-fracture electron micrographs showing representative reduction in complexity of tight junctions between cells lining the inner wall of Schlemm's canal as pressure is increased from 0 mm Hg (bottom) to 15 mm Hg (middle) to 45 mm Hg (top). Magnification scale bar = 0.2 μm (Ye *et al.*, 1997, Fig. 2).

dependence upon either cellular energy or protein synthesis (Meyer *et al.*, 2001). As such, a focal reduction of tight junction strand number to zero would be expected to self-repair rapidly.

Junctional simplification with increasing pressure may also lie at the heart of the distentions in the paracellular pathway found using cationized ferritin, but Epstein and Rohen (1991) do not mention the reduction in length of the paracellular cleft found by Ye *et al.* (1997). We know from numerous studies that as IOP increases, facility of outflow decreases. Facility of outflow is increased, however, when separations are produced between cells lining the inner wall of Schlemm's canal. Such separations have been produced with an array of chemical agents, including ethacrynic acid (Epstein *et al.* 1987);

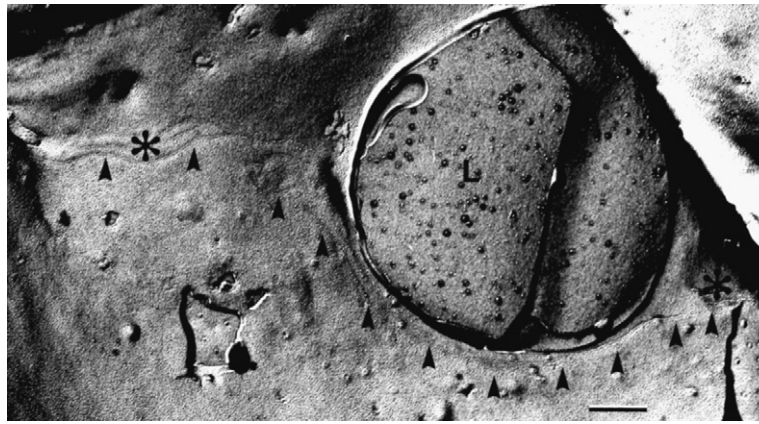

FIGURE 14 Freeze-fracture electron micrograph showing fracture plane through the lumen (L) of a giant vacuole. The resulting distention of the endothelial cells membranes diverted the course of the interendothelial tight junction joining two cells involved in formation of the vacuole (arrowheads). In the distended area, substantial simplification of the junctional structure is evident. Areas of the same junction on either side of the distended area continued to exhibit a more complex, multistranded junctional architecture (∗). Magnification scale bar = 0.5 μm (Ye *et al.*, 1997, Fig. 4).

alpha-chymotrypsin (Hamanaka and Bill, 1988); and EDTA (Bill *et al.*, 1980; Hamanaka and Bill, 1987). Clearly in all such instances, the intercellular junctions of the inner wall have been disrupted, but in a more dramatic way than the focal changes discussed above in possible relation to border pores.

Further studies of these matters are clearly imperative if the roles of pores and paracellular pathway are to be understood and possibly unified into an encompassing model of aqueous flow across the inner wall of Schlemm's canal. The findings from studies of "B" pores and the studies of the paracellular pathway would suggest that an improved understanding of tight junction regulation in the inner wall endothelium should be a priority because these junctions could be the common element limiting flow through each of these. Clearly, if "I" pores are not artifactual, however, a broader concept will be required in order to unify these findings into a single physiological model.

Understanding the actual mechanisms underlying normal aqueous outflow, and the altered outflow in glaucoma, would lead to the development of medical therapies to treat glaucoma at the source of the problem rather than by reducing the formation of aqueous humor—the nutritive fluid upon which the cornea, lens, and trabecular meshwork depend for metabolic support.

VII. SUMMARY

Unraveling the mystery of most diseases often begins with a simple comparison at the light microscopic level between the affected tissue in its normal and diseased state. The differences that are found serve to guide investigators to the ultimate cause of the disease. At this point, the differences found between the trabecular meshworks of age-matched normals and glaucomatous human eyes are very few—regardless of the method of analysis. This provides ample room for further investigation but is simultaneously a great source of frustration for both the basic scientist and the clinician. Much as glaucoma is a disease of increased resistance, the disease process itself remains resistant to giving up its secrets. Outflow resistance is the inverse of facility of outflow. Although our search for the source of outflow resistance in both the normal and glaucomatous eye must continue, the fluid mechanics of the process can still be simplified to their clinical essence using the formula developed by Goldmann: $F = C_{tm}\ (P_i – P_e) + F_u$, where F equals aqueous flow in μl/min; P_i and P_e represent intraocular and episcleral venous pressure, respectively, in mm Hg; F_u represents the component of outflow traveling the unconventional route; and the value C_{tm} remains the measurable but elusive facility of outflow—the mirror of outflow resistance.

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