The Pore Density in the Inner Wall Endothelium of Schlemm's Canal of Glaucomatous Eyes

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Purpose. In a prior study, it has been reported that glaucomatous eyes have a significantly lower density of pores in the inner wall of Schlemm's canal than do normal eyes. However, in that study the glaucomatous eyes were fixed at much lower flow rates than the normal eyes, and that is now known to affect inner wall pore density. The objective of the current study was to compare the inner wall's pore density in glaucomatous and normal eyes, accounting for the effects of fixation conditions.

Methods. Outflow facility was measured in enucleated glaucomatous human eyes. Eyes were fixed under constant flow conditions, microdissected to expose the inner wall of Schlemm's canal, and prepared for scanning electron microscopy (SEM). The density and diameter of the two subpopulations of pores in the inner wall, intracellular and intercellular (or "border") pores, were measured. Data were compared with those in previous studies of normal eyes.

Results. As previously reported, pore density decreased with increasing postmortem time and increased with increasing volume of fixative passed through the outflow pathway and with increasing fixation time. Linear regression analysis indicated that glaucomatous eyes had less than one fifth the number of pores than normal eyes have, after accounting for the influence of volume of fixative perfused through the eyes (835 pores/mm^2 in normal eyes versus 160 pores/mm^2 in glaucomatous eyes). A nonlinear regression of pore density versus fixative volume produced a pore density at zero fixative volume that was not statistically different from zero. If true, this implies that all (or nearly all) inner wall pores observed by SEM are fixation artifacts. The density of intracellular pores and the diameter of these pores correlated with the density and diameter of the border pores, respectively.

Conclusions. Inner wall pores are reduced in glaucomatous eyes. If pores are physiological structures, the elevated intraocular pressure characteristic of glaucoma may be explained by decreased porosity of the inner wall endothelium. Both border and intracellular pores seem to be induced in a similar fashion by fixation. The unlikely possibility that all inner wall pores are fixation-induced cannot be excluded. If so, a fundamental reassessment of the mechanism by which aqueous humor crosses the inner wall endothelium is necessary. (*Invest Ophthalmol Vis Sci.* 2002;43:2950–2955)

The precise route and mechanism by which aqueous humor crosses the inner wall endothelium of Schlemm's canal has been debated for more than 100 years.[1–4] When eyes are fixed by perfusion at pressure, it is observed that the inner wall of Schlemm's canal contains giant vacuoles that protrude from the endothelial layer into the lumen of the canal.[5–7] Associated with these giant vacuoles, but also occurring in other areas,[8] are pores passing through the inner wall. Many studies have shown that these inner wall pores could generate, at most, a small fraction of aqueous humor outflow resistance,[9–12] although the possibility of an indirect influence of inner wall pores on outflow facility has been proposed.[13]

Allingham et al.[14] reported a linear relationship between pore density and aqueous outflow facility and fewer inner wall pores in glaucomatous eyes than in normal eyes. This suggests that inner wall pores may have a role in determining outflow facility. However, the investigators used constant-pressure perfusion and constant-pressure fixation (both at 15 mm Hg). Recent findings[12] show that characteristics of inner wall pores depend on fixation conditions. In particular, the density of inner wall pores increases with the volume of fixative perfused through the outflow pathway. Because fixative flow rate across the inner wall is linearly proportional to outflow facility under constant pressure fixation conditions, the flow rate of fixative in the study by Allingham et al.[14] varied from eye to eye, being lower in the glaucomatous eyes. This caused glaucomatous eyes to receive lower total fixative volumes than normal eyes, which could explain the reduced pore density seen in glaucomatous eyes.

Our goal in the present study was to examine the pore density in glaucomatous eyes more definitively. We specifically wanted to know whether glaucomatous eyes have fewer inner wall pores, even after accounting for formation of pores due to fixation conditions.

Methods

The methods used in the present study are similar to those we have previously reported[12,15] and therefore will be only briefly summarized.

Three pairs of glaucomatous eyes were procured from eye banks. None of these eyes had had ophthalmic surgery, were from diabetic donors, or had a history of ocular disease (other than glaucoma). Because of confidentiality constraints, little further information was available concerning these eyes, but all three pairs of eyes had outflow facilities measured to be in the ocular hypertensive range (see Table 1).

The eyes were perfused within 25 hours of death, with Dulbecco's phosphate-buffered saline (Life Technologies, Grand Island, NY) with added 5.5 mM glucose. According to the method of Sit et al.,[12] eyes were perfused at a constant flow rate of 2 μL/min until a stable pressure was obtained. An exception was made in two of the six eyes, which had outflow facilities so low that the perfusion pressures were capped (at 50 mm Hg in one eye and 64.5 mm Hg in the other) to prevent pressure-induced damage. After outflow facility was measured, the anterior chamber was exchanged with a modified Karnovsky's

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TABLE 1. Characteristics and History of Donor Eyes Obtained for the Present Study

Eye	Age (y)	Ocular History/Medications	Fixation Pressure (mm Hg)	Prefixation C (μL/min per mm Hg)	Postfixation C (μL/min per mm Hg)	Total Pore Density (n/mm^2)
45	83	Glaucoma; no medications	64.5	0.01	0.01	232
46	83	Glaucoma; no medications	32.4	0.06	0.05	2577
47	85	Glaucoma; no information	16.3	0.12	0.08	482
48	85	Glaucoma; no information	50.0	0.03	0.014	128
49	74	Glaucoma for 20 years; eye drops	23.0	0.09	0.04	741
50	74	Glaucoma for 20 years; eye drops	26.0	0.075	0.03	519

C; outflow facility.

fluid,[15] and each eye was fixed at a constant pressure equal to the final pressure recorded just before anterior chamber exchange for that eye.

At the conclusion of fixation, the inner wall of Schlemm's canal was prepared for scanning electron microscopy (SEM), and pore densities and diameters were measured by using previously described methods.[15] Briefly, probable pores were identified from SEM montages made at 1,000×, and the original samples were then rescanned at 10,000× to verify that each putative pore was not artifactual. Tissue from all four quadrants was examined in all eyes. Typical total areas measured were 50,000 μm^2 per quadrant, totaling 200,000 μm^2 per eye. Openings in the endothelium were classified as artifactual openings and thus disregarded if the edges were rough, torn, irregular or notched. Border pores were defined to be those pores observed by SEM to intersect with a cell margin. Cell margins were usually easily identified, and therefore the categorization of pores as intercellular or intracellular was reasonably unambiguous. However, a small percentage of the pores (2%–3%) could not be definitively classified and were not included in either category. Failure to classify a pore was due to either a partially blocked field of view or ambiguity in the exact location of intercellular junctions. Because pores are typically elliptical, the major and minor axes were measured directly from the screen of the microscope at 10,000×. Length measurements were calibrated by means of two SEM calibration grids (SIRA Institute, Chislehurst, UK) with densities of 19.7 and 2160 lines per millimeter. Because circular pores appear elliptical when viewed in a tilted section, the pore diameter was assumed to be equal to the major axis of the pore.

The parameters measured in each eye included the patient's age (Age, in years), postmortem time to the start of the experiment (PM, in hours), time between the patient's death and enucleation (E, in hours), intracellular pore density of the inner wall endothelium (NI, in pores per square millimeter), border pore density (NB, in pores per square millimeter), total pore density of the inner wall endothelium (NT, in pores per square millimeter), average intracellular pore diameter (DI, in micrometers), average border pore diameter (DB, in micrometers), average total pore diameter (DT, in micrometers), outflow facility before fixation (C, in microliters per minute/mm Hg), outflow facility after fixation (CF, in microliters per minute/mm Hg), fixation pressure (IOP, in mm Hg), flow rate of buffer through outflow pathway before fixation (Q, in microliters per minute), volume of buffer perfused through outflow system before fixation (VB, in milliliters), volume of fixative perfused through outflow system (VF, in milliliters), and time that fixative was perfused through the outflow system (T, in minutes). As in our previous studies, an additional parameter, an estimated volume of fixative perfused VF' (in milliliters) = $0.6 \cdot Q \cdot T$, was calculated because the volume of fixative perfused was not directly measured in all studies.[15]

Statistical Analyses

In addition to the eyes we have described, our statistical analysis included data from our previous studies[12,15] and from Allingham et al.[14] for eyes in which postmortem time until the start of experiment was less than 25 hours. Thus, our total study group included 25 normal eyes and 10 glaucomatous eyes, broken down as follows: 6 normal eyes and 4 glaucomatous eyes from Allingham et al., 19 normal eyes from our previous studies,[12,15] and 6 glaucomatous eyes from the present study. Unless otherwise indicated, 35 eyes are included in all the analyses reported. It should be noted that because no distinction was made between intracellular and border pores by Allingham et al., only total pore density and diameter were available for that group of eyes. The correlations that were found in our earlier studies[15] were examined by analysis of variance to assess the difference between the normal eyes and the glaucomatous eyes. The residuals (the difference between the fitted value of the dependent parameter and its measured value) were examined and in all cases appeared random when plotted against the independent variables. Outliers of the fit and points with high leverage, as identified with a computer program (Systat for Macintosh, ver. 5.2.1, Chicago, IL), were also examined. If the externalized studentized residual (analogous to a t statistic) had a probability of occurrence less than $0.01/n$ (where n is the number of data points), this point was removed from the fit.[16] Points with high leverage (i.e., with a Cook distance greater than 1), were also removed.[16]

Comparison of means was performed with a two-sided Student's t-test. We also examined correlations between the two populations of pores. Specifically, we determined the Pearson correlation coefficient. The significance level in all studies was 0.05. Uncertainties reported are SEM.

RESULTS

Unless otherwise indicated, all results are based on the combined data sets of the present study, Allingham et al.,[14] Sit et al.,[12] and Ethier et al.[12,15] Also, in any data set in which an outlier(s) was identified, the data were analyzed both with and without the outlier(s).

Outflow Facility

Pre- and postfixation outflow facilities were correlated with the patient's age as previously reported ($P < 1 \times 10^{-9}$)[12,15] and the glaucomatous eyes had a lower age-matched facility than did the normal eyes ($P < 0.02$).

Perfusion with fixative decreased outflow facility in the normal eyes from a prefixation level of 0.21 ± 0.04 μL/min per mm Hg to a postfixation level of 0.14 ± 0.03 μL/min per mm Hg, a decrease of $42\% \pm 5\%$ ($n = 20$: series B of Sit et al.[12] and Ethier et al.[15] did not measure outflow facility after fixation); in the glaucomatous eyes ($n = 10$), this change was from 0.08 ± 0.01 to 0.050 ± 0.01 μL/min per mm Hg, a decrease of $37\% \pm 7\%$: the difference in the percentage of decrease between these groups was not statistically significant. This suggests that the fixation-induced facility reduction is approximately 40%, not 50%, as was previously estimated,[12] and justifies use of the factor 0.6 in the estimated fixative volume formula presented previously.

Total Pore Density and Diameter

Inner wall pore density increased as a function of estimated volume of fixative perfused, VF' (Fig. 1). We conducted a

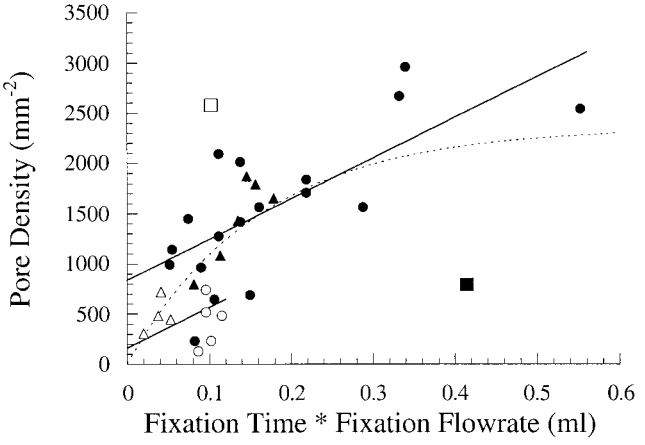

FIGURE 1. Total pore density as a function of estimated volume of fixative perfused in normal eyes (*filled symbols*) and glaucomatous eyes (*open symbols*). *Triangles*: data from Allingham et al.; *filled squares* and *circles*: data from Sit et al.; and *open squares* and *circles*: data from the present study. *Large squares*: outliers that were not included in the two linear fits shown: normal eyes (*top solid line*); glaucomas (*bottom solid line*). Also shown is a nonlinear fit (*dashed line*) to all the data. Data adapted, with permission from Allingham RR, de Kater AW, Ethier CR, Anderson PJ, Hertzmark E, Epstein DL. The relationship between pore density and outflow facility in human eyes. *Invest Ophthalmol Vis Sci.* 1992;33:1661–1669; and Sit AJ, Coloma FM, Ethier CR, Johnson M. Factors affecting the pores of the inner wall endothelium of Schlemm's canal. *Invest Ophthalmol Vis Sci.* 1997;38: 1517–1525.

two-way analysis of variance with total pore density as the dependent variable, and with one treatment variable being the presence or absence of glaucoma, and the second treatment variable being either volume of fixative perfused or postmortem time. Consistent with previous findings,[12] pore density increased with volume of fixative perfused ($P < 0.001$) and decreased with postmortem time ($P < 0.03$). Figure 1 also suggests that glaucomatous eyes have fewer pores than do normal eyes. The analysis of variance confirmed that this was the case; however, the result was only marginally statistically significant ($P < 0.065$).

Two of the data points were identified as outliers from the fit (Fig. 1, square symbols). Removal of these points led to much stronger correlations, with total pore density increasing with estimated volume of fixative perfused ($P < 2 \times 10^{-6}$), decreasing with postmortem time ($P < 0.02$) and with the glaucomatous eyes having less inner wall pores than the normal eyes ($P < 8 \times 10^{-4}$). The least-squares fit to the data with these outliers removed is shown in Figure 1.

Two variables closely related to *VF′* were the actual volume of fixative perfused (*VF*: not measured in five of the normal eyes studied) and the time of fixation (*T*). We have previously shown that all three of these variables correlate with the total pore density.[12] Conducting the analysis of variance using *VF* or *T* instead of *VF′* led to results similar to those in the *VF′* analysis, but we found that the glaucomatous eyes had significantly fewer pores than the normal eyes ($P < 0.03$), even without excluding the two outliers mentioned earlier. Of *VF′*, *VF*, and *T*, total pore density correlated most closely with *VF′* (*VF′*: $P < 0.001$;*VF*: $P = 0.01$; *T*: $P < 0.005$).

The diameter of the pores was found to increase with perfusion pressure ($P = 2 \times 10^{-4}$) and to decrease with the volume of buffer passed through the outflow pathway before fixation was started ($P = 6 \times 10^{-5}$). There was one point with high leverage that we had noted in our previous study[15]; however, even with exclusion of that point, the results were statistically significant ($P < 0.01$ and $P < 0.0002$, respectively). There was no difference between the diameter of the pores in normal and glaucomatous eyes ($P > 0.6$).

Intracellular and Border Pore Densities and Diameters

(Data in this section are from the present study and the studies of Sit et al.[12] and Ethier et al.[15]) The density of intracellular pores increased with *VF′* ($P < 0.008$) and decreased with postmortem time ($P < 0.003$). The glaucomatous eyes did not show a statistically significant difference from the normal eyes, but this was due to one glaucomatous eye (eye 46) that had a very high pore count. When this outlier was excluded, the density of intracellular pores was significantly lower in glaucomatous eyes than in normal eyes ($P < 0.025$).

Although the border pore density increased with *VF′* ($P < 0.004$), there was one outlier and one data point with large leverage (both in the normal eye data set). Removal of the point with large leverage eliminated the correlation with *VF′* ($P > 0.09$). No correlation was found with postmortem time or with pressure, although Ethier et al.[15] found a correlation between border pore density and pressure. The border pore density was marginally lower in the glaucomatous eyes than the normal eyes ($P < 0.053$ including the outlier eye 46; $P < 0.032$ otherwise), by two-sided Student's *t*-test. Similar results were found with *VF* and *T* instead of *VF′*. Figure 2 shows the density of both pores types as a function of fixation time.

The diameter of the intracellular pores and the border pores both increased with perfusion pressure and decreased with the volume of buffer passed through the outflow pathway before fixation was started. The significance levels of these correlations for the border pores ($P < 0.004$, $P < 3 \times 10^{-4}$, respectively) was somewhat lower than for the intracellular pores ($P < 0.007$, $P < 7 \times 10^{-4}$, respectively). There was no difference between the glaucomatous pore diameter and that of pores in the normal eyes for either pore type ($P > 0.2$).

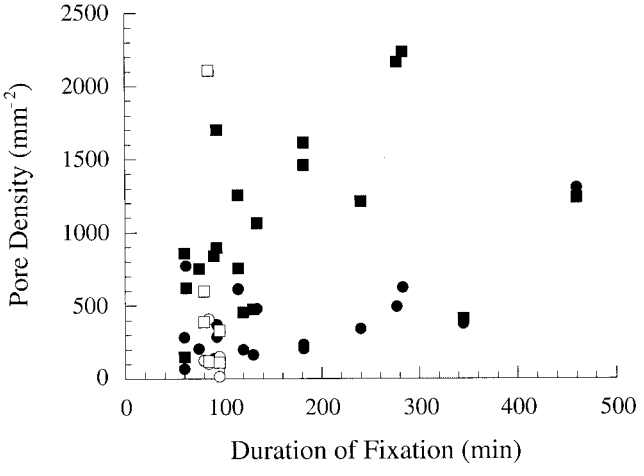

FIGURE 2. Density of intracellular (*squares*) and border (*circles*) pores as a function of fixation time in eyes. *Open symbols*: data from glaucomatous eyes; *filled symbols*: data from normal eyes. All data for the glaucomatous eyes in Figures 2 and 3 are from this study. Data for normal eyes were adapted, with permission from Allingham RR, de Kater AW, Ethier CR, Anderson PJ, Hertzmark E, Epstein DL. The relationship between pore density and outflow facility in human eyes. *Invest Ophthalmol Vis Sci.* 1992;33:1661–1669; Sit AJ, Coloma FM, Ethier CR, Johnson M. Factors affecting the pores of the inner wall endothelium of Schlemm's canal. *Invest Ophthalmol Vis Sci.* 1997;38: 1517–1525; and Ethier CR, Coloma FM, Sit AJ, Johnson M. Two pore types in the inner wall endothelium of Schlemm's canal. *Invest Ophthalmol Vis Sci.* 1998;39:2041–2048.

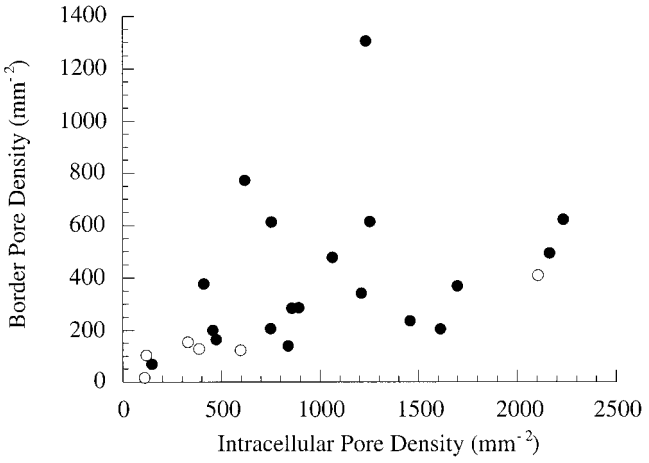

FIGURE 3. Intracellular pore density versus border pore density in normal eyes (*filled symbols*) and glaucomatous eyes (*open symbols*). All the data for glaucomatous eyes are from this study.

One of the more interesting findings of this study was the correlation between both the density ($r = 0.43$, $P = 0.03$) and the diameter ($r = 0.68$, $P < 2 \times 10^{-4}$) of the intracellular pores and the border pores. As Figure 3 shows, the densities of both pore types correlated in the normal and the glaucomatous eyes.

DISCUSSION

The mechanism by which fluid crosses the inner wall endothelium of Schlemm's canal has been debated for more than century. Schwalbe[1] and Leber[2] had a lively debate as to whether open communication existed between the anterior chamber and Schlemm's canal, with Leber contending that no pores existed and that the aqueous humor must pass through an intact membrane and Schwalbe insisting that there were pores passing through this membrane. Seidel[4,17] used tracer studies to confirm Schwalbe's contention of open communication and came to the conclusion that pores, not visible at the light microscopic level, must pass through the inner wall endothelium.

It was only with the advent of the electron microscope that these pores could be visualized. Examining serial sections, Holmberg[6] described pores in the inner wall endothelium of Schlemm's canal with diameters between 0.5 and 1.5 μm. With SEM, Bill and Svedbergh[9] characterized this pore population and found a density of 1840 pores/mm^2. By using hydrodynamic calculations, they concluded that these pores would generate, at most, 10% of the observed aqueous humor outflow resistance.

Normal Versus Glaucomatous Eyes

As discussed in the introduction, the role of inner wall pores remains controversial, nonetheless, in part because of recent findings of lower pore density in glaucomatous eyes[14] and the finding of Sit et al.[12] that inner wall pore characteristics depend on fixation conditions. We have extended the findings of these investigators by examining pore density in glaucomatous eyes that were fixed at significantly higher pressures than those examined by Allingham et al.[14] As Figure 1 shows, we found that even after accounting for the volume of fixative perfused, the glaucomatous eyes had a significantly lower pore density than did the normal eyes. If we assume that the intercept of the best linear fits to this data characterize the true in vivo pore density (i.e., at zero fixative volume), then the normal eyes have a "true" pore density of 835 pores/mm^2, whereas the glaucomatous eyes have a density of 160 pores/mm^2. In other words, glaucomatous eyes have only approximately one fifth the pore density of the normal eyes. This level of difference in pore density, if present in vivo, would almost certainly be physiologically significant. For example, the funneling theory[13] predicts that the effective resistance of the juxtacanalicular connective tissue (JCT)–inner wall is inversely proportional to pore density. Assuming that most outflow resistance is in the JCT–inner wall,[18] our data suggest that glaucomatous eyes have approximately a fivefold lower outflow facility than normal eyes, which is sufficient to explain the decreased outflow facility seen clinically in glaucoma.[19]

It would be difficult to use these data to make a more precise estimate of the true combined flow resistance of the JCT and the inner wall, because the magnitude of the funneling effect depends on the JCT resistance and the flow-wise length of this region—both unknowns.[13] Other theories predict that the flow resistance of the inner wall depends on pore density times pore diameter raised to the third power (Sampson's law) or the fourth power (Poiseuille's law).[15] In these cases, total flow resistance becomes sensitively dependent on the density of large pores. Because large pores are relatively infrequent, extrapolation of their density back to zero fixative volume has a very large degree of statistical uncertainty associated with it, which makes calculations of flow resistance based on Poiseuille's or Sampson's laws quite unreliable. A much larger data set is needed to make a reliable calculation.

In this study, we did not account for the possibility of a significant number of pores on the outer wall of Schlemm's canal. Vacuoles are occasionally seen on the outer wall, but we have no information about pore density in the outer wall. If there were evidence that a significant amount of filtration happened in the outer wall, or that the outer wall filtered more in glaucomatous eyes than in normal eyes, this question would have to be examined more closely.

Another complicating factor in this study was the potential collapse of Schlemm's canal in eyes fixed at high pressure, which occurred in some of the glaucomatous eyes perfused at constant flow. Collapse of Schlemm's canal could have obliterated pores, leading to lower average densities. However, there was no statistical difference in pore density between glaucomatous eyes perfused at constant pressure (Allingham et al. data set[14]) and those perfused at constant flow (new eyes in this study). This suggests that if the collapse of Schlemm's canal occurred, it was not significant, or was possibly offset by overformation of pores in noncollapsed regions.

Are All Pores Artifacts?

Another interpretation of Figure 1 is possible. There could be a nonlinear relationship between volume of fixative perfused and the inner wall pore density. We performed a least-squares fit of pore density to a simple nonlinear model: $C_0 + C_1 \cdot \exp(-VF'/V_0)$ where C_0 ,C_1, and V_0 are parameters determined by the best fit. The result is the dashed line in Figure 1.

It is noteworthy that the intercept C_0 is not statistically different from zero, indicating that another interpretation of Figure 1 is that most of or all of the pores in the inner wall are artifactual. If this were true, it would require a major revision of current thinking regarding how fluid crosses the inner wall of Schlemm's canal and where the major site of aqueous outflow resistance is located.

However, there are a several observations that argue against this interpretation. As mentioned earlier, Seidel[4,17] perfused tracers through the outflow pathway and found that micrometer-sized particles were able to traverse the aqueous outflow system. This finding has been confirmed by a number of inves-

tigators.[20–23] For example, Johnson et al.[23] found that approximately 50% of 0.5-μm microspheres successfully passed through the outflow pathway of enucleated human eyes. It is hard to understand how such a high fraction of particulates could successfully pass through the outflow pathway, except through pores.

A second argument in favor of pores, or some microsized flow structure, is the very high hydraulic conductivity of the inner wall endothelium. Johnson and Erickson[24] have pointed out that, based on the aqueous humor flow rate and a pressure drop of approximately 5 mm Hg, the hydraulic conductivity of the inner wall endothelium is the highest of any endothelium in the body, including fenestrated structures such as the renal glomerulus. Compared with nonfenestrated endothelia, the inner wall endothelium has a hydraulic conductivity that is at least 100 times larger. It seems that the most likely way to explain such a high hydraulic conductivity of the inner wall endothelium is that the fluid must pass through some porelike structure.

However, until histologic methods of preparation can be developed that do not artifactually generate pores, several interpretations of our data are possible.

Fixative-Induced Mechanisms of Pore Formation

Some clues to the mechanism of pore formation may be found from the correlation between pore density and volume of fixative perfused. This volume is the product of the flow rate of fixative and the duration of fixation. It seems reasonable to hypothesize that fixation at flow generates stresses in the inner wall, due both to the shrinkage of tissue after fixation[25,26] and to the pressure-induced stretching of the inner wall of Schlemm's canal. We further hypothesize that the magnitude and duration of this stress both affect formation of pores. Specifically, a higher fixative flow rate (leading to a greater transendothelial decrease in pressure) and longer fixation times lead to greater magnitude and duration of stress, respectively. Thus, in this scenario, it would be expected that the pore density would be a function of the product of the magnitude and duration of stress, leading to the observed correlation of pore density with fixation volume.

This scenario is consistent with an important finding of this study: the correlation between the density of intracellular pores and of border pores in both normal and glaucomatous eyes. The pore diameters of these two populations also correlated. Although it has been previously speculated that one of the two pore types may be artifactual, these correlations suggest that these two pore types have a common mechanism of formation—perhaps that just mentioned.

If this scenario is true, border pores may simply be enlargements of the paracellular route identified in ferritin tracer studies by Epstein and Rohen,[27] or perhaps border pores coexist normally with this paracellular route.[28] Because of resolution limitations and the tendency for inner wall cells to overlap somewhat at their borders, our experimental technique probably does not allow us to see paracellular flow routes that are less than approximately 0.2 μm in diameter. However, the correlation of border pores with intracellular pores suggests that fixation-induced stresses have a common influence on both of these pathways.

In summary, the major finding of this study is that pore density of the inner wall endothelium of glaucomatous eyes is less than one fifth that found in normal eyes. This may be responsible for the elevated intraocular pressure associated with glaucoma. However, an unlikely but alternate explanation of our data is that most or all inner wall pores are artifactual. Either of these two interpretations of the data would be a major change in the understanding of the generation of aqueous outflow resistance. Our data strongly indicate that at least one of these interpretations is correct. Future studies should evaluate different methods of examining the inner wall of Schlemm's canal, including methods that avoid fixation entirely.

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