Hydraulic Conductivity of Fixed Retinal Tissue After Sequential Excimer Laser Ablation

Barriers Limiting Fluid Distribution and Implications for Cystoid Macular Edema

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Objectives: To measure the hydraulic conductivity (HC) of human retina and to determine the presence and location of high-resistance barriers to fluid movement through the retina.

Methods: Forty-one pairs of human eyes were investigated using an HC chamber. Once baseline HC had been determined, the effect of ablating through varying thickness of retina from the vitreous or photoreceptor surface using an excimer laser (193 nm) was investigated. Tissue samples were then processed for histological investigation.

Results: The HC of fixed intact human retina was $2.54 \times 10^{-10}$ m/s per pascal at 539 Pa (range, $0.6 \times 10^{-10}$ to $3.3 \times 10^{-10}$ m/s per pascal; SD, $0.6 \times 10^{-10}$ m/s per pascal [1 mm Hg equals 133 Pa]). Ablation from either surface resulted in little change in HC until a critical depth was reached, at which point there was an order of magnitude increase. The critical depth was approximately 170 µm from the inner limiting membrane when ablating from the vitreous surface and 70 µm from the inner limiting membrane when ablating from the photoreceptor surface. Histological specimens showed that these barriers were the synaptic portion of the outer plexiform layer, and the inner plexiform layer, respectively.

Conclusions: The 2 high-resistance barriers to fluid flow through the retina are the synaptic portion of the outer plexiform layer, and the inner plexiform layer.

Clinical Relevance: These observations help to explain the distribution of cystoid macular edema seen in histological studies and with optical coherence tomography.

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Cystoid macular edema (CME) is a common sequel to many ocular conditions, including traumatic, vascular, inherited, and inflammatory diseases of the eye. Its fundoscopic appearance may vary, but typically it may be identified as round or ovoid cysts around the fovea. The cysts are characterized by an altered light reflex with a decreased central reflex and a thin, highly reflective edge. The retina may or may not show elevation or increased thickness. Visualization of the geometry and distribution of cysts may be enhanced by fluorescein angiography, where the cysts become hyperfluorescent over varying times.

The recent introduction of optical coherence tomography shows the cysts as areas of low or no signal, with occasional high-signal elements bridging the retinal layers. Histological studies show the cysts to be areas of retina in which the cells have been displaced. The cysts are presumed to result from the abnormal accumulation of fluid within the retina. For such an accumulation to occur, abnormalities must be present in one or both elements of the vascular supply of the retina. The location and distribution of such fluid will depend on the physical constraints imposed by the structure of the adjacent retina.

The retina has 2 sources of metabolic supply, the retinal capillary system and the choriocapillaris. Both systems demonstrate barrier functions that are referred to as the inner and outer blood-retinal barrier (BRB), respectively. The inner BRB is formed by the endothelial cells lining the retinal capillaries and junctional complexes between such cells. By contrast, the retinal pigment epithelium (RPE) and similar junctional complexes between adjacent cells form the outer BRB.

To maintain retinal metabolism, there must be movement of fluid and selected metabolites across these barriers, together with removal of catabolites. Fluid movement may arise as a result of abnormal barrier leakage and may be driven by a combination of active transport, diffu-

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MATERIALS AND METHODS

TISSUE PREPARATION

Forty-one pairs of human donor eyes were obtained from the United Kingdom Transplant Support Service Eye Bank at Bristol, England. Postmortem time varied from 24 to 56 hours. The median age of the donors was 69 years (range, 18-91 years). A full-thickness circumpapillary incision was made 5 to 7 mm behind the limbus. The vitreous, lens, and anterior segment were discarded. A 2-mm trephine was used to isolate the retina from the optic disc, and the neuroretina was gently teased away from the underlying RPE before being transferred to phosphate-buffered saline solution containing the following antibiotic and antimycotic agents: penicillin (100,000 U/L), streptomycin (100 mg/L), and amphotericin B (250 µg/L) (Sigma-Aldrich Corp, Poole, England).

Pilot experiments were undertaken using 57 trephined specimens from 22 pairs of eyes to develop a reproducible system for the measurement of retinal HC. Initial studies involved using freshly trephined retinal specimens. However, the tissue proved too friable and broke up even at very low-pressure differentials. A subsequent series of pilot studies established that HC could be measured if the retina was supported on a defined artificial membrane; however, tissue breakup still occurred in an unacceptable number of specimens. A final study was therefore undertaken using fixed retinal tissue together with a defined support membrane to ensure reproducible measurements in large numbers of specimens. They also determined that fixation resulted in no significant change in HC.

In all subsequent experiments, the isolated retina was fixed by means of immersion in 2.5% glutaraldehyde buffered with 0.1-mol/L sodium cacodylate containing calcium chloride, 10 mg/mL (final pH, 7.4), for 1 hour. With the use of a dissecting microscope, four 8-mm trephined specimens of neuroretina were isolated from the macula extending to the midperiphery. These were placed on 150-µm-thick nitrocellulose filters with 8-µm pores (Millipore Corporation, Bedford, Mass). In individual experiments, the trephined specimens were placed with the vitreous or photoreceptor surface against the filter, causing exposure of the photoreceptor or vitreous surface, respectively.

HC CHAMBER AND MEASUREMENT OF FLOW

The HC chamber used in the present series of experiments was identical to that previously described, except that the tissue-mounting cassette was constructed in transparent plastic with a central lumen of 4 mm. The retina and nitrocellulose samples were mounted in the cassette as previously described, and the cassette was inserted into the chamber such that the nitrocellulose surface was always distal to the direction of flow. Procedures for introducing fluid into the cassette were as previously described, except that the phosphate-buffered saline solution was degassed with the use of a vacuum. The assembled chamber was maintained at 37°C. Tissue was exposed to pressures varying from 343 to 1715 Pa by altering the height of the fluid reservoir (1 mm Hg equals 133 Pa). For each pressure at which flow was measured, a 30-minute equilibration was first allowed, during which the reservoir height slowly changed. The fluid height was then returned to this pressure and the position of the meniscus was noted at 3-minute intervals for 20 minutes.

LASER EXPOSURE PROCEDURE

An excimer laser (Apex Plus; Summit Technology, Boston, Mass) was used in the phototherapeutic keratectomy mode with a radiant emission of 180 mJ/cm², but with a...
beam diameter of 3 mm. From each pair of eyes, up to 8 trephined specimens were obtained and mounted in the transparent plastic cassettes. One sample was then mounted in the HC chamber with the vitreous surface exposed and the baseline flow determined. Measurements were then repeated on a second sample with the photoreceptor surface exposed. Subsequent samples were placed under the operating microscope of the excimer laser. The surface of the neuroretina was then carefully blotted using eye sponges (Visispear; Visitec, Sarasota, Fla). The phototherapeutic keratectomy software was programmed into the laser control system, the helium-neon aiming beams of the excimer system were focused on the surface of the retina, and the ablation sequence was initiated. Three specimens were ablated on the vitreous surface and 3 on the photoreceptor surface. In early experiments, specimens received 10, 20, or 30 pulses, whereas in the later experiments values were steadily increased up to 150, 200, and 250 pulses. After ablation, the cassettes were immersed in phosphate-buffered saline solution before being mounted in the chamber, and measurements of flow were undertaken. A total of 99 trephined specimens from 22 pairs of eyes were examined. Fifty-nine trephined specimens were mounted with the vitreous surface outward, of which 38 were ablated with excimer laser, and 40 trephined specimens were mounted with the photoreceptor surface outward, of which 32 were ablated with excimer laser. Finally, all specimens were removed from the cassette and processed for light microscopy (LM) and scanning electron microscopy (SEM). Thirteen additional samples were not included in the results because of holes detected during flow measurement or subsequent morphologic examination.

MORPHOLOGIC FEATURES

Samples were rinsed briefly in 0.1-mol/L sodium cacodylate containing 7.5% sucrose before they were fixed for 1 hour in 2% osmium tetroxide in 0.2-mol/L sodium cacodylate. Samples were then hemisected. Half were processed for LM, and the other half for SEM. The LM samples were dehydrated in ethanol and embedded in epoxy resin. Semithin (1 µm) sections were cut on an ultramicrotome (Huxley; Leica, Milton Keynes, England) and stained with toluidine blue. The SEM sections were dehydrated in acetone, dried in a critical point drier, sputter coated with gold, and examined in an SEM (510S; Hitachi, Wokingham, England).

CALCULATION OF FLOW AND HC

Flow, defined as the rate of volume change per unit of time per unit of surface area, was calculated from $F = \frac{(x \times C)}{(t \times A)}$, where $F$ indicates flow; $x$, distance moved by capillary column (in meters); $C$, manometer calibration constant; $t$, time (in seconds); and $A$, exposed membrane area ($1.26 \times 10^{-3}$ m² for the 4-mm-diameter tissue cassette).

The manometer calibration constant was determined by introducing a known weight of mercury into the capillary tube of the reservoir, lowering the tube into a horizontal position, and measuring the length of the mercury thread with the traveling microscope. From the density of mercury (13000 kg/m³), the constant was calculated to be $1.17 \times 10^{-4}$ m³/m².

The HC was calculated from $HC = F/P$, where $P$ indicates pressure (in pascals). The units of HC are cubic meters per second × square meters per second per pascal, which simplifies to meters per second per pascal. As no empirical studies of the pressure reduction across the retina have been published, it was assumed that pressure reduction would be similar to that across the Bruch membrane and choroid (estimated at 535 Pa); therefore, the results in the present report will be expressed at a similar pressure.

The HC of fixed intact human retina was measured to be $2.54 \times 10^{-10}$ m/s per pascal (range, $0.6 \times 10^{-10}$ to $3.3 \times 10^{-10}$ m/s per pascal; SD, $0.6 \times 10^{-10}$ m/s per pascal) at 539 Pa. The average values for the HC measured in 4 paired samples of fixed and unfixed retina were $2.75 \times 10^{-10}$ m/s and $2.97 \times 10^{-10}$ m/s per pascal, respectively (ranges, $2.47 \times 10^{-10}$ to $3.1 \times 10^{-10}$ and $2.85 \times 10^{-10}$ to $3.2 \times 10^{-10}$ m/s per pascal, respectively; SDs, $0.3 \times 10^{-10}$ and $0.1 \times 10^{-10}$, respectively; $P = .30$ by $t$ test) at 539 Pa. For a given eye, there was no difference in HC measured when pressure was applied to the vitreous surface or the photoreceptor surface (15 eyes; $P = .64$ by $t$ test). There was no correlation between HC and time from death to fixation or time from enucleation to fixation ($R^2 = 0.013$ [P = .66] and $R^2 = 0.117$ [P = .13], respectively). There was no correlation between HC and age ($R^2 = 0.055$ [P = .20]).

RESULTS
There was a logarithmic reduction in HC with increased pressure \( (R^2=0.98 \ [P<.001]; \textbf{Figure 1}) \).

Irrespective of the surface of the retina receiving ablation pulses, there was a linear relationship between the depth of tissue removed and the number of excimer pulses applied \( (R^2=0.91 \ [P<.001]) \). The average ablation depth per pulse was 0.5 µm.

The results of measuring HC after ablation are shown in \textbf{Figure 2}. Ablation from either surface resulted in little change in HC until a critical depth was reached, at which point there was an order-of-magnitude increase. The critical depth was approximately 170 µm from the ILM when ablating from the vitreous surface and 70 µm from the ILM when ablating from the photoreceptor surface.

Results of histological examination showed that these barriers were the OPL and the IPL, respectively (\textbf{Figure 3}). Sections showed that the synaptic portion of the OPL was always totally ablated such that the inner connecting fibers of the photoreceptor cells (Henle fiber layer) were exposed. Thus, the synaptic portion of the OPL presented the outer barrier.

**COMMENT**

The measurements of HC reported in this study support the hypothesis that the plexiform layers of the retina are regions of high resistance to the movement of fluid under pressure. These findings identify 2 of the 3 elements in the horizontal structural framework that would be required to produce the distribution of cysts seen in histological specimens. The remaining element is at the location of the OLM. It should be remembered that HC did not change significantly until considerable amounts of the IPL had been ablated. This observation probably reflects a lack of sensitivity of the measuring
technique used, given an inability to detect change as this inner barrier was traversed. This concept is further supported by the histological findings that, in ablations from the vitreous surface, the spatially confined synaptic portion of the OPL alone was always completely ablated before barrier loss was detected. This layer is typically 10 µm thick. Given the 0.06-µm thickness of the OLM and the apparent insensitivity of the present method, it is perhaps not surprising that its demonstrable barrier properties were not elucidated by our method using a 0.5-µm ablation rate per pulse.²⁵,²⁶

Under normal physiological conditions, fluid must move across the entire retina and through the high-resistance barriers; however, there is evidence that diffusion limits for metabolic supply in the system are approximately 150 µm. This would explain the thickness of the retina within the capillary-free zone where it rarely exceeds this value, and it would also explain cell loss in relation to vascular closure in either the retinal or choroidal supply. In large areas of capillary closure, all the layers internal to the OPL are lost and replaced by gliosis,²⁷ whereas choroidal infarction may lead to loss of RPE and inner nuclear layer. By contrast, RPE disease led to cysts accumulating in the outer retina in the Henle fiber layer.⁴ Tso and Shih also showed with horseradish peroxidase that leakage through the RPE in monkeys was constrained by the OPL. These observations are consistent with the evidence of the plexiform layers being high-resistance barriers to flow, as presented herein.

Although HC increased as the retina was ablated, there was no incremental increase on passing through the first of the 2 plexiform layers. Ablating through significant thickness of the retina would lead to the pressure being dissipated across a thinner section of retina. This would be equivalent to increasing the pressure across intact retina, which causes tissue compression. This compression of the remaining plexiform layer would enhance its barrier properties and thus exaggerate the increase in flow on ablating through it. In addition, the current technique may not be sufficiently sensitive to detect a difference in the barrier properties between the 2 plexiform layers. Hence, if each layer confers a similar rate-limiting barrier to fluid movement, ablation of both layers would be required before an increase in flow is detected.

There was no significant difference in HC measured at various postmortem times up to 50 hours and no significant differences generated by time between enucleation and death. In addition, no measurable difference was apparent between fixed and unfixed specimens. Previous work has suggested that vacuolation and cytoplasmic swelling occur after 10 hours post mortem but that, at least up to 24 hours post mortem, these changes are reversible.³³ The effect at a cellular level of fixation in 2.5% glutaraldehyde with its attendant protein cross-linking is uncertain, but such fixation would arrest any further postmortem changes. Fisher compared the HC of rat lens capsule before and after fixation with glutaraldehyde. He found the HC at low-pressure levels of fixed lens capsule to be about 0.6 times that of unfixed tissue, but he found that fixation stabilized the HC of the lens capsule, probably by making the capsule less deformable. Similar findings have been shown in kidney glomeruli.³⁵ Although caution must be expressed in applying the actual measurements presented in this article to the clinical situation, the concepts and locations of the barriers should hold.

To our knowledge, studies of the HC of human retina have not previously been published. Fatt and Shantha investigated rabbit retina, but it was not possible to transform their data to the units used in this study. Pederson described unpublished data showing the results of a study using dog retina with an HC of 0.38 × 10⁻¹⁰ m/s per pascal (converted from 0.03 µL/min per millimeters
of mercury per square centimeter), approximately 7 times less than the HC in this study, which would be within species and technique variation but might also reflect the freshness of their tissue. The HC of intact human retina showed no change with age. This compares with the exponential decrease of the HC with age in human Bruch membrane (Figure 4). In the young, the HC of the Bruch membrane is much greater than that of retina, and as a consequence, this would promote retinal apposition in the presence of a flow of fluid from vitreous to choroid. Although the pressure difference, and thus the flow, across the retina from vitreous to choroid is likely to be small, Fatt and Shanthinath estimated that a pressure difference of as little as 0.52 × 10⁻³ mm Hg would be sufficient to keep the retina firmly attached.

In conclusion, we have demonstrated the HC of intact human retina and have shown that 2 of the major barriers to fluid flow through the retina are the IPL and the synaptic portion of the OPL. These observations help to provide an explanation for the distribution of CME seen in histological studies.

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