Localization of the Site of Major Resistance to Fluid Transport in Bruch's Membrane

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**Purpose.** To determine the site of highest resistance to the movement of water across Bruch's membrane in humans.

**Methods.** A hydraulic conductivity chamber was designed that enabled us to measure flow across Bruch's membrane while ablating its subepithelial aspect using an excimer laser (193 nm). When resistance was lost, samples were fixed and processed for electron microscopy.

**Results.** Changes in the hydraulic conductivity of Bruch's membrane in response to the excimer-mediated sequential removal of tissue layers was studied in four control eyes of donors 26, 46, 61, and 76 years of age and in one eye of an 83-year-old donor with age-related macular degeneration. The number of laser pulses required to abolish the resistance in Bruch's membrane was found to be dependent on the age of the donor. The ablation rate was approximately 0.11 μm per pulse. Loss of resistance correlated with removal of layers internal to the layer of elastin.

**Conclusions.** This study indicates that the inner collagenous layer imparts the major resistance to fluid movement between the retinal pigment epithelium and the choroid. Aging changes in the ultrastructure of these compartments could be responsible for the decrease in hydraulic conductivity observed in early life in previous studies. Invest Ophthalmol Vis Sci. 1997;38:762–767.

To explain the etiology of pigment epithelial detachment in the absence of new vessels, Bird and Marshall postulated that with increasing age, deposition of lipids within Bruch's membrane would lead to the generation of a hydrophobic barrier, an attendant decrease in hydraulic conductivity, and, in extreme cases, a pooling of fluid beneath the retinal pigment epithelium (RPE). Histochemical studies showing an accumulation of lipids within Bruch's membrane gave empirical support for this concept and showed significant deposition of lipids in later life. However, in two recent studies using two different techniques, although hydraulic conductivity was shown to decrease with age, the decay was exponential—the major loss occurred in early life, long before lipid deposition was significant. These findings suggest that during early life, other changes must occur in the structure or chemistry of Bruch's membrane that influence fluid transport. In a study of the structure of more than 90 eyes from donors ranging from 6 to 100 years of age, Bruch's membrane was shown to increase in thickness in a linear fashion. In a study of sublaminal changes of Bruch's membrane, no significant changes were found in any specific layer in early life. Given the hydraulic conductivity findings and the inability to correlate these with morphologic changes in the membrane between birth and age 40, we wondered whether a method could be devised to fractionate Bruch's membrane systematically in a controlled fashion to determine the site of high resistance.

When the argon fluoride excimer laser (193 nm) is used to ablate the cornea, it has been shown that—depending on the hydration characteristics—at a radiant exposure of 180 mJ/cm², between 0.22 and 0.25 μm of tissue is removed per pulse. This ablation takes place across the entire area on which the beam falls; typically, such areas measure millimeters in diameter.

If the ablation rate of Bruch's membrane is similar to that of the cornea, it should be possible to ablate progressively the exposed subepithelial surface of Bruch's membrane while hydraulic conductivity is measured. The resistance to flow should drop to near zero immediately on ablation of the high-resistance barrier, and thus it should be possible to locate the removal of the barrier to within 0.25 μm. The current report describes the results of a pilot study to investigate the potential of this technique.

**METHODS.** Tissue Preparation. Human eyes from donors 26, 46, 61, and 76 years of age were obtained from the Bristol Eye Bank, and one eye from an 83-year-old donor with age-related macular degeneration was obtained through the Macular Disease Society Eye Donor Scheme. Postmortem time varied between 24 and 48 hours. In all five eyes, a full-thickness circumferential incision was made 5 to 7 mm behind the limbus. Vitreous, lens, and anterior segments were discarded. The neuroretina was teased gently away from the underlying RPE and was isolated from the optic nerve by scissors. To enable the macular region to be used in an independent series of experiments, a second full-thickness circumferential incision was made.
made to isolate the macula and to provide an annular region of peripheral RPE-choroid and sclera 8 mm wide. This annulus from the periphery was incised and uncoiled to give a rectangular strip of tissue. The isolated preparation was transferred to Dulbecco’s phosphate-buffered saline (DPBS; Sigma Chemical, Poole, United Kingdom), and the RPE cells were brushed gently away from the inner aspect of Bruch’s membrane with a camel-hair brush. Finally, Bruch’s membrane and the underlying choroid were teased gently away from the sclera using blunt dissection.

**Hydraulic Conductivity Chamber.** To measure hydraulic conductivity and simultaneously to irradiate the sample with excimer laser radiation, it was necessary to design a chamber that differed from the one previously described but that retained the stainless steel cassette. The modified chamber is shown in schematic form in Figure 1. It consisted of a base component constructed of aluminum, the stainless steel cassette previously described, and a capping plate also constructed of aluminum. The capping plate was locked into position by three screws and compressed the O-rings mounted on the tissue cassette. Fluid could be introduced through the lumen of the chamber by a brass connector.

The lumen of the chamber measured 6 mm in diameter. The bore diameter of the cassette measured 3.5 mm, and that of the capping plate measured 16 mm. Fluid was introduced into the system from a reservoir consisting of a vertical capillary tube through a three-way valve. The valve was connected to the brass connector by plastic tubing, and the third part of the three-way valve was connected to a syringe. Fluid movement was determined by monitoring the location of the meniscus in the reservoir using an electronically recording traveling microscope (in-house construction). All procedures were carried out using Dulbecco’s phosphate-buffered saline.
Tissue Mounting and Measurement of Flow. Tissue samples 8 mm wide and 10 mm long were isolated from the midtemporal region of the original strips. They were located and clamped in the stainless steel cassette as previously described. The lumen of the base chamber was filled with fluid from the syringe; care was taken to avoid air bubbles. Chamber and syringe were located and maintained in the same horizontal plane by use of a spirit level.

The tissue cassette, complete with tissue diaphragm, was inserted into the upper part of the base chamber so that the inner aspect of Bruch's membrane was uppermost. Again, care was taken to avoid air bubbles as fluid was displaced to compensate for the cassette volume.

The capping plate was then placed in situ and locked into position. The assembled chamber was placed on a laboratory jack located centrally in the focal plane of the excimer laser. The valve was set, coupling the syringe with the reservoir, and the pressure was adjusted to 24 cm H2O. Then the valve was repositioned to couple the reservoir to the chamber. Every 2 minutes for 10 minutes, measurements were taken of the changes in meniscus location.

Laser Exposure Procedure. An Excimed UV 200 (Summit Technology, Boston, MA) excimer laser was used in the phototherapeutic keratectomy mode with a beam diameter of 6 mm and a radiant emission of 180 mj/cm². The software was programmed to deliver a train of three pulses at a pulse repetition rate of 10 Hz.

Using the operating microscope of the laser, the exposed surface of Bruch's membrane was blotted carefully using spills of hardened filter paper (Whatman no. 50) inserted through the aperture of the capping plate. Before each firing of the laser, the three-way valve was located to connect the syringe to the reservoir, and the meniscus was normalized to 24 cm H2O. The valve was repositioned to couple the reservoir to the chamber. Every 2 minutes for 10 minutes, measurements were taken of the changes in meniscus location.

Calculation of Flow. Flow (F), defined as the rate of volume change per unit time per unit surface area, was calculated from:

\[ F = \frac{(x \times C)}{t \times A} \]  

where x = distance moved by capillary column in millimeters, C = manometer calibration constant, t = time in seconds, and A = exposed membrane area (m²), giving 9.621 \times 10^{-6} for the 3.5 mm diameter tissue cassette.

The calibration constant was determined by introducing a known weight of mercury into the capillary tube of the reservoir, and was calculated to be 1.077 \times 10^{-6} m³/m. The reservoir was located in a horizontal position, and the length of the mercury thread was measured using the traveling microscope. From the density of mercury (13,000 kg/m³), the constant was calculated to be 1.077 \times 10^{-6} m³/m, with t = time in seconds and A = exposed membrane area (m²), giving 9.621 \times 10^{-6} for the 3.5 mm diameter tissue cassette.

Hydraulic conductivity (HC) was calculated from:

\[ HC = \frac{F}{P} \]  

Pressure (P) was expressed in pascals.

RESULTS. The effect of excimer laser ablation on flow through Bruch's membrane is shown in Figure 2. In the eyes from the two younger donors, six pulses of laser energy were required to cause a precipitous drop in resistance. By contrast, at least twice this number were required for the same effect in eyes from older donors. In eyes from the youngest three donors, successive laser pulses induced a progressive increase in flow. In eyes from the 76-year-old donor and the 86-year-old donor with age-related macular degeneration, the first six pulses resulted in minimal change in flow (Fig. 3).
FIGURE 2. The effect of laser ablation of Bruch's membrane on flow. Flow was determined in samples of Bruch's membrane obtained from the peripheral fundus of four donors. The bar for zero pulses corresponds to baseline flow before ablation. Total loss of barrier resulted in flow rates that could not be measured, and these are represented as $400 \times 10^{-7} \text{ m}^3/$ second $\cdot$ m$^2$ in the bar charts. Data show that a higher number of pulses and, hence, a greater depth of tissue removal are required to abolish membrane resistance in the samples from older donors.

When flow was maximal and was plotted as $400 \times 10^{-7} \text{ m}^3/$ second $\cdot$ m$^2$, in reality it was so fast that it was unmeasurable and represented total loss of barrier.

The ultrastructural studies of each specimen showed a common finding. Loss of barrier was coincident with loss of RPE basement membrane, along with most, if not all, of the inner collagenous layer. Typical ablation showed exposed areas of the layer of elastin, together with the intact outer collagenous layer and the basement membrane of the choriocapillaris (Fig. 4). The ablation of tissue was not identical throughout the 3.5 mm of exposed membrane because, in some areas of eyes from older donors, the membrane had broken down completely over the lumen of some capillaries. In other areas, larger portions of intact inner collagenous layer could be determined. By measuring the amount of tissue removed for a given number of pulses, it was determined that the ablation rate per pulse was typically 0.11 $\mu$m. In the eye from the donor with age-related macular degeneration, findings were
FIGURE 4. Electron micrographs of isolated Bruch’s membrane preparation from the 46-year-old donor. (A) Unablated area showing the layers of Bruch’s membrane. (B) Sample from the ablated area showing removal of layers internal to the layer of elastin. BM = basement membrane of the retinal pigment epithelium; IC = inner collagenous layer; E = layer of elastin; OC = outer collagenous layer; EM = basement membrane of the endothelium of the coriocapillaris. Bar = 0.5 μm.

similar to those seen in the normally aged eyes, and, again, resistance seemed to be lost when the ablation reached the elastin layer.

DISCUSSION. In this pilot study, we have demonstrated the efficacy of excimer laser ablation in progressively fractionating Bruch’s membrane. Our initial evidence indicates the site of high resistance of Bruch’s membrane to be located internal to the elastin layer. In this study, the ultimate resolution of the technique was not used because we chose to fire laser pulses in batches of three. This batch modality was a conscious decision induced by the time taken for the programming sequence of the laser. Firing from one pulse to the next took at least 1 minute in programming and testing the laser. It proved impossible to deliver single pulses, preprogramming for 10 pulses, and trying to select single pulses with the foot switch. If the ablation rate of Bruch’s membrane was similar to that obtained for the cornea, the three-pulse sequence would reduce ultimate resolution of barrier location from 0.25 μm to 0.75 μm. Given this degradation of potential resolution, we can state only that the high-resistance barrier lies somewhere internal to the elastin layer (that is, within the inner collagenous layer). In future studies, the manufacturer’s help will be elicited to enable us to deliver single pulses without prolonging interpulse intervals. From measurements of tissue ablated in the current study, an 0.11 μm average ablation per pulse was obtained. Theoretically, this would improve barrier resolution to 0.1 μm; however, this relatively low ablation rate could be an artifact induced by high hydration levels in the tissue. Because the experiment demanded passage of water through the system, it was extremely difficult to maintain the specimen without a surface water layer during the ablation process. With experience, blotting the surface water layer became more efficient and eventually results became consistent. The dependence of resistance on the inner layer of Bruch’s membrane is demonstrated clearly in Figure 2. In the eyes from the two younger donors, successive laser pulses led to a progressive loss of resistance. By contrast, in the eyes from the older donors, loss of small amounts of the inner layers gave rise to little or no change. This suggests that in the older eyes, either the immediate subepithelial deposits played little or no role in resistance or that resistance in this tissue was so high throughout...
its thickness that removing small amounts showed no change. Given the limited resolution of the current study, it suggests that the major barrier to resistance must reside within the inner collagenous layer.

To date, there is no morphologic evidence that the inner collagenous layer undergoes major changes throughout the first four decades of life, nor is there any physiological evidence of significant changes in Young's modulus of elasticity of Bruch's membrane in the young. In the older concept of subretinal neovascularization, the loss of elasticity of Bruch's membrane through calcification was postulated as a mechanism for inducing breaks in the system, and such breaks were suggested as the causal agent for penetration by choroidal vessels. Although no empirical evidence exists for the loss of elasticity with increasing age, the breaks in Bruch's membrane seen in the current study between intercapillary columns and across vascular components suggest that even under the low hydrostatic pressure used, the elastic constants were exceeded. To support this concept, it should be noted that the occurrence is seen more commonly in older eyes.

The uneven nature of ablation in some specimens and the breaks in others did not confound the overall finding that the high-resistance barrier resides within the inner collagenous layer of Bruch's membrane.

**Key Words**
ageing, age-related macular degeneration, Bruch's membrane, excimer laser, hydraulic conductivity

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**References**


