Studies of Scleral Biomechanical Behavior Related to Susceptibility for Retinal Ganglion Cell Loss in Experimental Mouse Glaucoma

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PURPOSE. To study anatomical changes and mechanical behavior of the sclera in mice with experimental glaucoma by comparing CD1 to B6 mice.

METHODS. Chronic experimental glaucoma for 6 weeks was produced in 2- to 4-month-old CD1 (43 eyes) and B6 mice (42 eyes) using polystyrene bead injection into the anterior chamber with 126 control CD1 and 128 control B6 eyes. Intraocular pressure (IOP) measurements were made with the TonoLab at baseline and after bead injection. Axial length and scleral thickness were measured after sacrifice in the CD1 and B6 animals and compared to length data from 78 eyes of DBA/2J mice. Inflation testing of posterior sclera was conducted, and circumferential and meridional strain components were determined from the displacement response.

RESULTS. Experimental glaucoma led to increases in axial length and width by comparison to fellow eyes (6% in CD1 and 10% in B6; all $P < 0.03$). While the peripapillary sclera became thinner in both mouse types with glaucoma, the remainder of the sclera uniformly thinned in CD1, but thickened in B6. Peripapillary sclera in CD1 controls had significantly greater temporal meridional strain than B6 and had differences in the ratios of meridional to effective circumferential strain from B6 mice. In both CD1 and B6 mice, exposure to chronic IOP elevation resulted in stiffer pressure-strain responses for both the effective circumferential and meridional strains (multivariable regression model, $P = 0.01$ – 0.03).

CONCLUSIONS. Longer eyes, greater scleral strain in some directions at baseline, and generalized scleral thinning after

glaucoma were characteristic of CD1 mice that have greater tendency to retinal ganglion cell damage than B6 mice. Increased scleral stiffness after glaucoma exposure in mice mimics findings in monkey and human glaucoma eyes. (*Invest Ophthalmol Vis Sci.* 2013;54:1767–1780) DOI:10.1167/iov.12-10952

Both mean intraocular pressure (IOP) level,¹ IOP fluctuation,² and peak IOP³ are closely associated with incident human glaucoma and its progressive worsening. IOP mechanically deforms the optic nerve head (ONH) through a pressure differential across the ONH that causes posterior bowing of the lamina cribrosa and through tensile stresses generated in the adjacent scleral tissues that cause expansion of the scleral canal. These stresses contribute to permanent excavation of ONH tissues, a defining clinical feature of human glaucoma.^{4,5} ONH deformation affects retinal ganglion cell (RGC) axons, astrocytes, blood vessels, and (in human and nonhuman primates) ONH connective tissues. Anterograde and retrograde RGC axonal transport are interrupted at the ONH leading to axon degeneration and RGC somal death by apoptosis^{6,7} in human glaucoma, as well as in experimental monkey and rodent eyes. While vascular, glial, and immune factors contribute to RGC death in glaucoma, the contribution of IOP-generated stress is supported by abundant evidence and is potentially amenable to therapeutic intervention.

Ocular connective tissues are potential mediators of human glaucoma damage. First, the ONH zones that suffer more RGC axon injury, the superior and inferior poles, have a lower density of connective tissue support. This has led to a hypothesis that links connective tissue structure to the typical pattern of visual field defects seen in glaucoma.^{8–12} Second, persons with axial myopia are more susceptible to open-angle glaucoma (OAG).¹³ This may relate in part to the greater stress in the sclera and ONH that is likely to result from their larger globe diameter and thinner sclera. Third, corneal hysteresis measured by an ocular response analyzer has been suggested as a risk factor for OAG progression.¹⁴ Fourth, two reports in human OAG patients have estimated that scleral rigidity is greater than in control eyes by indirect in vivo measurements.^{15,16}

Because the ONH is a complex and relatively small structure, testing its specific mechanical behavior is only feasible indirectly.¹⁷ By contrast, studies of scleral anatomy and physiology are possible and are highly relevant to what occurs at the ONH. Biomechanical models^{18,19} show that the IOP-generated stresses in the sclera are critical in producing strain at the ONH.²⁰ A recent report²¹ stated, “The sclera is an important factor in ONH biomechanics, and recent work strongly suggests that the biomechanics of the posterior sclera and lamina cribrosa are tightly coupled.” Variations in scleral

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mechanical properties could be one explanation for the fact that half of the patients with OAG suffer injury in the normal IOP range.²² The mechanical behavior of the sclera, initially studied by uniaxial strip testing,^{23–25} has been more realistically modeled using in vitro inflation testing with two- and three-dimensional analysis of intact posterior sclera in human, bovine, monkey, tree shrew, and mouse eyes, including those subjected to experimental glaucoma or induced myopia.^{21,26–31} These reports have generally found increases in scleral stiffness with glaucoma.

Mouse IOP elevation models provide data relevant to human glaucoma and offer research avenues not possible in monkey or human eyes, including but not limited to the practical applicability of genetic alteration of mouse subtypes and the use of large sample sizes in experimental studies. Mammalian eyes that are subjected to experimental increases in IOP have neuronal, glial, and associated tissue alterations that are phenotypically similar to human glaucoma.^{32,33} Furthermore, lowering of IOP slows the progressive loss of RGC in both animal and human glaucoma.^{34,35} While mouse eyes differ in details of ONH anatomy from primates, they share the site of glaucoma injury and the selective death of RGC. Sun et al.³⁶ demonstrated that astrocytes in the mouse ONH simulate the structure of the collagenous lamina cribrosa in primate eyes. The mouse sclera has collagens, elastin, and other molecules, as in human sclera,³⁷ though its thickness and diameter are a tenth of the size of the thickness and diameter in human eyes.³⁸ While mouse eyes increase their axial length with chronic IOP increase, so do rat, monkey, and human infants with chronic glaucoma. Experimental mouse glaucoma data can relevantly validate the role of scleral structure and its response to chronic IOP elevation in ways not possible with other approaches.

We previously determined that CD1 mice are more susceptible to RGC death than B6 mice in experimental glaucoma.^{39,40} We produced experimental glaucoma in these two types of mice and report both baseline scleral data and changes induced after chronic experimental glaucoma in the anatomy and biomechanical behavior of the sclera. A better understanding of scleral biomechanics in glaucoma can improve our ability to predict which eyes will worsen more rapidly and may lead to new therapeutic approaches.

METHODS

Animals

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocol MO10M159 approved and monitored by the Johns Hopkins University School of Medicine Animal Care and Use Committee. CD1 albino mice (Charles River Laboratories, Wilmington, MA) and B6 pigmented mice (Jackson Laboratories, Bar Harbor, ME) were used. There were 128 control or fellow eyes and 42 glaucoma eyes from B6 mice and 126 control or fellow eyes and 43 glaucoma eyes from CD1 mice. For comparison with these two mouse types and their experimental glaucoma changes, we studied DBA/2J mice that develop spontaneous glaucoma by 1 year of age (Jackson Laboratories), measuring axial length and scleral thickness in 51 eyes at 2 to 4 months of age (prior to development of glaucoma), 20 eyes at 10 to 12 months, and 7 eyes from 15- to 26-month-old mice.

Bead Injections for Glaucoma

For anterior chamber injections to produce glaucoma, mice were anesthetized by an intraperitoneal injection of 50 mg/kg of ketamine, 10 mg/kg of xylazine, and 2 mg/kg of acepromazine and received

topical anesthesia of 0.5% proparacaine hydrochloride eye drops (Akorn, Inc., Buffalo Grove, IL). Two bead injection protocols were used, as recently reported.³⁹ In one protocol, the 4 + 1 method, we injected 2 μ L of 6- μ m diameter beads, then 2 μ L of 1- μ m diameter beads (Polybead Microspheres; Polysciences, Inc., Warrington, PA), followed by 1 μ L of viscoelastic compound (10 mg/mL sodium hyaluronate, Healon; Advanced Medical Optics, Inc., Santa Ana, CA) through a glass cannula pulled to a tip diameter of 50 μ m connected by polyethylene tubing to a Hamilton syringe (Hamilton, Inc., Reno, NV). The 4 + 1 protocol was used in 34 eyes each of B6 and CD1 mice (2–4 months of age at injection). In the other protocol, the 2 + 3 protocol, we injected a total of 2 μ L of the 6- μ m beads followed by 3 μ L of viscoelastic compound. The 2 + 3 protocol was used in eight B6 and nine CD1 mouse eyes (12–19 months of age). We estimated that the final concentration in the 4 + 1 protocol was 3×10^6 beads/ μ L for 6- μ m beads and 1.5×10^7 beads/ μ L for 1- μ m beads. The two different protocols did not induce differences that were relevant to the analyses presented here, since groups were being compared with regard to a property or measurement only when the same method was used in both groups.

Intraocular Pressure Measurement

Prior to IOP measurement, animals were anesthetized by inhalation of isoflurane, using the RC²-Rodent Circuit Controller (VetEquip, Inc., Pleasanton, CA). This instrument supplies oxygen from an attached tank at 50 to 55 pounds per square inch. Oxygen is mixed with isoflurane and sent at a speed of 500 mL/min, delivering 2.5% of isoflurane in oxygen to the animal. Two minutes after the animal was sedated, IOP measurements were made using the TonoLab tonometer (TioLat, Inc., Helsinki, Finland), recording the mean of six readings with optimal variability grade. We measured baseline IOP prior to injection, at 10 minutes after injection, and weekly to sacrifice at 6 weeks after injection.

Axial Length and Width Measurement

Animals with induced glaucoma that did not undergo inflation testing received intraperitoneal injection of general anesthesia before sacrifice by exsanguination, followed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.2). IOP was set at 15 mm Hg with a needle connected to a fluid-filled reservoir to produce standard conditions for axial length and width measurement. The measurements were performed with a digital caliper (Instant Read Out Digital Caliper; Electron Microscopy Sciences, Hatfield, PA). The length was measured from the center of the cornea to a position just temporal to the optic nerve, and both nasal-temporal width and superior-inferior width were measured at the largest dimension at the equator, midway between the cornea and optic nerve. Eyes that underwent biomechanical inflation testing were first enucleated after sacrifice, were not treated with aldehyde fixation, and were measured before inflation testing for axial length and width using a digital caliper (Instant Read Out Digital Caliper; Electron Microscopy Sciences), as previously described.

Inflation Test Methods and Analysis

The inflation test method has been previously described in detail.²⁶ In brief, the eye was glued to a fixture at the limbus and inflated through pressure-controlled injection of a saline solution. Digital image correlation (DIC) was used to locate the scleral edge as seen from a superior view, extending from the fixture to the optic nerve both nasally and temporally (Fig. 1A). The coordinates for a series of locations along the sclera were obtained from DIC at the baseline pressure (undeformed configuration) and after displacement produced by inflation (deformed configuration). The strains were calculated directly from the DIC displacements. In this analysis, the term

FIGURE 1. Schematic for scleral strain analysis. Schematics for strain analysis indicate the meridional and circumferential orientations with Φ and θ , respectively. (A) Representative schematic of an inflation-tested right eye, where R_k indicates the regions for scleral analysis. (B) Representative superior view of the sclera with curvilinear coordinate s , which is used to locate a point along the scleral edge. (C) Representative posterior view of the sclera indicating the two material directions used for strain calculations. (D) Representative superior view of the undeformed (solid line) and deformed (dashed line) scleral edge, indicating the undeformed position, $\mathbf{X}(s)$, the deformed position, $\mathbf{x}(s)$, the displacement vector, $\mathbf{u}(s)$, and the diameter D of the undeformed cross-section at s .

meridional referred to the direction along the scleral edge, while circumferential referred to the direction parallel to the equator.

Experimental Method. Inflation testing used enucleated, unfixed whole mouse eyes glued with cyanoacrylate to a fixture. The anterior chamber was connected through a 30-gauge needle and tubing to a programmable transducer-pump manifold and immersed in phosphate-buffered saline at 22°C. The preparation permitted analysis of the posterior 2/3 of the globe. A CCD video camera (Grasshopper, model Gras-20S4M-C; Point Grey Research, Inc., Richmond, BC, Canada) attached to a dissecting microscope (Stereomicroscope Stemi 2000-CS; Carl Zeiss Microscopy, LLC, Thornwood, NY) viewed the eye from superiorly, recording scleral edge images every 2 seconds, which were processed by DIC software⁴¹ to extract the two-dimensional (2D) displacement field of selected points along the scleral edge. The error in the displacement measurement was calculated previously as $\pm 0.46 \mu\text{m}$. This included contributions from the uncertainty in the pixel-distance calibration, $\pm 0.36 \mu\text{m}$, and the inherent error of the DIC correlation, $\pm 0.1 \mu\text{m}$.²⁶ To characterize the nonlinear, time-dependent material behavior, testing began at a reference pressure, P_0 , determined for each eye as the minimum pressure at which the sclera was no longer wrinkled, typically 6 to 8 mm Hg. The specimen was first subjected to two load-unload cycles from P_0 to 30 mm Hg at a rate of 0.25 mm Hg/s. The pressure was returned to P_0 and held for 10 minutes after each unloading to ensure full recovery of the displacements. A ramp hold test was then conducted, at a rate of 0.25 mm Hg/s, from P_0 to 30 mm Hg. The specimen was held at 30 mm Hg for 30 minutes before the pressure was brought back to P_0 for a recovery period of 20 minutes. The present analysis was applied to the loading portion of the first load-unload cycle. We successfully carried out inflation tests on 23 glaucoma CD1 eyes, 20 CD1 control eyes, 17 glaucoma B6 eyes, and 21 B6 control eyes. Unsuccessful inflation tests had obvious leakage from cannulation, eyes that detached from the fixture, or technical failure to complete the protocol. Among the successful inflations, we were able to apply the analytical model to 20

glaucoma CD1 eyes, 20 CD1 control eyes, 12 glaucoma B6 eyes, and 20 B6 control eyes.

Strain Analysis. The following describes the analytical method developed to calculate the meridional and circumferential strains of the mouse sclera from the data of the inflation experiments. At any given pressure step, the DIC algorithm provided the 2D reference (undeformed) coordinates of select points along the nasal and temporal edge at the reference pressure, as well as the 2D displacement vectors. The points were chosen by first identifying the location of the ONH then defining a series of subsequent points every 0.1 mm along the scleral edge toward the fixture. We defined for each point a rectangular subset (35×35 pixels) in the reference image that contained part of the dark sclera and part of the whiter background to create a natural speckle pattern (see Supplementary Material and Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10952/-/DCSupplemental>). Each pixel corresponded to a real area of $13.9 \times 13.9 \mu\text{m}^2$. The DIC algorithm used the distribution of gray values in the subset to determine the position of the point in subsequent images of the deforming specimens to calculate the in-plane displacements.

The displacements and reference coordinates were used to calculate the meridional and effective circumferential strains (defined in following text). In developing the analysis, we assumed that the scleral edge deforms within the plane. We did not assume that the scleral shell is axisymmetric; thus the configuration and displacements of the nasal and temporal edge were allowed to differ. To calculate the pressure-strain response, we defined two different coordinate systems: (1) a Cartesian coordinate system ($\mathbf{e}_1$, $\mathbf{e}_2$), in which $\mathbf{e}_1$ was parallel to the fixture (Fig. 1A), and (2) a curvilinear coordinate system following the scleral edge as shown in Figures 1B and 1C. The coordinate s denotes the arc length measured from the apex. Because the scleral shell was not assumed to be axisymmetric, different arc length coordinates s were used to parameterize the undeformed nasal and temporal edges. For both, $s = 0$ indicates the position of the ONH. This location could be determined consistently between specimens and

enabled regional comparisons among specimens. The meridional strains for the nasal and temporal halves of the sclera were analyzed separately.

To determine the e_2 axis of the Cartesian coordinate system, a line was drawn to connect the two apex points on the nasal and temporal edges, where the optic nerve margins joined the sclera. The e_2 axis was defined as the line bisecting the two apex points extending to the fixture. The e_1 axis was defined as being perpendicular to the e_2 axis and passing through a point where the scleral edge met the fixture. Once the Cartesian coordinate system was constructed, DIC reference positions and displacements were determined for the Cartesian coordinate system at each pressure step, using a dense grid along both scleral edges. From the reference positions, we defined for the temporal and nasal edges the referenced arc length coordinates s along the scleral edge and four scleral regions starting from the peripapillary sclera (Fig. 1A).

To calculate the strain of the scleral edge, we model each scleral edge as a deforming one-dimensional continuum curve. The coordinates of the deformed positions for the curvilinear coordinate system are given by

$$\mathbf{x}(s) = \mathbf{X}(s) + \mathbf{u}(s), \quad (1)$$

where $\mathbf{x}(s)$ and $\mathbf{X}(s)$ are the coordinates of the deformed and undeformed positions, respectively, and $\mathbf{u}(s)$ is the displacement vector (Fig. 1D). The tangent of the deformed meridian is defined as

$$\mathbf{t}(s) = \frac{d\mathbf{x}}{ds} = \frac{d\mathbf{X}}{ds} + \frac{d\mathbf{u}}{ds}, \quad (2)$$

where $\mathbf{T}(s) = d\mathbf{X}/ds$ is the unit tangent vector of the undeformed curve, and $ds = \sqrt{dX_1^2 + dX_2^2}$. The stretch of the meridian at the point s can be calculated from the magnitude of the deformed tangent vector, $\lambda_\Phi(s) = \|\mathbf{t}\|$. The Green-Lagrange strain of the curve, defined as the meridional strain, can be calculated from the stretch as

$$E_{\Phi\Phi}(s) = \frac{1}{2} (\lambda_\Phi^2(s) - 1) = \frac{d\mathbf{X}}{ds} \cdot \frac{d\mathbf{u}}{ds} + \frac{1}{2} \frac{d\mathbf{u}}{ds} \cdot \frac{d\mathbf{u}}{ds}. \quad (3)$$

To evaluate Equation 3 for the meridional strain, we first obtained an analytical description of the nasal and temporal scleral edges by fitting the reference coordinates for $\mathbf{X}$ of each edge to a generalized ellipse of the form:

$$X_1(v) = a \sin v \cos \gamma - b \cos v \sin \gamma + X_{c1},$$

$$X_2(v) = a \sin v \sin \gamma - b \cos v \cos \gamma + X_{c2}. \quad (4)$$

The parameters a and b are the major and minor axes of the ellipse, γ is the counterclockwise rotation angle of the principal axis of the rotated ellipse, (X_{c1}, X_{c2}) are the coordinates of its center, and v is a free parameter representing a counterclockwise angle from the major axis. Applying the chain rule, the tangent vector of the undeformed curve can be evaluated as $\mathbf{T}(s) = d\mathbf{X}/ds = (d\mathbf{X}/dv) (dv/ds)$, where $ds = \sqrt{dX_1^2 + dX_2^2} = \sqrt{(X_1')^2 + (X_2')^2} dv$, $X_1' = dX_1/dv$, and $X_2' = dX_2/dv$. This allows the components of the tangent vector to be evaluated as

$$T_1(s) = \frac{X_1'}{\sqrt{(X_1')^2 + (X_2')^2}}, \quad T_2(s) = \frac{X_2'}{\sqrt{(X_1')^2 + (X_2')^2}}. \quad (5)$$

At each pressure step, the DIC method determines the Cartesian displacement components u_1 and u_2 at each point $\mathbf{X}$. The displacement components were fitted to a sixth order polynomial as a function of the free parameter v in Equation 4, using the Matlab (Matlab R2010b; Mathworks, Natick, MA) function *polyfit*, to obtain an analytical expression for $u_1(v)$ and $u_2(v)$. Applying the analytical displacements and reference coordinates in Equations 4 to Equation 3 and carrying out the chain-rule, the meridional strain ($E_{\Phi\Phi}$) can be evaluated as

FIGURE 2. Analytical versus discrete strain calculations. Comparing a smoothed (blue) and discrete (black) method of strain calculation for the temporal edge of a representative CD1 specimen. The discrete method discretized the scleral edge into line segments and used central difference to calculate the stretch of each line segment, while the smoothed method modeled the scleral edge as an elliptical curve undergoing 2D displacements in the plane. R_k indicates the regions, which are defined as every four points along the scleral edge.

$$E_{\Phi\Phi}(s) = \frac{1}{(X_1')^2 + (X_2')^2} \left(X_1' u_2' + X_2' u_1' + \frac{1}{2} ((u_1')^2 + (u_2')^2) \right), \quad (6)$$

where $u_1' = du_1/dv$ and $u_2' = du_2/dv$. The method was applied separately to calculate the meridional strain for each scleral edge. To validate this method for select specimens, the scleral edge was discretized into line segments connecting the reference positions $\mathbf{X}$. The meridional stretch was calculated discretely using central difference as $\lambda_\Phi(s_i) = \|\mathbf{x}_{i+1} - \mathbf{x}_{i-1}\| / \|\mathbf{x}_{i+1} - \mathbf{x}_{i-1}\|$, and applied to calculate the strain as $E_{\Phi\Phi}(s_i) = \frac{1}{2} (\lambda_\Phi^2(s_i) - 1)$ (Fig. 2). The analytical and discrete strain calculations yielded similar results. The analytical method provided a smoother strain field, while the discrete method was more susceptible to experimental noise.

The 2D DIC system was unable to image the out-of-plane displacement component. This prevented rigorous calculation of the circumferential strain in the same manner as for the meridional strain. However, an estimate for the circumferential strain was calculated from the change in the distance, d , between a point on the nasal edge and a corresponding point on the temporal edge with the same coordinate s . The result is referred to here as the effective circumferential strain. The effective circumferential strains ($\bar{E}_{\theta\theta}$) were calculated from the ratio of the deformed diameter d to the undeformed diameter D as follows (Fig. 1D):

$$\bar{E}_{\theta\theta}(s) = \frac{1}{2} \left[\left(\frac{d(s)}{D(s)} \right)^2 - 1 \right], \quad (7)$$

The nasal and temporal edges of each specimen were not significantly different from each other (Fig. 3). Thus, the definition for D provides a reasonable approximation for the diameter. The effective circumferential strain would equal the local circumferential strain for an axisymmetric scleral shell.

For statistical comparisons, we defined four regions, R1 to R4, as consisting of four consecutive points along the scleral edge, excluding the first two points closest to the ONH and the last two points closest to the fixture (Fig. 2). The strains within a region were then averaged to provide a single pressure-strain curve for each region for each of three strain measures: the effective circumferential strain, the temporal meridional strain, and the nasal meridional strain. The three averaged

FIGURE 3. Temporal versus nasal scleral edge. This figure shows the superimposed temporal and nasal scleral edges for (A) a CD1 mouse and (B) a B6 mouse. The nasal and temporal edges of each specimen were not significantly different from each other. Thus, the definition for D provides a reasonable approximation for the diameter.

strain measures were compared regionally for each specimen at each pressure step of interest. Region 1 was that closest to the ONH (peripapillary), and regions 2, 3, and 4 were sequentially in the direction of the anterior eye (Fig. 1A).

Scleral Thickness Measurements

For measurement of scleral thickness, the superior quadrant of fresh unfixed sclera was cut from the limbus to the peripapillary area and placed in buffer. Three strips from this quadrant, measuring 0.33 mm wide and 2.5 mm long, were cut from the peripapillary area to the limbus with a sharp blade. Each strip was further divided into six portions, every 0.4 to 0.5 mm, designated as section 1 (peripapillary)

to section 6 (limbal area; Fig. 4). Using an eyepiece micrometer, three measurements of sclera thickness were then made in each of the six sections of the three strips from an eye, with the mean for each section reported here. Parallel measurements done on fresh, unfixed scleral segments by confocal microscopy showed that the thickness obtained was consistent between the two methods (data not shown). Some eyes had scleral thickness measured without prior inflation testing, while others were measured after inflation testing. The potential effect of such prior testing was measured in the biostatistical analysis and taken into account as a potential confounder in regression models.

Tissue Fixation and RGC Axon Loss Quantification

Tissues were fixed after inflation testing by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.2). To assess RGC damage, we estimated axon loss in optic nerve cross-sections by a quantitative sampling technique.^{42,43} After initial paraformaldehyde fixation, the optic nerve was removed and postfixed in 1% osmium tetroxide, dehydrated in alcohol, and stained with 1% uranyl acetate in 100% ethanol for 1 hour. Nerves were embedded in epoxy resin and 1- μ m cross-sections were digitally imaged to measure each optic nerve area. Then, five 40 $\times$ 40 μ m, randomly selected $\times$ 100 images were made (Cool Snap camera, Metamorph Image Analysis software; Molecular Devices, Downingtown, PA), comprising 9% of the overall nerve area. Masked observers edited nonaxonal elements from each image to estimate true axon density. The average axon density per square millimeter was multiplied by the individual nerve area to estimate the axon number. Experimental eyes were compared to the mean axon number in pooled fellow eye nerves to yield percent axon loss.

Statistical Analysis

The following data were tabulated and compared statistically between treated and control eyes: IOP average level, IOP exposure over time (positive integral = area under the IOP versus time curve in the treated eye that exceeded the area under the IOP versus time curve in the control eye), axial length and widths, axon count, and strains from inflation testing. Mean values were compared with parametric statistical tests for data that were normally distributed and median values with nonparametric testing for those whose distributions failed normality testing. Multivariable regression models, using a generalized estimating equation (GEE) approach when multiple measurements on each mouse were included, were used to compare pressure-strain behavior between the two types of mice and between glaucoma and

FIGURE 4. Schematic of locations for scleral thickness measurements. This figure shows the schematic of an inflation tested right eye, where S_k indicates the sections delineated by the six locations of scleral thickness measurements. The first four scleral thickness measurement sections (S1-S4) approximately correspond to the position of the four regions analyzed during inflation testing Figure 1A (R1-R4). The regions corresponding to sections 5 and 6 were not measured during inflation testing as the fixture and glue obstruct the view of these areas. The **bold dashed line** indicates the typical position of the fixture and the **dotted line** indicates the limbal margin.

TABLE 1. Axial Length/Width and Scleral Thickness Data*

	<i>n</i>	Length†	Width S-I	Width N-T	Section 1‡	Section 2	Section 3	Section 4	Section 5	Section 6
Glaucoma B6	42	3.68 (0.39)	3.44 (0.35)	3.51 (0.37)	55.6 (9.8)	48.4 (7.6)	48.4 (9.1)	41.7 (8.4)	45.1 (8.9)	53.5 (9.5)
B6 control	128	3.37 (0.12)	3.27 (0.13)	3.29 (0.15)	61.1 (8.0)	47.7 (6.8)	41.0 (6.3)	38.1 (6.2)	38.6 (6.7)	53.7 (8.3)
Glaucoma CD1	43	3.84 (0.25)	3.61 (0.19)	3.74 (0.20)	48.7 (9.0)	41.1 (6.9)	36.8 (6.5)	34.5 (6.2)	35.6 (4.7)	43.0 (5.8)
CD1 control	126	3.53 (0.19)	3.48 (0.14)	3.52 (0.15)	55.1 (7.7)	46.0 (7.1)	40.4 (6.0)	38.1 (5.4)	38.4 (5.4)	44.9 (8.0)

n, number of eyes; S-I, superior-inferior direction; N-T, nasal-temporal direction.

* Values are given as mean (standard deviation).

† Length and width measurements are given in millimeters.

‡ Scleral thickness measurements from sections 1 through 6 are given in micrometers.

control data, and to compare outcome parameters such as axial length/width and axon count (GraphPad InStat; GraphPad Software, Inc., LaJolla, CA; and SAS 9.2; SAS Institute, Cary, NC). Strain curves for each region and each measure of strain were estimated using three separate GEE models. The first model (control eyes only) estimated the curve for CD1 control eyes and B6 control eyes and the difference between B6 and CD1 control eyes. The second model (glaucoma eyes only) estimated the curve for CD1 glaucoma eyes and B6 glaucoma eyes. For the third model, the difference in strain between the control eye and the glaucoma eye for a mouse was used as the independent variable in order to estimate the difference between glaucoma eyes and control eyes for each strain measurement and to compare the B6 difference and the CD1 difference. The working correlation matrix for the repeat measurements at seven pressures was assumed to have an autoregressive structure, in which measurements taken closer in time have higher correlation. For each strain ratio, separate GEE models were used to obtain estimates for control eyes and glaucoma eyes. The working correlation matrix for the repeat measurements at seven pressures for each of four regions was assumed to have an exchangeable structure, in which any two repeat measurements had the same correlation.

RESULTS

Normal Axial Length/Width

Control CD1 mice had significantly longer and wider eyes than did B6 mice ($P < 0.0001$, multivariable model adjusting for age and previous inflation testing; Table 1). The CD1 controls overall were 4.6% longer than control B6. In multivariable regression models, for both CD1 and B6 combined, older eyes and eyes that were measured without aldehyde fixation were significantly longer (multivariable regression $R^2 = 0.37$, $P < 0.0001$ [age], $P = 0.003$ [fixation]). There was a slightly greater width for both mouse types in the nasal-temporal meridian than in the superior-inferior one. This difference was not significant in B6 control eyes ($P = 0.2$), and while significant in CD1 controls, the difference was only 1.2% ($P = 0.03$, t -test; Table 1).

B6 control eyes were significantly longer in older mice than in younger mice, and eyes that were measured without aldehyde fixation were longer than those with fixation (multivariable model, $R^2 = 0.21$, $P < 0.0001$ [age], $P = 0.0009$ [fixation], $n = 121$ eyes). There was also an increase in both width measurements with age (nasal-temporal and superior-inferior; Table 2). In 122 control CD1 mouse eyes, axial length and both widths were significantly larger in older mice (multivariable regression, $R^2 = 0.31$, $P < 0.0001$), but these parameters were unaffected by fixation ($P = 0.10$). The mean axial length in eyes measured prior to fixation for B6 eyes was 3% greater than those measured after fixation (3.41 mm compared with 3.31 mm, $n = 77$, 51); in CD1 eyes mean axial length was 3.53 mm for both eyes with and without prior fixation.

Normal Scleral Thickness

Among all three mouse types, the sclera was thickest at the peripapillary area (section 1), second thickest at the limbus (section 6), and thinnest in the midsclera (Tables 1–3). Normal B6 eyes had significantly thicker peripapillary and limbal sclera than CD1 eyes, but similar scleral thickness to CD1 in the midscleral sections (multivariable regression model controlling for age, the P value for peripapillary or limbal sclera was < 0.0001 ; Fig. 5). DBA/2J eyes at 2 to 4 months of age were not significantly different from B6 in scleral thickness in any section.

Comparing the scleral thicknesses to axial length and width by mouse type, the thickness of the peripapillary sclera in B6 controls was significantly related to the width of the eye, such that the wider the eye, the thinner the peripapillary sclera (regression model, adjusted for age and inflation test status, $P = 0.03$ [peripapillary thickness]). Axial length (as opposed to width) was not significantly related to peripapillary scleral thickness in B6. There was no significant relationship between either axial length or width and peripapillary scleral thickness in CD1 control eyes (multivariable regression model, $P = 0.9$ [peripapillary sclera]).

In B6 mice, we acquired more extensive age-related data. These showed that as animals became older in the first year of

TABLE 2. Mean Axial Length/Width and Scleral Thickness: B6 Control Mice by Age*

<i>n</i>	Age†	Length‡	Width S-I	Width N-T	Section 1§	Section 2	Section 3	Section 4	Section 5	Section 6
72	2 to 4	3.34	3.2	3.23	59.3	48.5	41.8	38.7	40.8	54.9
25	5 to 7	3.36	3.31	n.a.	64.4	43.9	37.3	36.5	35.3	50.3
24	10 to 12	3.46	3.34	3.36	65.2	49.4	42.5	37.8	35.9	58.0
7	15 to 26	3.44	3.53	3.59	55.1	45.7	39.4	37.5	35.9	40.9

n.a., data are not available.

* Young DBA/2J mice (2–4 months old, prior to developing glaucoma) had similar axial length to the B6 controls (Table 3).

† Age is given in months.

‡ Length and width measurements are given in millimeters.

§ Scleral thickness measurements from sections 1 through 6 are given in micrometers.

TABLE 3. Mean Axial Length/Width and Scleral Thickness: DBA/2J Mice by Age

<i>n</i>	Age*	Length†	Width S-I	Width N-T	Section 1‡	Section 2	Section 3	Section 4	Section 5	Section 6
51	2 to 4	3.37	3.27	3.29	56.9	44.3	38.8	36.9	38.0	51.9
20	10 to 12	3.83	3.69	3.71	55.6	42.3	37.2	34.9	37.2	54.2
7	15 to 26	3.9	3.57	3.6	56.5	43.0	37.2	37.2	38.3	48.7

* Age is given in months.

† Length and width measurements are given in millimeters.

‡ Scleral thickness measurements from sections 1 through 6 are given in micrometers.

life, axial length and width increased, and scleral thickness either remained the same or increased. However, in mice over 1 year of age, while length and width remained stable or increased somewhat, scleral thickness declined to levels even below those seen in the youngest animals. For example, in the peripapillary area (section 1), thickness was significantly related to age (nonparametric ANOVA, $P = 0.0002$; Table 3), with increasing mean thickness from 2 to 4 months to 5 to 7 months and between 2- to 4-month and 10- to 12-month eyes ($P < 0.05$, $P < 0.01$, respectively). By contrast, the oldest, 15- to 26-month-old animals had significantly thinner sclera in section 1 than either 5- to 7-month-old or 10- to 12-month-old mice ($P < 0.05$, $P < 0.01$, respectively), but not significantly different from 2- to 4-month-old mice ($P > 0.05$). A similar pattern occurred in the limbal area (section 6), in which the 15- to 26-month sclera was significantly thinner than the youngest 2- to 4-month-old or the 10- to 12-month-old mice ($P < 0.01$, $P < 0.001$, respectively). This pattern of scleral thickening from young to adult animals with subsequent thinning in elderly animals has been reported by Girard and coworkers in monkeys.²⁷ Coudrillier et al.³⁰ also reported that older age was predictive of a thinner sclera in human donor eyes, with the average scleral thickness decreasing 15% between 40 and 90 years of age in normal human eyes. A different pattern was seen in the midsclera (sections 2–5); the youngest mice, 2 to 4 months of age, had significantly thicker sclera than any of the older three groups, but from 5 months onward the groups did not differ (e.g., section 2, nonparametric ANOVA, $P < 0.001$ for 2–4 months compared with 5–7 months, other differences $P > 0.05$).

FIGURE 5. Normal scleral thickness: B6, CD1. Blue (CD1 control) and red (B6 control) indicate the mean scleral thickness from sections 1 (peripapillary) through 6 (limbus) and corresponding standard deviations (flagged vertical bars).

IOP and Axon Data for Bead-Induced Glaucoma Eyes

The IOPs in bead-injected glaucoma eyes from both types of mice were significantly higher than in control eyes. The positive integral IOP difference between bead-injected and control fellow eyes was not significantly different between the two types of mice. The median for CD1 was 118 mm Hg-days and for B6 it was 104 mm Hg-days (means: 134 ± 149 and 174 ± 113 , respectively, $P = 0.3$, *t*-test).

For the inflation studies included here, in the protocol utilized, the globes were removed, the optic nerve excised, and inflation tests performed. Then, the tissues were immersed in fixative. Previously, we showed that immersion fixation is not ideal for counting optic nerve axon loss compared with fixation by perfusion of fixative through the vasculature immediate after sacrifice by exsanguination under anesthesia.³⁹ In fact, delayed immersion fixation leads to significantly higher variability in axon counts, which makes the determination of differences in axon loss between groups much more difficult. Therefore, it was not surprising that the variance in axon numbers in the B6 and CD1 nerves that were evaluated here was twice as high as in perfusion-fixed specimens. The mean axon loss for the study nerves was $25 \pm 23\%$ compared to their fellow eye nerves (median loss = 20%; $P < 0.0001$, Wilcoxon rank sum test), showing that the glaucoma model produced significant damage. However, due to the higher variance in axon counts compared to ideal fixation, we did not detect a significant difference between CD1 and B6 nerves with the present sample, which had only 50% power, to have determined a difference between the mouse types as large as that seen in our prior work with much larger numbers of animals and more ideal perfusion fixation.

Effect of Experimental Glaucoma on Axial Length/Width

There was a significant increase in axial length and in both width measurements in CD1 and B6 mice after 6 weeks of glaucoma. Likewise, axial length significantly increased in DBA/2J mice by 10 months of age or older ($P < 0.0001$ for all, *t*-test; Tables 1, 3, and 4). The length increase was 8.8% for CD1 and 9.2% for B6, while in 10- to 12-month-old DBA/2J, length was 13.7% greater than in 2- to 4-month-old mice. The width increase in the nasal-temporal meridian was 6.2% (CD1) and 6.8% (B6), but only 3.8% (CD1) and 5.1% (B6) in the superior-inferior meridian. Regression models adjusting for age, prior aldehyde fixation, and IOP exposure showed no significant difference between the CD1 and B6 mice in the changes induced by bead glaucoma in axial length or width ($P > 0.05$ for all).

Effect of Experimental Glaucoma on Scleral Thickness

After chronic IOP elevation, the changes in scleral thickness differed in the two mouse types with induced bead glaucoma.

TABLE 4. Percent Change in Scleral Anatomy with Glaucoma

	Length	Width S-I	Width N-T	Section 1	Section 2	Section 3	Section 4	Section 5	Section 6
CD1	8.8*	3.8*	6.2*	-11.7*	-10.7*	-9.0†	-9.3†	-7.2‡	-4.1
B6	9.2*	5.1*	6.8*	-9.0†	1.6	5.8	9.5‡	16.7*	-0.3

n = 34 pairs of eyes from each strain of mice.

* *P* < 0.0001.

† *P* < 0.001.

‡ *P* = 0.003.

In both types of mice, peripapillary scleral thickness became significantly thinner and the limbal sclera did not change significantly (Table 4; differences were not significantly related to positive integral IOP). However, in CD1 mice, every area of the sclera became thinner, and for all but the limbal measure the thinning was statistically significant (Fig. 6; Table 4; *P* = 0.008 for significance due to multiple comparisons). By contrast, the B6 mice actually developed thicker sclera—significantly so in sections 4 and 5 (*t*-tests, adjusted for positive integral IOP exposure, Table 4; Fig. 6). By contrast, the DBA/2J mice did not develop either thicker or thinner sclera (data not shown).

Mechanical Behavior

The averaged pressure-strain curves measured for control CD1 and B6 eyes for nasal meridional strain, temporal meridional strain, and the effective circumferential strain exhibited a nonlinear, strain-stiffening response typical of collagenous tissues (Fig. 7). In the statistical models that compared the pressure-strain response by region across mouse types, we used the slope of the pressure/strain relation denoted as the change in strain per unit change in pressure, with the pressure data converted to a log scale to produce assumptions of linearity for comparisons (Tables 5, 6). In this metric, a larger ratio of strain to log pressure indicates a more compliant response. In control eyes, CD1 showed significantly greater temporal meridional strain than B6 in three of the four regions (multivariable regression with GEE approach, Table 5; typical data shown for region 1, peripapillary area; Fig. 7). In both types of mice, the glaucoma eyes were stiffer than controls, with statistically significant stiffening in the majority of regional data for the three parameters of strain, nasal meridional, and temporal meridional ($E_{\Phi\Phi}$), and effective circumferential ($\bar{E}_{\theta\theta}$) (Table 6, representative data from Region 1; Fig. 8). The degree of stiffening did not differ significantly

between CD1 and B6 eyes in any region, and in each of the three strain measures, the B6 eyes remained numerically stiffer than CD1 after exposure to IOP increase.

We compared the pressure-strain response of each type of mouse as a ratio of each of the two meridional strains to the effective circumferential strain, using GEE multivariable models (Table 7). At baseline, both types of mice had significant differences in a comparison of meridional temporal to effective circumferential strain, but in the opposite direction (i.e., meridional temporal greater than circumferential for CD1 and the reverse for B6). With glaucoma, the strain ratio for CD1 sclera changed to be not different from 1 in the temporal meridional to circumferential value, while the B6 eyes retained a ratio significantly less than 1.

For the nasal meridional to circumferential ratio, CD1 sclera had a value not different from 1 at baseline, which significantly increased in the glaucoma eyes (Table 7). For B6, the nasal/circumferential ratio was not significantly different from 1 in control or glaucoma eyes.

DISCUSSION

CD1 mice are more susceptible than B6 mice to death of RGC in experimental glaucoma induced by bead injection, as shown in two prior reports^{39,40} by both RGC cell body and axon loss in hundreds of eyes. This difference provides the opportunity to explore possible factors that determine susceptibility. In previous research, we found that young DBA/2J mice (prior to developing spontaneous glaucoma) have RGC damage in the experimental bead model that falls between that of CD1 and B6 mice. We explored the hypotheses that either the baseline state of the sclera or the scleral response to chronically elevated IOP, or both, are associated with this variability in susceptibility to glaucoma injury.

The greater susceptibility in CD1 mice was associated with the following baseline features compared with B6: longer eyes, thinner sclera in the critical peripapillary area, greater baseline temporal meridional strain, and greater temporal meridional than effective circumferential strain. For both theoretical and empirical reasons, a larger eye would be expected to be at greater risk for IOP-related damage. The larger the diameter of a spherical shell, the greater the stresses are in its wall, all other factors equal. Consistent with this concept, persons with myopia, who generally have longer eyes, are known to be at greater risk for OAG.¹³ However, it is clearly too simplistic to consider that axial length/width is the sole factor involved in glaucoma susceptibility. Scleral tissues can also vary in thickness, composition, and biomechanical behavior, leading to greater or lesser strain. To illustrate how axial length alone may not be the dominant factor, we found that older B6 mice have longer eyes with similar scleral thickness, yet are less susceptible to RGC death than younger B6.⁴⁰ In addition, mice with an induced mutation in collagen 8, which have longer eyes than control B6, also have less susceptibility to RGC loss than wild type.⁴⁴ We are now carrying out further studies of the changes in scleral anatomy and their relationship to the

FIGURE 6. Change in scleral thickness with experimental glaucoma. Blue (CD1) and red (B6) bar graphs indicate the change in scleral thickness after glaucoma.

FIGURE 7. Pressure versus strain, region 1: CD1 control versus B6 control. *Blue* (CD1 control) and *red* (B6 control) curves illustrate the mean pressure-strain (solid line) and corresponding standard deviation (flagged horizontal line) for (A) the temporal meridional strain, (B) the nasal meridional strain, and (C) the effective circumferential strain.

inflation responses of mouse eyes with experimental glaucoma. The peripapillary sclera is a site of great interest in glaucoma pathogenetic research, so it is intriguing that the more susceptible CD1 mice have a thinner sclera and greater temporal meridional strain at baseline at this site prior to induction of glaucoma. Human scleral thickness varies by location in a manner similar to that seen in mice.^{38,45} The peripapillary area has been studied histologically and found to have collagen and elastin fibers oriented in a circumferential ring around the ONH in human,^{46–48} rat,⁴⁹ and mouse eyes.⁴⁷ The increased stiffness from these circumferential fiber reinforcements may partially protect the tissues of the ONH from the stress concentrations caused by the presence of the more compliant ONH by reducing the scleral canal expansion in response to IOP elevation.⁵⁰ At the same time, the fiber reinforcements may cause the tissues of the ONH to be more susceptible to damage from posterior bowing in response to IOP elevation. The degree of circumferential fiber alignment decreases significantly away from the ONH in mice and in human eyes.^{51–53} Models of scleral behavior in human eyes consistently indicate that the peripapillary area is an important element determining stress on the ONH and is tightly coupled to effects in the lamina cribrosa.^{20,54} Unless a thinner peripapillary sclera was somehow compensated by a greater resistance to deformation, it would represent a second factor increasing strain at the ONH.

It is equally likely that the response of the sclera to IOP during and after exposure to higher IOP in experimental glaucoma is an additional factor in glaucoma damage. In that regard, we found some responses that were consistent between mouse types and some that were different. The findings that were similar were increase in length and width of the eyes, thinning of the peripapillary sclera, and increase in stiffness in both material orientations (circumferential and meridional). The most apparent differences were that the CD1 mice developed uniformly thinner sclera than B6 mice after

glaucoma induction and relative changes of the meridional and effective circumferential strain response that were different from those of B6.

In both CD1 and B6 mice, extended IOP elevation led to thinner sclera in the peripapillary area and to larger axial length and width. These irreversible deformations of the normal scleral and ONH anatomy illustrate a behavior that is observed in infant human eyes with glaucoma,⁵⁵ and in other animal models, but not in adult human eyes. As we previously reported⁴⁰ in these two types of mice, as well as in DBA/2J mice, IOP length increase is similar among mouse types with experimental bead glaucoma, despite substantial differences in RGC damage. Therefore, neither peripapillary thinning nor elongation of the eye per se was closely correlated with differential susceptibility. Thinning in the peripapillary area could distort or alter the choroid near the ONH, leading to changes in the “crescent” zones observed to be more common or to enlarge with glaucoma.⁵⁶ Widening of the peripapillary opening for the ONH in human glaucoma has been documented.¹¹ Studies of blind secondary glaucoma human eyes found no definite thinning of the peripapillary sclera compared to normal.⁵⁷

The response to glaucoma did differ in scleral thickness away from the peripapillary sclera, with CD1 mice uniformly becoming thinner, while DBA/2J remained relatively constant in thickness, and the B6 mice actually developed thicker sclera. This matches the relative susceptibilities to RGC loss among the three mouse types in axon loss after 12 weeks of experimental bead glaucoma.^{39,40} Girard et al.²¹ found no scleral thickness changes in a small number of glaucoma monkey eyes, and though that research group⁵⁸ found that the equatorial sclera was thinner in some monkey glaucoma eyes, the peripapillary sclera was not found to thin with glaucoma in monkeys.¹⁷ Coudrillier et al.³⁰ studied human glaucoma eyes, finding that glaucoma specimens that exhibited optic nerve damage had a significantly thicker sclera than either age-

TABLE 5. Pressure Strain Data, Region 1: B6 Control versus CD1 Control

Measure of Strain	Group	No. of Eyes	Change in Strain per Unit Change in Log Pressure Estimate (95% CI)	Difference between Groups	
				Estimate (95% CI)	P Value
Temporal $E_{\Phi\Phi}$	B6 control	20	0.010 (0.004, 0.016)	−0.022 (−0.036, −0.008)	0.002
	CD1 control	20	0.032 (0.019, 0.045)		
Nasal $E_{\Phi\Phi}$	B6 control	20	0.031 (0.022, 0.041)	−0.001 (−0.02, 0.018)	0.91
	CD1 control	20	0.032 (0.016, 0.048)		
$\bar{E}_{00}$	B6 control	20	0.017 (0.014, 0.019)	−0.003 (−0.008, 0.001)	0.16
	CD1 control	20	0.02 (0.016, 0.024)		

CI, confidence interval.

TABLE 6. Pressure Strain Data, Region 1: Control versus Glaucoma

Measure of Strain	Group	No. of Eyes	Change in Strain per Unit Change in Log Pressure Estimate (95% CI)	Difference between Glaucoma and Control for Each Strain			P Value Comparing B6 Difference with CD1 Difference
				No. of Mice	Estimate (95% CI)	P Value	
Temporal $E_{\Phi\Phi}$	B6 glaucoma	12	0.007 (0.002, 0.012)	9	0.001 (−0.01, 0.012)	0.87	0.004
	B6 control	20	0.010 (0.004, 0.016)				
	CD1 glaucoma	20	0.012 (0.005, 0.02)	13	−0.026 (−0.040, −0.011)	0.001	
	CD1 control	20	0.032 (0.019, 0.045)				
Nasal $E_{\Phi\Phi}$	B6 glaucoma	12	0.013 (0.005, 0.021)	9	−0.008 (−0.019, 0.003)	0.17	0.57
	B6 control	20	0.031 (0.022, 0.041)				
	CD1 glaucoma	20	0.018 (0.007, 0.028)	13	−0.014 (−0.033, 0.004)	0.13	
	CD1 control	20	0.032 (0.016, 0.048)				
$E_{\Theta\Theta}$	B6 glaucoma	12	0.011 (0.007, 0.015)	9	−0.005 (−0.01, 0.001)	0.10	0.70
	B6 control	20	0.017 (0.014, 0.019)				
	CD1 glaucoma	20	0.012 (0.008, 0.016)	13	−0.006 (−0.013, 0.001)	0.09	
	CD1 control	20	0.02 (0.016, 0.024)				

matched normal controls or undamaged glaucoma specimens. These findings and the present data make it possible that remodeling of the sclera is a contributing feature to susceptibility to glaucoma damage.⁵⁹

The strain response of both CD1 and B6 were stiffer after glaucoma, despite the differences in scleral thickness change. The relative increase in stiffness was similar in both types of mice, suggesting that this was not the explanation for differential susceptibility. It is unclear whether the stiffening after glaucoma is beneficial or detrimental. In a previous report,²⁶ we inflation tested seven 2-month-old B6 mice and six 11-month-old B6 mice, determining the stiffness by pressure-induced displacement in the peripapillary sclera. The load-

unload tests of younger specimens were significantly more compliant than for the older specimens, while the axial lengths and widths of the older specimens were also significantly larger than the younger specimens without a difference in scleral thickness. Clearly, the behavior of the sclera is complex, meriting detailed study, not only of inflation behavior and macroscopic anatomy, but of fibril orientation, composition, and other molecular rearrangements with age and during disease. We are presently engaged in such studies.

Stiffening of the ONH and sclera has been reported in other models and in living and postmortem human glaucoma eyes. Zeimer and Ogura⁶⁰ used an inflation method with postmortem glaucoma eyes and found that the ONH was stiffer and that

FIGURE 8. Pressure versus strain, region 1: CD1 control versus CD1 glaucoma, B6 control versus B6 glaucoma. Blue (CD1 control) and green (CD1 glaucoma) curves illustrate the mean pressure-strain (solid line) and corresponding standard deviation (flagged horizontal line) for (A) the temporal meridional strain, (B) the nasal meridional strain, and (C) the effective circumferential strain. Red (B6 control) and black (B6 glaucoma) curves illustrate the mean pressure-strain (solid line) and corresponding standard deviation (flagged horizontal line) for (D) the temporal meridional strain, (E) the nasal meridional strain, and (F) the effective circumferential strain.

TABLE 7. Strain Ratios of Meridional to Effective Circumferential Inflation Behavior

Strain Ratio*	Mouse Strain	Treatment	No. of Eyes	Estimate (95% CI)	P Value, H_0 : Ratio = 1
Temporal $E_{\Phi\Phi}/\bar{E}_{00}$	CD1	Control	20	1.36 (1.15, 1.61)	0.0004
		Glaucoma	20	0.86 (0.62, 1.19)	0.35
	B6	Control	20	0.76 (0.68, 0.84)	<0.0001
		Glaucoma	12	0.70 (0.56, 0.89)	0.003
Nasal $E_{\Phi\Phi}/\bar{E}_{00}$	CD1	Control	20	1.03 (0.85, 1.26)	0.75
		Glaucoma	20	1.34 (1.15, 1.57)	0.0002
	B6	Control	20	0.82 (0.66, 1.02)	0.08
		Glaucoma	12	0.95 (0.80, 1.13)	0.59

* Calculations for the strain ratios include data from all four regions. Temporal $E_{\Phi\Phi}/\bar{E}_{00}$ is the ratio of the temporal meridional strain to the effective circumferential strain. Nasal $E_{\Phi\Phi}/\bar{E}_{00}$ is the ratio of the nasal meridional strain to the effective circumferential strain.

stiffness was greater with greater RGC damage. Testing of living human eyes by indirect methods also suggests that glaucoma eyes have stiffer responses.^{15,16} Coudrillier et al.³⁰ compared 24 normal and 11 glaucoma pairs of postmortem eyes, finding that the glaucoma scleras had a different strain response in the peripapillary sclera characterized by a stiffer meridional response and slower circumferential creep rates than normal. Glaucoma eyes were not significantly different from normal eyes in stresses and strains in the midposterior sclera. Girard et al.²¹ studied eight monkey glaucoma eyes, determining that stiffness increased with moderate glaucoma damage, though the response was variable. They caution, “reporting a single stiffness value for the sclera does not represent its biomechanical response well. Scleral stiffness is a function of IOP (nonlinearity), orientation (anisotropy), and location (heterogeneity). This complexity should be taken into account when evaluating the contribution of scleral biomechanics to glaucoma pathogenesis.” Roberts et al.⁶¹ modeled behavior of the ONH in three early glaucoma monkey eyes from connective tissue volume fractions. They hypothesized that scleral stiffening in glaucoma may shield the ONH somewhat by an increased load carried in the sclera. From the human data suggesting increased stiffness, we could not distinguish between two alternative hypotheses. One hypothesis is that compliant sclera increases susceptibility to glaucoma, and as damage occurs, the sclera becomes stiffer than normal. In this scenario, a compliant response at baseline would increase strain at the ONH and make damage more likely. The stiffness found in damaged glaucoma eyes would be explained as a response occurring during the chronic glaucoma process in the sclera. An alternative hypothesis is that stiffer eyes at baseline are more susceptible and become even stiffer during disease. This scenario would result if stiffness of the sclera increased strain within the ONH. Both hypotheses are compatible with existing human data. If the monkey and mouse experimental glaucoma data are relevant to the human disease, then greater stiffness is at least an effect of glaucoma. Whether eyes that are more compliant at baseline are more or less susceptible is as yet unsettled. In part, this is due to our inability at present to directly measure changes in the ONH tissues before and after induced glaucoma. Scleral canal expansion is determined more by scleral properties and responses, but outward bowing of the ONH is also influenced by properties of the ONH itself, and the two are both contributors to damage.

It will be vital to determine what molecular changes underlie alterations in scleral biomechanics in glaucoma. We and others have studied scleral fibrillar collagens and elastin,^{62,63} particularly in the peripapillary area,^{4,64} in normal and glaucomatous human eyes. The diameter distribution and orientation of fibrillar collagens in the ONH is unchanged in human OAG eyes, though elastin is

either normal⁶⁵ or possibly somewhat degraded⁶⁶ and definitely has an altered appearance.^{67,68} Collagen density decreased by 17% in both the ONH and peripapillary sclera as measured in seven human and three monkey glaucoma eyes, but collagen fibril diameter distribution was not different from controls. The orientation and response of connective tissue molecules in sclera and ONH in glaucoma have been studied in monkeys with experimental glaucoma.²¹ Substantial reorganization and new synthesis of collagens are seen in the monkey glaucoma ONH, but not with simple optic atrophy, suggesting that they are IOP mediated.³² We have measured the orientation of fibrillar elements in human normal and glaucoma eyes using wide-angle x-ray scattering.⁶⁹ ONH and peripapillary scleral elastin differs in individuals of African and European descent, perhaps representing a risk factor for higher OAG prevalence in individuals of African descent.⁷⁰ Mutations in the lysyl oxidase-like protein 1 (LOXL1) gene are associated with exfoliation glaucoma,⁷¹ providing impetus to study the connective tissue molecules that may be altered in this syndrome.^{72,73} Further research is needed into the microstructure of scleral connective tissues.

It will be useful to measure the biomechanical behavior of human eyes in vivo, both to monitor the baseline state of the eye as a risk factor for future development of glaucoma and to assess progression of disease. Some methods to assess corneal biomechanics have been recently developed^{14,74,75} that could be applied to these questions. Of even greater relevance would be methods to measure scleral compliance in vivo.

The present research should be assessed in light of several weaknesses. The mouse model of glaucoma utilized here, while having similarities to the human disease, is short term compared with the chronicity of human glaucoma. Studies of the behavior of eyes ex vivo may not duplicate precisely the behavior in life. A key assumption of the strain calculation is that points on the scleral edge deform within a plane. Significant local twisting or rotation can occur with uneven gluing and in the presence of material anisotropy characterized by preferential collagen alignment in orientations other than the circumferential and meridional directions. Wide angle x-ray scattering (WAXS) measurements of the human posterior sclera⁵³ show that the degree of collagen alignment is the strongest in the peripapillary sclera and occurs along the circumferential orientation. The degree of collagen alignment decays rapidly away from the peripapillary region. Our preliminary transmission electron microscopy measurements of collagen orientation show similar results for the mouse posterior sclera. It is likely that the out-of-plane displacements caused by local twisting or rotation are small. The scleral thickness and specimen surface are nonuniform. The latter is because it is nearly impossible to uniformly remove the extraocular tissues from the surface of the small mouse sclera.

This results in a unique natural speckle pattern for the scleral edge. Large local rotation or twisting would have changed significantly the local speckle pattern of the scleral edge and caused the DIC algorithm to lose correlation. Our method for modeling mouse eye inflation behavior would benefit from a fully three-dimensional view of the sclera to provide more comprehensive regional data for strains and stresses. We are presently developing a method by which to do this. Furthermore, there can never be a perfect model of connective tissue behavior and that used here may not ideally approximate the true state of the tissues. We are engaged in detailed study of the ultrastructure and proteomic content of sclera to extend the features of the model. Finally, differences between types of mice may be related to features other than or in addition to the biomechanical behavior of sclera.

In summary, we identified differences between CD1 and B6 mice in the baseline anatomy and inflation behavior of their sclera and in scleral response to chronic IOP elevation. These differences between mouse types may underlie the differential susceptibility to RGC death from experimental glaucoma in the two types of mice. With further detailed study of the molecular bases of these differences, it is feasible that therapeutic approaches to decreasing neuronal loss in glaucoma can be developed.

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