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Susceptibility to glaucoma damage related to age and connective tissue mutations in mice

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ABSTRACT

The purpose of this research was to study the effects of age and genetic alterations in key connective tissue proteins on susceptibility to experimental glaucoma in mice. We used mice haploinsufficient in the elastin gene (EH) and mice without both alleles of the fibromodulin gene (FM KO) and their wild type (WT) littermates of B6 and CD1 strains, respectively. FM KO mice were tested at two ages: 2 months and 12 months. Intraocular pressure (IOP) was measured by Tonolab tonometer, axial lengths and widths measured by digital caliper post-enucleation, and chronic glaucoma damage was measured using a bead injection model and optic nerve axon counts. IOP in EH mice was not significantly different from WT, but FM KO were slightly lower than their controls ($p = 0.04$). Loss of retinal ganglion cell (RGC) axons was somewhat, but not significantly greater in young EH and younger or older FM KO strains than in age-matched controls ($p = 0.48$, 0.34, 0.20, respectively, multivariable regression adjusting for IOP exposure). Older CD1 mice lost significantly more RGC axons than younger CD1 ($p = 0.01$, multivariable regression). The CD1 mouse strain showed age-dependence of experimental glaucoma damage to RGC in the opposite, and more expected, direction than in B6 mice in which older mice are more resistant to damage. Genetic alteration in two genes that are constituents of sclera, fibromodulin and elastin do not significantly affect RGC loss.

1. Introduction

Glaucoma is the second leading cause of world blindness (Quigley and Broman, 2006) and older age (Burgoyne and Downs, 2007) and myopia (Boland and Quigley, 2007) are found to be risk factors for its prevalence. There is substantial evidence that the state of ocular connective tissues and their response to the stress of intraocular pressure (IOP) are key determinants of susceptibility to glaucoma damage to retinal ganglion cells (RGC) (Burgoyne et al., 2005; Quigley and Addicks, 1981). If we can determine the features of ocular connective tissues that make glaucoma more likely or more damaging, new treatments could be directed to altering those features (Quigley and Cone, 2013; Strouthidis and Girard, 2013). With increasing age, human eyes develop changes in the connective tissues of the sclera (Friberg and Lace, 1988) and lamina cribrosa of the optic nerve head (Quigley, 1977). Scleral thickness decreases with age in humans (Coudrillier et al., 2012) and monkeys (Girard et al., 2009), though older reports suggested an increase with age in humans (Watson and Young, 2004). There is an associated increase in scleral stiffness with age as measured by *ex vivo* strip (Friberg and Lace, 1988) and inflation testing (Coudrillier et al., 2012). Initial testing of the effect of age on scleral stiffness in mice confirmed that the sclera also was stiffer in older than in younger B6 mice (Myers et al., 2010). The determinants of this age-related change have been studied in animals and include alterations in proteoglycans (Rada et al., 2000) or increased cross-linkage of extracellular matrix components (Schultz et al., 2008). In initial studies of the effect of experimental glaucoma in mice, we have reported and confirmed the surprising finding that older B6 mice are less susceptible to RGC loss with chronic elevated IOP than younger B6 mice (Cone et al., 2010; Cone et al., 2012).

Myopic eyes have, in general, not only larger than normal axial length, but thinner sclera and decreased stiffness (Curtin and Teng, 1958; Curtin, 1969; McBrien et al., 2009). While there have been many studies of induced myopia/axial length increase in a variety of animal models (Rada et al., 2006), the relation between myopia and its scleral alterations on the one hand and susceptibility to

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glaucoma injury on the other has not been studied in detail in animals. If the sclera were considered as a theoretical thin-walled sphere, axially longer eyes would be at a disadvantage in withstanding the same IOP as a smaller eye, since it would be expected that the stress would be greater. However, this simple relation does not take into account scleral thickness, baseline behavior of the sclera biomechanically, and the dynamic response of the sclera, all of which are probably important. Reasoning that axially longer mouse eyes might have different susceptibility to experimental glaucoma, we have tested several strains and compared scleral thickness, scleral inflation behavior, and RGC loss with comparable chronic IOP elevation. In the first such comparison, we found that albino CD1 mice, which have larger eyes than B6 mice, are more susceptible to RGC loss than B6 (Cone et al., 2010; Nguyen et al., 2013). Next, we studied another mouse strain with axially longer eyes, the Aca23 mutant, with a mutation in Collagen 8α2 (Steinhart et al., 2012). Interestingly, these mutants were significantly less susceptible to glaucoma damage than wild type littermates.

These initial findings suggest that the common beliefs that glaucoma damage would occur more easily in all older or in all larger eyes are not supportable, at least in a murine glaucoma model. We need to understand better what features of age and myopia may contribute to susceptibility to glaucoma injury. The state of scleral connective tissues may be one area that affects this susceptibility. In the present study, we include study of two further mouse strains with genetic deficiency in key components of scleral connective tissue. One of the strains is haploinsufficient for elastin (Aszodi et al., 2006) (designated EH and developed on a B6 background) and the other is a knockout of fibromodulin (Chakravarti et al., 2003; Jepsen et al., 2002; Svensson et al., 1999) (designated FM KO and produced in CD1 mice). EH mice have abnormal biomechanical responses in major connective tissues, such as arterial walls (Carta et al., 2009). Fibromodulin is a small interstitial proteoglycan thought to participate in the assembly of the extracellular matrix as it interacts with type I and type II collagen fibrils and inhibits fibrillogenesis *in vitro*. It is reported to regulate TGF-β activity (Kalamajski and Oldberg, 2007). The FM KO mice have thinner sclera and smaller collagen fibril diameter, potentially altering their susceptibility to IOP-induced damage. In the FM KO strain, we studied mice at two distinct ages. Neither strain, EH or FM KO, showed significantly different RGC loss from their wild type controls, but there was significantly greater damage in older than in younger CD1 mice – the opposite of our prior finding in B6.

2. Methods

2.1. Animals

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved and monitored by the Johns Hopkins University School of Medicine Animal Care and Use Committee. Wild type (WT) CD1 albino mice were obtained from Charles River Laboratories, Wilmington, MA, USA, and WT B6 mice were obtained from Jackson Laboratories, Bar Harbor, ME, USA. The fibromodulin knockout mice (FM KO) mice on a CD1 background were obtained from co-investigator, Dr. Shukti Chakravarti, and have collagen structural defects including reduced strength of skin and tendon. These animals also showed distinct sclera architecture abnormalities including a reduction of sclera thickness and a decreased number of lamellae. The lamellae which were present were less organized and defined than in WT. The absence of fibromodulin from the sclera of these animals has been confirmed by Western blot. Also noted during that analysis was a marked increase in lumican in the sclera of these animals (Chakravarti et al., 2003). A second mouse strain (on a B6 background) used were haploinsufficient for elastin (EH) and develop supravalvular aortic stenosis as well as larger than normal eyes. Northern blot analysis showed a 47% decrease in elastin mRNA in EH mice as compared to WT. Also, it was found that elastic lamellae were about 50% thinner than in WT in the aorta indicating a structural abnormality (Li et al., 1998). Masked histological analysis in the peripapillary sclera showed no difference in the circumferential pattern or content of elastin between EH and WT mice (data not shown). The homozygous knockout for elastin does not survive more than a few days after birth.

2.2. Bead glaucoma

We induced experimental glaucoma by employing a protocol previously published by this laboratory (Cone et al., 2012). First, we anesthetized the animal intraperitoneally with 50 mg/kg of ketamine, 10 mg/kg of xylazine, and 2/kg mg of acepromazine. Proparacaine hydrochloride eye drops (Akorn Inc., Buffalo Grove, IL) were used for additional ocular topical anesthesia. Then, we injected 2 μl of 6 μm diameter beads (Polybead Microspheres®; Polysciences, Inc., Warrington, PA), then 2 μl of 1 μm diameter beads, followed by 1 μl of viscoelastic compound (10 mg/ml sodium hyaluronate, Healon; Advanced Medical Optics Inc., Santa Ana, CA). The injection was made with a glass cannula with tip diameter of 50 μm connected by polyethylene tubing to a Hamilton syringe (Hamilton, Inc., Reno, NV) into which all 3 components of the injection were loaded in series.

2.3. IOP Measurements

IOP measurements were taken prior to bead injection, immediately after the injection, and at 3 days, 1 week, and weekly thereafter until sacrifice 6 weeks after injection. For IOP measurements, mice were sedated with inhalation of isoflurane delivered by means of the RC2 – Rodent Circuit Controller (VetEquip, Inc., Pleasanton, CA), delivering 2.5% isoflurane in oxygen to the animal via a sealed immersion box, then through a nose cone. The TonoLab tonometer (TioLat, Inc., Helsinki, Finland) was used to perform IOP measurements. The tonometer performs 6 separate pressure measurements and then provides the mean of those measurements for optimal reproducibility. Only measurements indicated as optimal reproducibility were utilized.

2.4. Axial length/width measurements

In this study, we measured length in aldehyde-fixed eyes. Normal mice and experimental glaucoma mice were sacrificed by exsanguination and perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.2). The optic nerve was collected 1 mm distal to the globe. IOP was set at 15 mmHg 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, USA). The axial length was measured from the center of the cornea to a position just temporal to the optic nerve insertion. The axial width was measured nasal—temporal and superior—inferior at the largest dimension at the equator, midway between the cornea and optic nerve (Fig. 1).

2.5. Optic nerve axon count method

To quantify RGC loss, we quantified axon loss in optic nerve cross-sections using a validated sampling method (Levkovitch-