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Experimental scleral cross-linking increases glaucoma damage in a mouse model☆

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ABSTRACT

The purpose of this study was to assess the effect of a scleral cross-linking agent on susceptibility to glaucoma damage in a mouse model.CD1 mice underwent 3 subconjunctival injections of 0.5 M glyceraldehyde (GA) in 1 week, then had elevated intraocular pressure (IOP) induced by bead injection. Degree of cross-linking was measured by enzyme-linked immunosorbent assay (ELISA), scleral permeability was measured by fluorescence recovery after photobleaching (FRAP), and the mechanical effects of GA exposure were measured by inflation testing. Control mice had buffer injection or no injection in 2 separate glaucoma experiments. IOP was monitored by Tonolab and retinal ganglion cell (RGC) loss was measured by histological axon counting. To rule out undesirable effects of GA, we performed electroretinography and detailed histology of the retina. GA exposure had no detectable effects on RGC number, retinal structure or function either histologically or electrophysiologically. GA increased cross-linking of sclera by 37% in an ELISA assay, decreased scleral permeability (FRAP, $p = 0.001$), and produced a steeper pressure–strain behavior by *in vitro* inflation testing. In two experimental glaucoma experiments, GA-treated eyes had greater RGC axon loss from elevated IOP than either buffer-injected or control eyes, controlling for level of IOP exposure over time ($p = 0.01$, and 0.049, multivariable regression analyses). This is the first report that experimental alteration of the sclera, by cross-linking, increases susceptibility to RGC damage in mice.

1. Introduction

Glaucoma is the second leading cause of blindness worldwide (Quigley and Broman, 2006), and the incidence and progression of open angle glaucoma (OAG) are closely related to mean intraocular pressure (IOP) (Bengtsson and Heijl, 2005) and to its variability (Nouri-Mahdavi et al., 2004; Boland and Quigley, 2007). IOP acts a mechanical load on the lamina cribrosa through the translaminar pressure differential and the tensile hoop stresses in the sclera. These two effects combine to produce a stress and deformation state that leads to alterations in ONH glial and connective tissues, damage to RGC axons (Quigley et al., 1981), and ultimately to ONH excavation, a defining clinical feature of glaucoma (Quigley et al., 1983; Burgoyne et al., 2005). Histological and physiological evidence suggests that both anterograde and retrograde RGC axonal transport are interrupted soon after a change in IOP from baseline levels, in human eyes (Quigley and Green, 1979), and in monkey (Gaasterland and Kupfer, 1974) and rodent eyes (Morrison et al., 1990).

Mammalian eyes that are subjected to experimental IOP increase have neuronal, glial, and associated tissue alterations that are phenotypically similar to those of human glaucoma (Morrison et al., 1990, 1997). Furthermore, lowering of IOP protects against progressive worsening of both animal and human glaucoma (Morrison et al., 1998; Heijl et al., 2002). IOP generates stress and strain in the sclera and lamina cribrosa, the level of which depends on the mechanical properties of the tissues. Variation in the mechanical properties of the ocular connective tissues may explain

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why half of individuals with OAG suffer injury at physiological IOP levels (Quigley and Broman, 2006). Biomechanical models (Norman et al., 2011; Sigal et al., 2005, 2011; Girard, 2009; Sigal et al., 2011; Coudrillier et al., 2012) suggest that the mechanical behavior of the sclera significantly influences the stress and deformation of the lamina cribrosa (LC) and may be a critical mechanical driver of glaucomatous damage to RGC axons. The candidate genes and their products that affect glaucoma damage may include those determining scleral composition and its response to IOP-related stress. Corneal hysteresis and myopia with its thinner sclera have been implicated as glaucoma risk factors (Boland and Quigley, 2007; Congdon et al., 2006).

Mechanical testing of normal human (Woo et al., 1972, Coudrillier et al., 2012; Fazio, 2013), porcine (Girard et al., 2008), monkey (Girard et al., 2009) and rabbit (Greene et al., 1979) scleral tissue established that the sclera deforms nonlinearly in response to IOP elevation and creeps at constant elevated IOP. The structure of the mouse sclera is similar in several respects to that of human sclera (Gelman et al., 2010; Watson and Young, 2004). The sclera becomes thinner with aging (Girard et al., 2009; Coudrillier et al., 2012) and there are decreases with age in decorin, biglycan and elastin (Watson and Young, 2004; Rada et al., 2000). An increase in glycation of collagen fibrils with age may be a factor in increasing their cross-sectional area (Keeley et al., 1984; Malik et al., 1992). Increased mechanical stiffness of the sclera with age has been reported in human (Avetisov et al., 1984; Friberg and Lace, 1988; Coudrillier et al., 2012; Geraghty et al., 2012), monkey (Girard et al., 2009), and mouse (Myers et al., 2010), in part due to increasing intermolecular collagen crosslinking with age (Curtin, 1969; Ihanamaki et al., 2001; Girard et al., 2009). Fazio et al. (2013) showed that the strains in the peripapillary sclera were significantly lower in older human specimens. The structure of the mouse lamina cribrosa is similar to the human sclera, in that axon bundles pass out through septae; however, the septae in the mouse consist of astrocytes, not connective tissue beams surrounded by astrocytes as in the monkey and human (Sun et al., 2009).

There is evidence that both human glaucoma and experimental animal models of glaucoma lead to increased scleral stiffness. Downs et al. (2005) showed that the stress relaxation behavior measured by strip tests of monkey sclera with induced glaucoma damage displayed a larger relaxation time and equilibrium modulus compared to the sclera of normal monkey eyes. Nguyen et al. (2013) found increased stiffness in mouse eyes exposed to elevated IOP. Hommer et al. (2008) measured *in vivo* ocular expansion caused by blood pressure pulsation and concluded that the eyes of glaucoma patients exhibited a higher ocular stiffness than those of non-glaucoma patients. Coudrillier et al. (2012) found that human glaucoma eyes were stiffer than normal in *post-mortem* inflation testing. It is critical to determine whether scleral stiffening as observed in human glaucoma eyes is a beneficial adaptation or a detrimental contributor to ONH injury. A stiffer sclera would decrease the expansion of the sclera canal, but would also potentially increase posterior bowing of the LC (Yang et al., 2009).

The aim of this work is to investigate the effect of scleral stiffness on the susceptibility to glaucoma damage. The concept of scleral crosslinking was introduced in 2004 by Wollensak et al. and further elaborated as a potential treatment of progressive myopia. This *in vivo* method (Wollensak et al., 2008a), was used to increase the stiffness of mouse sclera by subconjunctival injection of 0.5 M glyceraldehyde (GA), a known collagen crosslinking agent (Tessier et al., 2003; Danilov et al., 2008). We measured the alteration in biomechanical behavior caused by GA treatment using an *in vitro* inflation test (Myers et al., 2010) of normal and experimental glaucoma eyes compared to their contralateral controls. Corneal cross-linking treatment has been applied to human eyes with keratoconus, by activating riboflavin with ultraviolet light (Wollensak et al., 2003). Exposure of living rabbit corneas to GA increased cross-linkage and altered stress–strain behavior without significant damage to either the retina or to other ocular structures (Wollensak et al., 2005, 2008a, 2008b, 2009; Mattson et al., 2010; Terai et al., 2012). Cross-linking of sclera in excised pig eyes has shown increased stiffness (Thornton et al., 2009). To our knowledge, this is the first test of the effect of an experimental scleral modification on glaucoma damage.

2. Methods

2.1. Mice

We used 381 CD1 albino, female mice that were 2 months of age at the start of experiments (Charles River, Inc., Wilmington, MA). Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All protocols were approved and monitored by the Johns Hopkins University School of Medicine Animal Care and Use Committee. For individual study breakdown, please see Table 2.

2.2. GA exposure

Mice were anesthetized with a mixture of ketamine (Fort Dodge Animal Health, Fort Dodge, IA), xylazine (VedCo Inc., Saint Joseph, MO), and acepromazine (Phoenix Pharmaceuticals, Burlingame, CA) at 50, 10 and 2 mg/kg, respectively. The dosage and time of anesthesia was controlled and has been standardized as previously published (Cone et al., 2010, Gelman et al., 2010, Nguyen et al., 2013, Cone-Kimball., 2013). There is no reason to believe that variation in anesthesia was a factor in the comparative IOP data within animals or across groups of animals. Investigators were masked during injection to the content of the injected material, masked during IOP measurements after bead injection, and masked during all data acquisition including axon loss assessment. The GA experimental and control animals were treated in masked fashion on the same days, interchangeably, so that any difference in the state or effect of anesthesia would be random and would not lead to any systematic bias. A pilot opening was made in the conjunctiva with a 30 gauge needle and injections of GA or buffer were made with a mouse tail vein catheter (SAI Infusion Technologies, Libertyville, IL) connected to a 1 cc syringe. The largest volume that could be injected was 0.4 cc of 0.5 M GA (Sigma–Aldrich, St. Louis, MO, USA) dissolved in 0.1 M phosphate buffer (0.1 M Na_3PO_4, pH = 7.2). At all steps of the experiment the solutions and tissues were coded to mask the participants. The solutions were slowly injected over 5–10 s, the cannula was removed after 1 min, and excess solution was blotted with a cotton swab. IOP was measured immediately, at 3 days, and at one week. Animals receiving subconjunctival injections had 3 injections of either GA or buffer over a 7 day period- Day 0, Day 3, and Day 7. In glaucoma experiments, elevated IOP was induced by bead injection one week after the third subconjunctival injection. For assessment of GA effects, some eyes received only 1 or 2 GA injections, or lower concentrations of GA (0.1 and 0.3 M). We completed inflation studies on groups of 5–10 animals using these lower concentrations and found that the effects on stiffness in inflation testing were less consistent and insufficient to be statistically significant (data not included). At 0.5 M GA, the stiffening effects after 2 or 3 injections were significant (Fig. 2), without causing toxicity. Some effects of GA were also tested by soaking eyes after enucleation in 0.5 M GA for 1–24 h. Exposure to glutaraldehyde (not glyceraldehyde, a different molecule) in 3 tested concentrations was found to be toxic or lethal (data not shown).