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Research article

Expansions of the neurovascular scleral canal and contained optic nerve occur early in the hypertonic saline rat experimental glaucoma model

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ABSTRACT

Purpose: To characterize early optic nerve head (ONH) structural change in rat experimental glaucoma (EG).

Methods: Unilateral intraocular pressure (IOP) elevation was induced in Brown Norway rats by hypertonic saline injection into the episcleral veins and animals were sacrificed 4 weeks later by perfusion fixation. Optic nerve cross-sections were graded from 1 (normal) to 5 (extensive injury) by 5 masked observers. ONHs with peripapillary retina and sclera were embedded, serial sectioned, 3-D reconstructed, delineated, and quantified. Overall and animal-specific EG versus Control eye ONH parameter differences were assessed globally and regionally by linear mixed effect models with significance criteria adjusted for multiple comparisons.

Results: Expansions of the optic nerve and surrounding anterior scleral canal opening achieved statistical significance overall ($p < 0.0022$), and in 7 of 8 EG eyes ($p < 0.005$). In at least 5 EG eyes, significant expansions ($p < 0.005$) in Bruch's membrane opening (BMO) (range 3–10%), the anterior and posterior scleral canal openings (8–21% and 5–21%, respectively), and the optic nerve at the anterior and posterior scleral canal openings (11–30% and 8–41%, respectively) were detected. Optic nerve expansion was greatest within the superior and inferior quadrants. Optic nerve expansion at the posterior scleral canal opening was significantly correlated to optic nerve damage ($R = 0.768$, $p = 0.042$).

Conclusion: In the rat ONH, the optic nerve and surrounding BMO and neurovascular scleral canal expand early in their response to chronic experimental IOP elevation. These findings provide phenotypic landmarks and imaging targets for detecting the development of experimental glaucomatous optic neuropathy in the rat eye.

1. Introduction

Mouse and rat experimental glaucoma models are increasingly utilized to study the mechanisms of chronic intraocular pressure (IOP) induced optic nerve injury. While the non-human primate experimental glaucoma model (Burgoyne, 2015b) benefits from optic nerve head (ONH) anatomy and physiology that is similar to the human ONH, the model is impractical for studies that require large sample sizes. By contrast, rodent models (Crowston et al., 2015; Fernandes et al., 2015; Morgan and Tribble, 2015; Morrison et al., 2015; Overby and Clark, 2015; Pang et al., 2015) display pathophysiologic changes that are comparable to human glaucoma, despite substantial differences in ONH anatomy.

The suitability of rodent models is based upon several lines of reasoning. First, a series of previous reports have determined that early (if not the earliest) damage to the retinal ganglion cell (RGC) axons occurs within the ONH tissues in all induced and spontaneous forms of rodent (Morrison, 2005; Morrison et al., 2011, 2008, 1997,

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1998; Schlamp et al., 2006) and primate (Burgoyne et al., 2004; Downs et al., 2007; Yang et al., 2007a, 2007b) chronic IOP elevation. Second, to a certain degree, the rodent ONH demonstrates characteristics that are analogues to the primate ONH (Morrison et al., 2011). These include similar ultrastructural relationships between astrocytes and axons (Morrison et al., 1995, 1997) as well as similar cellular changes (Hernandez et al., 1990; Johnson et al., 1996; Morrison et al., 1990), neural canal expansion (Chauhan et al., 2002; Guo et al., 2005) and posterior deformation of the ONH surface in response to chronic IOP elevation (Chauhan et al., 2002). Likewise, chronic models of IOP elevation in rats have also demonstrated a predilection for early superior optic nerve injury (Dai et al., 2012; Huang and Knighton, 2009; Li et al., 2015; Morrison, 2005; Morrison et al., 1997; WoldeMussie et al., 2001) (Morrison JC et al. *IOVS* 2002; ARVO E-Abstract 2885). Understanding the basis for this regional susceptibility in rats may provide insight into mechanisms of regional susceptibility in humans.

Third, the anatomy of the rodent ONH is also very different from the human and non-human primate (Morrison et al., 2011). Understanding these differences and recognizing how they contribute to species-related differences in age-related RGC axon loss (Cepurna et al., 2005) and age-related differences in the susceptibility of axon transport to acute (Kong et al., 2009) and chronic IOP elevation (Morrison et al., 1997) (Morrison JC et al. *IOVS* 2007; 48: ARVO E-Abstract 3662; Johnson EC et al. *IOVS* 2008; 49: ARVO E-Abstract 4059) should provide important knowledge about these same phenomena in human eyes.

We recently performed high-resolution (1.5 cubic micrometer voxel) tridimensional (3D) histomorphometric reconstruction of the optic nerve head (ONH) and peripapillary sclera from both the normal control (Control) and experimental glaucoma (EG) eyes of eight brown Norway rats that had undergone 4 weeks of unilateral chronic IOP elevation. In an initial publication (Pazos et al., 2015), we described the 3D histomorphometric anatomy of the 8 Control rat ONHs and characterized the differences between the normal control rat and primate ONH (Figs. 1–7 of our previous report) (Pazos et al., 2015). That study was the first to describe the rat ONH as consisting of two scleral openings (a neurovascular and arterial) and clarify that the central retinal artery (CRA) does not accompany the central retinal vein (CRV) within the neurovascular canal, but passes through a separate, large, irregular opening, inferior to it, accompanied by the long posterior ciliary arteries (LCPAs) and their dense intra-scleral branches. Our study confirmed the presence of a previously described (Dai et al., 2012; Morrison et al., 1999; Sugiyama et al., 1999) vascular plexus that is continuous from the choroid to the optic nerve sheathes and surrounds the optic nerve within the neurovascular scleral canal. It additionally confirmed the anatomic proximity of the RGC axon bundles and a prominent extension of Bruch's Membrane (BM) superiorly that is unique to that region.

The purpose of the present report is to 3D characterize overall and animal specific, global and regional EG versus Control eye differences within the 8 rats of our previous report (Pazos et al., 2015).

2. Materials and methods

Table 1 defines all abbreviations. Supplemental Figs. 1–3 (corresponding to Figs. 1–3 of our previous publication (Pazos et al., 2015)) review the macroscopic and microscopic relationships of the rat ONH. See our previous report (Pazos et al., 2015) for more detailed illustration of the 3D relationships among the neural, vascular and connective components of the rat ONH. Throughout the present report, all right and left eye data are presented in right eye orientation and all *parameters* are italicized to distinguish the behavior of *measured parameters* from the behavior of the underlying anatomy they characterize (not italicized).

2.1. Animals and eyes

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Both the Control and EG eyes of 8 adult male brown Norway rats, between 9.5 and 10.5 months of age, were studied (Table 2).

2.2. Induction of chronic unilateral experimental IOP elevation and its measurement in rats

Animals were housed initially in standard lighting conditions, with lights automatically turned on at 6 AM and off at 6 PM. One week before the hypertonic episcleral vein injection in one eye (the treated eye), each animal was placed in constant light conditions (24 hour exposure to 40–90 lux) (Morrison et al., 2005; Pang et al., 2005). Following injection, each animal was followed for 4 weeks, and then sacrificed (see below). IOP readings in both the Control and EG eyes of each animal were made while awake (using 0.5% proparacaine hydrochloride as topical anesthesia to avoid the effects of general anesthetics) using a hand-held TonoPen tonometer. IOP was measured at least every other day, with a minimum of 14 IOP readings over the 4 weeks post saline injection. Mean IOP for both eyes of each animal was calculated as the area under the curve of an IOP vs days plot divided by the number of days. The Mean IOP Difference for each animal was defined to be the difference between the mean IOP of the EG versus the Control eye. The Peak IOP for each animal was defined to be the highest IOP in the EG eye.

2.3. Rat Euthanasia, fixation and injury grade analysis

All animals were sacrificed 4 weeks post-initial saline injection to the treated eye under isoflurane anesthesia by transcardial injection of heparin (1 ml/kg) containing 10 mg ml sodium nitroprusside followed by 1 L of 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Optic nerves were dissected, washed, dehydrated and embedded in Spurr's resin as previously described (Morrison et al., 1998). Using light microscopy, 5 masked observers graded the orbital optic nerve cross-section from each eye using a previously published scale from 1 (no injury) to 5 (active degeneration involving the whole nerve area) (Jia et al., 2000) and their grades were averaged to obtain the final injury grade for each nerve.

2.4. 3D Histomorphometric reconstruction of the ONH

The ONH and peripapillary sclera of each eye were trephined (3 mm diameter), embedded in paraffin, mounted to a microtome (RM2165; Leica, Wetzlar, Germany) and serial sectioned at 1.5 μm thickness from the vitreous surface through the ONH into the orbital optic nerve (Burgoyne et al., 2004). After each section was cut, the block surface was stained with a 1:1 (v/v) mixture of Ponceau S and acid fuchsin stains, then imaged at a resolution of 1.5×1.5 μm per pixel using a custom device (Burgoyne et al., 2004). For each ONH, 275 to 501 serial digital transverse section images were thus generated, aligned and stacked into a digital 3D reconstruction (Burgoyne et al., 2004; Downs et al., 2007; Yang et al., 2007a, 2009a, 2007b, 2011a).

2.5. 3D delineation of the rat ONH and peripapillary scleral landmark points

Each 3D ONH reconstruction was loaded into our custom Multiview 3D visualization and delineation software (based on the