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Combinatorial targeting of early pathways profoundly inhibits neurodegeneration in a mouse model of glaucoma

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ABSTRACT

The endothelin system is implicated in various human and animal glaucomas. Targeting the endothelin system has great promise as a treatment for human glaucoma, but the cell types involved and the exact mechanisms of action are not clearly elucidated. Here, we report a detailed characterization of the endothelin system in specific cell types of the optic nerve head (ONH) during glaucoma in DBA/2J mice. First, we show that key components of the endothelin system are expressed in multiple cell types. We discover that endothelin 2 (EDN2) is expressed in astrocytes as well as microglia/monocytes in the ONH. The endothelin receptor type A (*Ednra*) is expressed in vascular endothelial cells, while the endothelin receptor type B (*Ednrb*) receptor is expressed in ONH astrocytes. Second, we show that Macitentan treatment protects from glaucoma. Macitentan is a novel, orally administered, dual endothelin receptor antagonist with greater affinity, efficacy and safety than previous antagonists. Finally, we test the combinatorial effect of targeting both the endothelin and complement systems as a treatment for glaucoma. Similar to endothelin, the complement system is implicated in a variety of human and animal glaucomas, and has great promise as a treatment target. We discovered that combined targeting of the endothelin (Bosentan) and complement (*C1qa* mutation) systems is profoundly protective. Remarkably, 80% of DBA/2J eyes subjected to this combined inhibition developed no detectable glaucoma. This opens an exciting new avenue for neuroprotection in glaucoma.

Introduction

Glaucoma is one of the most common neurodegenerative diseases (Quigley, 1996) characterized by the death of retinal ganglion cells (RGCs) and degeneration of the optic nerve (reviewed in Burgoyne, 2011; Nickells et al., 2012). The optic nerve head (ONH) is a key site in glaucoma (e.g., Anderson and Hendrickson, 1974; Quigley and Anderson, 1976; Anderson and Hendrickson, 1977; Quigley and Anderson, 1977; Quigley and Addicks, 1980; Howell et al., 2007a). More effective therapies, particularly those that target damaging processes in the optic nerve head are required. A critical and early insult damages RGC axons in the ONH (Schlamp et al., 2006; Howell et al., 2007a; Burgoyne, 2011). Nevertheless, the earliest processes that damage RGC axons are not well defined. To better understand these early stages, we performed gene expression profiling from DBA/2J mice, a widely used mouse model of glaucoma (Howell et al., 2011a, 2012b). Our gene expression analyses identified a temporally ordered series of early glaucoma stages. Previous studies had suggested the importance of the endothelin system in glaucoma (reviewed in Chauhan, 2008; Good and Kahook, 2010; Prasanna et al., 2011) and our data showed that endothelin-2 (*Edn2*) was significantly upregulated compared to non-glaucoma eyes at early stages of the disease, prior to significant axon damage (Howell et al., 2011a). Cumulatively, this data suggest that components of the endothelin system maybe critical in the early progression of glaucoma in the optic nerve head.

The endothelin system is comprised of three ligands (endothelin 1, EDN1; EDN2 and endothelin 3, EDN3) that interact with two receptors, endothelin receptor type A (*Ednra*) and endothelin receptor type B (*Ednrb*) (Kedzierski and Yanagisawa, 2001). Endothelin ligands binding either to one or both of the endothelin receptors activate a variety of different responses within tissues (reviewed in Kedzierski and Yanagisawa, 2001). Upregulation of components of the endothelin system is described in human glaucoma and in animal models relevant

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to glaucoma. Endothelin 1 (EDN1) was elevated in the aqueous humor of primary open angle glaucoma (POAG) patients compared to normotensive individuals (Noske et al., 1997; Tezel et al., 1997). Injections of EDN1 or endothelin 2 (EDN2) can induce RGC loss in the retina and optic nerve head (Chauhan et al., 2004; Cioffi, 2005; Stokely et al., 2005; Sasaoka et al., 2006; Howell et al., 2011a). Intravitreous injections of EDN1 cause a dose-related decrease in the number of retrogradely labeled RGCs (Taniguchi et al., 2006). Furthermore, in response to optic nerve crush in rabbits, *Ednrb* is upregulated in activated astrocytes (Rogers et al., 2003). Infusion of Bosentan (an inhibitor of endothelin receptor type A, *Ednra* and endothelin receptor type B, *Ednrb*) reduced astrocyte activation in crushed optic nerves (Rogers et al., 2003). Also, *Ednrb* deficiency lessened neurodegeneration in a rat model with experimentally induced elevation of IOP (Minton et al., 2012).

Targeting early events in glaucoma is likely to have better therapeutic efficacy than targeting later events. However, the exact roles of the endothelin system in early stages of glaucoma have not been elucidated. Therefore, to begin to understand these roles, we have performed a detailed characterization of the endothelin system in DBA/2J glaucoma. As combinatorial treatment regimens targeted against multiple early events are likely to be more effective than monotherapy, we have also tested the effects of targeting the endothelin pathway alone and in combination with the complement pathway. The complement pathway is another promising target as it has been widely implicated in human and animal models relevant to glaucoma (Stasi et al., 2006; Steele et al., 2006; Johnson et al., 2007; Stevens et al., 2007). Furthermore, like the endothelin system, we have shown that the complement cascade is upregulated very early in DBA/2J glaucoma, prior to significant RGC axon damage (Howell et al., 2011a). Here, we show that combinatorial targeting of the endothelin system and the classical pathway of the complement cascade is more effective at reducing glaucomatous damage than separately inhibiting either process.

Materials and methods

Mouse strains, breeding and husbandry

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology statement on the use of animals in ophthalmic research and approved by The Jackson Laboratory Animal Care and Use Committee. Mice were housed in a 14-hour light/10-hour dark cycle under previously described conditions (Smith et al., 2000). All DBA/2J mice used were obtained from either The Jackson Laboratory production facility (Bar Harbor, ME) or from the John Lab research colony. This colony is routinely crossed with DBA/2J mice from The Jackson Laboratory production facility to prevent genetic drift. Details for D2.*C1qa*$^{+/-}$ mice have been described previously (Botto et al., 1998; Howell et al., 2011a). Cohorts of *C1qa*-deficient DBA/2J mice (D2.*C1qa*$^{-/-}$) were generated by intercrossing D2.*C1qa*$^{+/-}$ mice. Endothelin deficient mice for assessment of the *Edn2* riboprobes were obtained from our colony (Reinholdt et al., 2012).

Gene expression analysis

We previously collected a large dataset of gene expression changes in the ONH across different stages of glaucoma (Howell et al., 2011a, 2011b, 2012a). Briefly, we previously performed gene expression analysis on eyes from female DBA/2J mice at 4, 8 and 10.5 months of age. Eyes from mice at 4 and 8 months of age showed no glaucoma whereas eyes at 10.5 months of age had a range of glaucoma from no glaucoma to severe glaucoma (see Analysis of glaucomatous damage below). We used hierarchical clustering to determine temporally ordered, molecular stages of disease that were termed stages 1a, 1b, 1c, 2, 3, 4 and 5. Stage 1a was most similar to no glaucoma controls. Stages 1a–2 contained eyes with no morphological signs of axon damage (as judged by PPD staining). This dataset can be interrogated for specific genes to support many studies and is publicly available at GEO DataSets (GSE26299). We interrogated this dataset to determine the expression levels of *Edn1*, *Edn2*, endothelin 3 (*Edn3*), *Ednra* and *Ednrb*.

RNA in situ hybridization and immunofluorescence

RNA in situ hybridization was performed as previously described (Soto et al., 2008; Howell et al., 2011a), using 4% PFA perfusion-fixed, 12-μm-thick tissue sections. Digoxigenin-labeled (DIG-labeled) riboprobes for *Ednra*, *Ednrb* and *Edn2* were transcribed from cDNA clones (Open Biosystems clone ID: 2812426, 4971909, and 4512195, respectively). For anti-sense probes, *Ednra* and *Ednrb* plasmids were digested with *Sal*I, and the *Edn2* plasmid was digested with *Eco*R1. In vitro transcription was performed with T7 polymerase. For sense control probes, *Ednra* and *Ednrb* plasmids were digested with *Not*I and the *Edn2* plasmid was digested with *Hind*III. In vitro transcription was performed with SP6 polymerase. No signal was observed for the *Edn2* antisense probe in mice lacking *Edn2* (Supplemental Fig. S1) (Saida et al., 2002). In all cases, the sense control probe showed no signal (see Supplemental Figs. S1 and S2). Details for the *C1qa* riboprobe have been described previously (Howell et al., 2011a).

The detection of hybridized mRNA in sections was performed using the Cy-3 Tyramide Signal Amplification System (PerkinElmer). After in situ hybridization, the sections were incubated in the primary antibodies: rabbit anti-IBA1 (1:500, Wako), chicken anti-GFAP (1:500, Abcam), and rat anti-EMCN (1:50, Santa Cruz). Primary antibodies were diluted in a solution of 10% normal goat serum, 0.5% Triton X-100, and 0.5% BSA in 0.1 M PBS. For the secondary antibodies, goat anti-mouse Alexa Fluor 647, goat anti-rabbit Alexa Fluor 488, goat anti-chicken Alexa Flour 633 or goat anti-rat Alexa Flour 488 were used at a 1:1000 dilution (Invitrogen). The sections were then incubated with DAPI (Invitrogen) and mounted in Fluoromount (Sigma-Aldrich). Fluorescence was visualized using a SP5 confocal microscope (Leica). Images were processed in Fiji (formerly ImageJ). For each probe/antibody at least three sections taken from at least six eyes were assessed. With the exception of the control eyes from the D2.*Edn2*$^{-/-}$ mice (which were collected from pre-wean pups), all eyes assessed had no glaucoma and were from DBA/2J mice between 9 and 10.5 months of age (as judged by PPD staining, see Analysis of glaucomatous damage below).

Bosentan and Macitentan administration

Bosentan or Macitentan (Actelion Pharmaceuticals) was incorporated into standard mouse chow (Bosentan: 100 mg/kg, Test Diet; Macitentan: 30 mg/kg, Test Diet). DBA/2J mice were administered Macitentan from 6 months of age and assessed for glaucoma at 10.5 and 12 months of age. Results were compared to our previous study using Bosentan (Howell et al., 2011a). Number of eyes were: 10.5 months; control = 42, Bos = 54, Mac = 50 and 12 months; control = 58, Bos = 58, Mac = 54. D2.*C1q*$^{-/-}$ mice were administered with Bosentan from 6 months of age and assessed for glaucoma at 12 months of age and compared to our previous study of D2.*C1qa*$^{-/-}$ mice on regular chow and DBA/2J mice fed Bosentan-contain chow (Howell et al., 2011a). Number of eyes were: control = 58, Bos = 54, *C1qa* deficient = 56, Bosentan treated *C1qa* deficient = 40. As endothelin receptor antagonists can decrease blood pressure in hypertensive individuals, a separate cohort of 11 mice were administered Macitentan for 2 weeks, and blood pressures were measured using previously described procedures (Sugiyama et al., 2002). Blood pressure was slightly lower in the Macitentan treated group (BP, mm Hg ± SEM: 108.18 ± 1.2 treated; 111.97 ± 1.6 control, P = 0.034), but the slight decrease is not expected to affect neurodegeneration. Bosentan has no effect on blood pressure (Howell et al., 2011a).

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