Experimental Eye Research 150 (2016) 34–43

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Research article

Glial coverage in the optic nerve expands in proportion to optic axon loss in chronic mouse glaucoma

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ARTICLE INFO

Article history:
Received 21 July 2015
Received in revised form
21 December 2015
Accepted in revised form 23 January 2016
Available online 3 February 2016

Keywords:
Glaucoma
Neurodegeneration
Optic nerve
Axon loss
Remodeling
Gliosis
Microglia
Segmentation

ABSTRACT

Within the white matter, axonal loss by neurodegeneration is coupled to glial cell changes in gene expression, structure and function commonly termed gliosis. Recently, we described the highly variable expansion of gliosis alebosco@neuro.utah.edu in degenerative optic nerves from the DBA/2J mouse model of chronic, age-related glaucoma. Here, to estimate and compare the levels of axonal loss with the expansion of glial coverage and axonal degeneration in DBA/2J nerves, we combined semiautomatic axon counts with threshold-based segmentation of total glial/scar areas and degenerative axonal profiles in plastic cross-sections. In nerves ranging from mild to severe degeneration, we found that the progression of axonal dropout is coupled to an increase of gliotic area. We detected a strong correlation between axon loss and the aggregate coverage by glial cells and scar, whereas axon loss did not correlate with the small fraction of degenerating profiles. Nerves with low to medium levels of axon loss displayed moderate glial reactivity, consisting of hypertrophic astrocytes, activated microglia and normal distribution of oligodendrocytes, with minimal reorganization of the tissue architecture. In contrast, nerves with extensive axonal loss showed prevalent rearrangement of the nerve, with loss of axon fascicle territories and enlarged or almost continuous gliotic and scar domains, containing reactive astrocytes, oligodendrocytes and activated microglia. These findings support the value of optic nerve gliotic expansion as a quantitative estimate of optic neuropathy that correlates with axon loss, applicable to grade the severity of optic nerve damage in mouse chronic glaucoma.

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1. Introduction

The progressive decline and loss of optic nerve axons is characteristically associated with vision loss in all types of glaucoma (Casson et al., 2012). This neurodegenerative disease of the retina and optic nerve targets the retinal ganglion cells (RGCs), causing deterioration and destruction of their axons and somata through pathogenic mechanisms that are still unresolved (Quigley, 2011). Axonopathy is progressive and heterogeneous in multiple experimental models of glaucoma (Calkins, 2012; Nickells et al., 2012; Vidal-Sanz et al., 2012). Axonal degeneration is sectorial and asynchronous in models of induced intraocular pressure elevation (Salinas-Navarro et al., 2010; Soto et al., 2011), as well as in the DBA/2J mouse model of inherited, chronic glaucoma (Howell et al., 2007a; John et al., 1998; Schuettauf et al., 2004). Early in DBA/2J glaucoma, sparse degenerative axonal profiles are interspersed within nerves with preserved axon densities, and as disease advances, there is progressive increase in axon degeneration and loss that culminates in axon depletion and nerve scarring (Crish et al., 2010; Inman et al., 2006; Libby et al., 2005a). Given the variable severity and progressive nature of glaucomatous optic nerve degeneration, the assessment of nerve damage in animal models of glaucoma is largely based on grading or quantifying the numbers of persistent axons (reviewed in Nuschke et al., 2015).

Evaluation of optic axon decline and loss is key in rodent models of acute and chronic glaucoma that are used to define mechanisms of RGC and axonal degeneration, and potential neuroprotection. However, this poses challenges due to asynchronous and variable distribution of axon loss and atrophy. Axon drop out can be diffuse and uniform across the entire nerve (Sappington et al., 2010), focal and heterogeneous between fascicles (Morrison et al., 1997), or a

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http://dx.doi.org/10.1016/j.exer.2016.01.014
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combination of both topographies, as in the DBA/2J model (Schlamp et al., 2006). Direct counting of the number of persistent axonal profiles in semithin nerve cross-sections is the standard and most accurate method to determine glaucomatous axon loss, and much effort continues to be devoted to perfecting this analysis (Nuschke et al., 2015). In rodent models of glaucoma, actual axon counts in light micrographs are performed manually or by semi-automated analysis in nerve subareas distributed in a fixed patterned or within zones selected for their uniform damage (Buckingham et al., 2008; Chauhan et al., 2006; Chen et al., 2011; Crish et al., 2010; Ebneter et al., 2010, 2012; Howell et al., 2007a, 2011; Inman et al., 2006; Isaacs et al., 2014; Jia et al., 2000; Joos et al., 2010; Mabuchi et al., 2003; Marina et al., 2010; May and Mittag, 2006; Sappington et al., 2010; Scholz et al., 2008; Templeton et al., 2014). Alternatively, glaucomatous optic nerves are visually scored by degree and expanse of axon loss, damage, gliosis and scarring by trained observers (Chidlow et al., 2011). This method is commonly applied to grade optic nerve pathology in the DBA/2J mouse strain (Anderson et al., 2005; Harder et al., 2012; Howell et al., 2007b, 2012; Libby et al., 2005a; Pelzel et al., 2012; Schlamp et al., 2006; Son et al., 2010), and is complemented by estimation of the proportion of degenerative axonal profiles (Crish et al., 2010; Howell et al., 2012). Overall, defining disease stage and severity in glaucoma depends on accurate detection and estimation of the variable levels and patterns of axonal degeneration and loss in the optic nerve.

In human and experimental glaucoma, the progressive damage of optic axons is paralleled by gliosis (Anderson et al., 2005; Bosco et al., 2015; Crish et al., 2010; Dai et al., 2012; Hernandez, 2000; Libby et al., 2005a; Lye-Barthel et al., 2013; Qu and Jakobs, 2013; Sappington et al., 2010; Schlamp et al., 2006; Sofroniew, 2009; Son et al., 2010; Sun and Jakobs, 2012; Sun et al., 2009; Yang et al., 2012). Degenerating optic axons are replaced by reactive glial cells, infiltrating cells and extracellular matrix within atrophic nerve areas, in both human patients and in primate models (Jonas et al., 1995; Quigley et al., 1982; Radius and Pederson, 1984). In DBA/2J mice, severely degenerative nerves show extensive gliosis and scarring after 8 months of age, while the number of degenerating axon profiles more than doubles from 3 to 13 months of age (Crish et al., 2010; Schlamp et al., 2006). These conspicuous features have been useful for nerve damage grading (Anderson et al., 2005). However, the relative expansion of total glial territories as axons vacate the glaucomatous optic nerve has not been used as a proxy for axonal neurodegeneration.

In a recent study, we detected a highly variable distribution of optic nerve gliosis across DBA/2J mice (Bosco et al., 2015). Here, using this well-established model of chronic glaucoma, we evaluated whether the global expansion of glial and scarred nerve territories provides a quantifiable estimate of total axonal loss. For this purpose, we analyzed DBA/2J optic nerves representative of healthy, moderate and severe glaucoma, as well as Gpnmb$^{+/+}$ DBA/2J control mice. We quantified and mapped axon density by semiautomated counting methods, and in the same nerve cross-sections we applied image segmentation methods to isolate and measure degenerative axonal profiles and nerve area occupied by glial cells and scar tissue. These two methods of analysis of glaucomatous optic nerve degeneration, allowed us to detect a significant positive correlation between the expansion of nerve area occupied by glial cells and the reduction in mean axonal density. Applying segmentation analysis of nerve glial/scar areas and highly degenerative axons to a large cohort of DBA/2J optic nerves, we provide evidence that this semiquantitative method represents a practical and sensitive gauge to score progressive and variable glaucomatous pathology.

2. Materials and methods

2.1. Mice

DBA/2J and non-glaucoma control Gpnmb$^{+/+}$ DBA/2J were bred and housed at the University of Utah (females aged 10–11 months; Figs. 4–5), introducing new breeder mice purchased from Jackson Laboratories (Bar Harbor, ME) twice a year to prevent genetic drift. Additionally, DBA/2J mice (males and females aged 7, 12 and 13 months; Figs. 1–3) were obtained from P.J. Horner (University of Washington Harborview Medical Center, Seattle, WA). All mice were maintained in sterile conditions, on 12/12 light/dark cycles, and fed standard rodent diet. All animal protocols were approved by the local IACUC, and experiments performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Nerve histopathology

All optic nerves were collected from mice after transcardial perfusion with 4% paraformaldehyde (PFA) and overnight post-fixation within the exposed orbit, prepared as two pieces (each 1–1.5-mm in length), cut from the postlaminar region. One nerve segment was processed for 1- to 2-μm plastic cross-sections, stained with toluidine-blue and paraphenylenediamine (PPD) to increase contrast for light microscopy, and the contiguous segment was prepared for gelatin embedding, cross-cryosectioning, immunofluorescence and confocal imaging (see below). For plastic embedding, we used previously described processing methods (Calkins et al., 2005; Inman et al., 2006; Sappington et al., 2003), with slight optimization (see Supplemental Methods for detailed protocol).

2.3. Axon counts in light microphotographs

Axon counts for individual optic nerves were obtained as described previously (Buckingham et al., 2008; Crish et al., 2010; Inman et al., 2006; Joos et al., 2010; Sappington et al., 2010). Briefly, a single, plastic cross-section was imaged in its entirety for each nerve by creating a montage of 20–25 adjacent frames using a 100× oil-immersion objective, differential interference contrast optics (Provis AX70; Olympus, Melville, NY), a motorized stage and a digital charge-coupled device (CCD) camera. Image montages were minimally processed for contrast- and edge-enhancing using macro routines (ImagePro; Media Cybernetics, Silver Spring, MD). All axons with an identifiable myelin sheath were counted in each frame at 1,000× digital magnification using an additional macro routine. The axon density was calculated as the number of axons divided by frame area, and the axon density for each nerve was estimated as the mean of densities across frames.

2.4. Segmentation analysis of total glia/scar area and degenerative axon profiles

To identify and measure the relative nerve area corresponding to glial cells and highly degenerative axons, observers masked to nerve identity and axon counts used image segmentation methods recently described (Bosco et al., 2015). First, two observers with expertise in optic nerve histopathology, glaucomatous pathology and image analysis worked together to optimize intensity–threshold parameters to consistently isolate nerve areas occupied by all glial cell types, scar tissue, degenerative axons, blood vessels and meninges, which are explained in detail (Supplemental Methods). Nerve glial and scar areas are prominent and easily recognizable, requiring only minimal manual refinement to include astrocyte

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