Experimental Eye Research 121 (2014) 11–22

Contents lists available at ScienceDirect

Experimental Eye Research

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

Comparative quantitative study of astrocytes and capillary distribution in optic nerve laminar regions

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ARTICLE INFO

Article history:
Received 20 June 2013
Accepted in revised form 9 February 2014
Available online 19 February 2014

Keywords:
glia
astrocyte
lamina cribrosa
optic nerve head
axon
capillary

ABSTRACT

Retinal ganglion cell (RGC) axonal structure and function in the optic nerve head (ONH) is predominantly supported by astrocytes and capillaries. There is good experimental evidence to demonstrate that RGC axons are perturbed in a non-uniform manner following ONH injury and it is likely that the pattern of RGC axonal modification bears some correlation with the quantitative properties of astrocytes and capillaries within laminar compartments. Although there have been some excellent topographic studies concerning glial and microvascular networks in the ONH our knowledge regarding the quantitative properties of these structures are limited. This report is an in-depth quantitative, structural analysis of astrocytes and capillaries in the pre laminar, lamina cribrosa and post laminar compartments of the ONH. 49 optic nerves from human ($n = 10$), pig ($n = 12$), horse ($n = 6$), rat ($n = 11$) and rabbit ($n = 10$) eyes are studied. Immunohistochemical and high-magnification confocal microscopy techniques are used to co-localise astrocytes, capillaries and nuclei in the mid-portion of the optic nerve. Quantitative methodology is used to determine the area occupied by astrocyte processes, microglia processes, nuclei density and the area occupied by capillaries in each laminar compartment. Comparisons are made within and between species. Relationships between ONH histomorphometry and astrocyte-capillary constitution are also explored. This study demonstrates that there are significant differences in the quantitative properties of capillaries and astrocytes between the laminar compartments of the human ONH. Astrocyte processes occupied the greatest area in the lamina cribrosa compartment of the human ONH implicating it as an area of great metabolic demands. Microglia were found to occupy only a small proportion of tissue in the rat, rabbit and pig optic nerve suggesting that the astrocyte is the predominant glia cell type in the optic nerve. This study also demonstrates that there is significant uniformity, with respect to astrocyte and capillary constitution, in the post laminar region of species with an unmyelinated anterior optic nerve. This implicates an important role served by oligodendrocytes and myelin in governing the structural characteristics of the post laminar optic nerve. Finally, this study demonstrates that eyes with similar lamina cribrosa structure do not necessarily share an identical cellular constitution with respect to astrocytes. The quantitative properties of astrocytes in the pre laminar and lamina cribrosa regions of the rat, which has a rudimentary lamina cribrosa with only a few collagenous beams, shared more similarities to the human eye than the pig or horse. The quantitative properties of astrocytes and capillaries in the laminar compartments of the ONH provide a basis for understanding the pathogenic mechanisms that are involved in diseases such as glaucoma and ischemic optic neuropathy. The findings in this study also provide valuable information about the distinct advantages of different animal models for studying human optic nerve diseases. Utilisation of structural data provided in this report together with emerging in vivo technology may potentially permit the early identification of RGC axonal injury by quantifying changes in ONH capillaries and astrocytes.

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1. Introduction

In order to optimise resolution and maximise field of vision the dimensions of the optic nerve head (ONH) need to be geometrically

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http://dx.doi.org/10.1016/j.exer.2014.02.008
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confined. This places structural constraints on the organisation and density of non-neuronal elements that support the immense metabolic requirements of retinal ganglion cell (RGC) axons. Astrocytes and capillaries are the major non-neuronal elements that maintain RGC axonal viability in the ONH (Garthwaite et al., 2006; Trivino et al., 1996). There is good experimental evidence to demonstrate that RGC axons are perturbed in a non-uniform manner following ONH injury (Quigley and Green, 1979) and it is likely that the pattern of disease-induced RGC axonal modification bears important correlations to the spatial and quantitative properties of astrocytes and capillaries within laminar compartments. Understanding the quantitative properties of astrocytes and capillaries in the ONH may therefore provide valuable insights into cellular mechanisms that are important to RGC health and disease.

To date, there have been a number of detailed reports that have described the topographic characteristics of microvascular networks and glial cells in the ONH (Bussow 1980; Minckler et al., 1976; Morrison et al., 1999; Onda et al., 1995; Raff 1989; Rootman 1971; Simoens et al., 1996; Sugiyama et al., 1992; Trivino et al., 1996; Ye and Hernandez, 1995). However, few investigators have quantified the structural properties of astrocytes and capillaries in different laminar regions. This report is an in-depth quantitative, structural analysis of astrocytes and capillaries in the pre laminar, lamina cribrosa and post laminar compartments of the ONH. Optic nerve heads from five species (human, rat, rabbit, pig and horse) whose histomorphometric properties have been previously characterised and whose pattern of RGC axonal dysfunction following ONH injury have been previously delineated are compared. Relationships between ONH histomorphometry and astrocyte-capillary constitution are also explored in this report. The information attained from this comparative study may improve our understanding of ONH structure and function and may also improve our knowledge about possible disease mechanisms that are involved in glaucomatous and ischemic optic neuropathies. This comparative study may also provide some useful information about the distinct advantages of different animal models for studying human optic nerve diseases.

2. Materials and methods

2.1. General

All experiments were conducted and all laboratory animals treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All human tissue was handled according to the tenets of the Declaration of Helsinki. The study was approved by The University of Western Australia Animal Ethics and Human Ethics Committees.

2.2. Human and animal optic nerves

A total of 49 eyes from 5 mammalian species were studied. This included human ($n = 10$), pig ($n = 12$), horse ($n = 6$), rat ($n = 11$) and rabbit ($n = 10$) optic nerves.

All human eyes were obtained from the Lions Eye Bank of Western Australia (Lions Eye Institute, Western Australia) after removal of corneal buttons for transplantation. Human donor eyes used for this research had no documented history of eye disease. The mean age of human donors was 49.2 ± 5.8 years (age range, 23–62 years). We examined 5 right eyes and 5 left eyes from a total of 5 male and 1 female donor. The average post mortem time before eyes were enucleated was 11.2 ± 1.1 h (range 7–14 h).

Horse eyes were attained from the Equine Centre at Murdoch Veterinary Hospital following euthanasia of animals for untreatable surgical diseases. Pig eyes were acquired from the local abattoir. Brown Norway rat and rabbit eyes were acquired from animals that underwent euthanasia in unrelated scientific experiments.

2.3. Tissue preparation

Prior to enucleation a scleral suture was placed at the 12 o'clock position to allow orientation of the optic nerve during tissue processing. For human eyes the extra-ocular muscles and position of the macula were also used to orient the nerve when a superior stitch was not applied prior to enucleation. All eyes were carefully dissected to expose the optic disk and the first centimetre of optic nerve before fixation in 4% paraformaldehyde and cryoprotection with 30% sucrose. Tissue for all studies was mounted in optimal cutting temperature compound (Tissue-Tek 4583, Product No. 62550-12, Japan) and longitudinally sectioned into 12 μm specimens on a cryotome set at −20 °C. To avoid the potential tilting of sections the optic nerve was aligned parallel to the blade on the cryostat during sectioning. Longitudinal sections were cut along the sagittal plane beginning in the superior portion of each optic nerve and proceeding to the inferior part of the nerve. Specimens were numerically labelled as they were sectioned so that we could determine from which region of the optic nerve they were derived. Specimens from the middle portion of the optic nerve were studied in all species.

2.4. Staining protocol and immunohistochemistry

Optic nerve sections adjacent to that used for immunohistochemical studies were stained using the Van Gieson protocol. This allowed us to reliably partition each optic nerve into different laminar regions during quantitative analysis.

Spatial relationships between glia, capillaries and nuclei were examined by multiple-labelling methods that incorporated previously reported immunohistochemical protocols (Alroy et al., 1987; Christie and Thomson, 1989; Fischer and Kissel, 2001; MacEachern et al., 1997; Meyer et al., 1999; Miller et al., 1989; Qu and Jakobs, 2013; Ramirez et al., 1994; Schnitzer, 1988; Villain et al., 2002; Yu et al., 2010).

Human, horse and pig endothelia were labelled using a rabbit polyclonal Factor VII related antigen/Willibrand Factor antibody (NeoMarkers Cat. #RB-281-A, 1:80) and astrocytes labelled using a mouse anti-glial fibrillary acidic protein antibody (anti-GFAP, Sigma G3893, Saint Louis, Missouri USA; 1:400). The rat and rabbit endothelia were labelled using a biotinylated Griffonia (Bandeiraea) simplicifolia lectin I Isolectin B4 glycoprotein (GSL-I; Vector Laboratories B1205; 1:50) and astrocytes labelled using the mouse anti-GFAP antibody. The rat, rabbit and pig microglia were labelled using a goat anti-Iba1 antibody (Abcam ab5076, 1:500). Visualisation of labelling was via Alexa Fluor 488 conjugated donkey anti-rabbit antibody (Invitrogen A21206; 1:200), Alexa Fluor 546 conjugated donkey anti-goat antibody (Life Technologies A11056; 1:200), Alexa Fluor 647 conjugated donkey anti-mouse antibody (Invitrogen A31571; 1:200) and Streptavidin FITC antibody (DakoCytomation F0422; 1:50). Nuclei were counterstained with Hoescht 33342 trihydrochloride (Sigma B2261; 1.25 ng/ml).

Specimens were prepared and stained as follows. Air dried sections were hydrated in three 5 min changes of phosphate buffered saline with 0.05% Tween 20 (PBS-Tween 20). Ice cold 3% hydrogen peroxide in methanol was then applied for 10 min to permeabilise the tissue and then washed off in three 5 min changes of PBS-Tween 20. For human, horse and pig specimens the sections were then incubated in 10% donkey serum (Sigma D9663) in PBS for an hour. The rat and rabbit specimens were incubated in a mixture of 5% swine serum and 5% donkey serum made up in 10 mM HEPES for an hour. After tipping off the blocking sera, the corresponding

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