STRUCTURAL BASIS OF GLAUCOMA: THE FORTIFIED ASTROCYTES OF THE OPTIC NERVE HEAD ARE THE TARGET OF RAISED INTRAOCULAR PRESSURE

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ABSTRACT

Increased intraocular pressure (IOP) damages the retinal ganglion cell axons as they pass through the optic nerve head (ONH). The massive connective tissue structure of the human lamina cribrosa is generally assumed to be the pressure transducer responsible for the damage. The rat, however, with no lamina cribrosa, suffers the same glaucomatous response to raised IOP. Here we show that the astrocytes of the rat ONH are 'fortified' by extraordinarily dense cytoskeletal filaments which would make them ideal transducers of distorting mechanical forces. The ONH astrocytes are arranged as a fan-like radial array, firmly attached ventrally to the sheath of the ONH by thick basal processes, but dividing dorsally into progressively more slender processes with only delicate attachments to the sheath.

At one week after raising the IOP by an injection of magnetic microspheres into the anterior eye chamber, the fine dorsal processes of the ONH astrocytes are torn away from the surrounding sheath. There is no indication of distortion or compression of the axons. Subsequently, despite return of the IOP towards normal levels, the damage to the ONH

progresses ventrally through the astrocytic cell bodies, resulting in complete loss of the fortified astrocytes and of the majority of the axons by around 4 weeks. We propose that the dorsal attachments of the astrocytes are the site of initial damage in glaucoma, and that the damage to the axons is not mechanical, but is a consequence of localised loss of metabolic support from the astrocytes (Tsacopoulos and Magistretti, 1996).

INTRODUCTION

Glaucoma is one of the most important conditions leading to irreversible blindness (Quigley and Broman, 2006). The key cause of glaucoma is a rise in intraocular pressure (IOP). This damages the short umyelinated segments of the retinal ganglion cell (RGC) axons as they pass through the narrow region - optic nerve head (ONH) – between leaving the retina and acquiring their myelinated sheaths in the optic nerve (Morrison et al., 2005; Soto et al., 2008; Hernandez et al., 2008; Quigley et al., 1981).

In man and primates, the optic nerve head (ONH) is traversed by a dense meshwork of tough connective tissue – the lamina cribrosa – which would be a candidate for transferring damaging mechanical forces to the RGC axons (Quigley and Addicks, 1981; Quigley et al., 1983). Within and between the beams of the lamina cribrosa, the RGC axons run through a territory of astrocytes (Anderson, 1969). Raised IOP (experimental, (Morrison et al., 2008), or genetic (Sun et al., 2009)) causes similar damage in the ONH of smaller mammalian species, such as rat and mouse. In these species, however, there is no lamina cribrosa, and the predominant cellular elements of the ONH are the astrocytes.

In the present study of the rat ONH we have examined the structure and arrangement of these astrocytes to see what extent their cytoskeletal structure and arrangement provides anatomical indications of how these elements could act as mechanical transducers and we have examined the sequence of structural changes produced by raising the IOP from levels in the mid 20's mm Hg to the mid 40's by injection of magnetic microspheres into the anterior eye chamber (Samsel et al., 2010).

We propose two new possibilities for the pathogenesis of glaucoma – that the specialised astrocytes of the ONH are the elements which are damaged by the mechanical stresses of raised IOP, and that the damage to axons is metabolic, caused by loss of the astrocytic processes needed for support of the energy metabolism of the axons.

MATERIAL AND METHODS

Adult female Albino Swiss rats (n= 53; weight 220-250g, age 8-12 weeks) were raised in a light and temperature controlled room. All animals were handled according to UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act 1986 and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction and measurement of raised intraocular pressure (IOP)

Rats were deeply anaesthetized with isoflurane, also received 0.5% proparacaine hydrochloride eye drop topical. For unilateral induction of high intraocular pressure, 20µl of

a 30mg/ml suspension of 5µm diameter magnetic microspheres (Aldehyde-terminated Magnetic, MagicBeads™, Chi Scientific) were injected into right anterior chamber with a 33 gauge needle. After injection a small magnetic rod was used to direct the magnetic microspheres into the drainage area of the canal of Schlemm in the peripheral angle of the anterior chamber (Samsel et al., 2010). IOP was recorded using a tonometer (Tono-Pen XL, Reichert Inc, Germany) at 1 day before injection of magnetic microspheres (baseline), 1 day later, and then once a week, under local anaesthesia induced by instillation of one drop of 0.5% proparacaine hydrochloride (Alcaine, Alcon Laboratories, Couvreur, Belgium). The mean of 8 consecutive readings was taken as the IOP. After survivals of 1 (n=28), 2 (n=19), 3 (n=10) and 4 (n=10) weeks and n=28 unoperated control eyes, the rats were prepared for either resin embedding (for semithin and electron microscopy; n=35) or cryostat sectioning (n=18).

Resin embedding procedure

Under deep pentobarbital anaesthesia rats were transcardially perfused with 50ml of 0.1M phosphate buffered saline (PBS) followed by a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (PB). The optic nerves including optic nerve heads were dissected out and postfixed in 2% osmium tetroxide for 1-2 hours. Dehydrated tissues were embedded in epoxy resin, and a nick was made in the edge of the trimmed resin block to identify the temporal side of the nerve. Serial 1.5µm semithin sections were cut, either (a) longitudinally, through the ONH from the retinal end to the commencement of the optic nerve, or (b) transversely (cross-sections), and stained with 1% methylene blue and Azur II. Ultrathin sections were cut and stained with 25% uranyl acetate in methanol for 2min and Reynold's lead citrate for 15min.

Cryostat procedure

Rats were perfused with 50ml of 0.1M PBS followed by 500ml of 4% paraformaldehyde in 0.1M PB and heads were left in the fixative overnight. The eyeballs and attached optic nerve were removed and immersed in 10%, then 20% sucrose solution until the tissue sank. Continuous adjacent 14µm cryostat sections were cut in a plane transverse to the long axis of the optic nerve.

1.5µm thick semithin sections were immersed in matured sodium ethoxide for 8min, washed with ethanol 4 times, marinated in 8% formic acid in distilled water for 10min, washed with distilled water several times, and blocked with 5% milk, 0.1%Triton X100 in PBS.

14µm fixed cryosections were further fixed in 4% paraformaldehyde for 20min, washed several times in PBS, blocked in 1% bovine serum albumin (BSA; Sigma) in PBS with 0.2% Triton X-100 and transferred to primary antibody solution. The antibody solution was diluted with 1% BSA, 0.2% Triton X-100 and 0.005% sodium azide in PBS.

The sections were incubated in the following primary antibodies overnight at 4ºC in a humidified chamber. Rabbit anti-glial fibrillary acidic protein (1:100 for resin sections, 1:500 for cryostat sections; GFAP; Dako Cytomation Ltd., Cambridgeshire, UK), mouse anti- ß-III-tubulin (TUJ1, 1:500; Sigma-Aldrich Company Ltd, Dorset, UK) for cryostat sections or rabbit anti-neurofilament heavy chains and light chains (1:100; Serotec, Kidlington, UK) for resin sections. After primary incubation sections were washed and incubated in species specific secondary antibodies conjugated with either Alexa-546 or Alexa-488 (Invitrogen,

Paisley, UK) at room temperature for 2 hours. Fluorescent images were visualized and captured using a TCS SP1 Leica confocal microscope.

Axon counts

Under the x40 objective photographs were taken across the cross sectional area of 1.5µm thick resin sections of the optic nerve at a level 2mm from the retina. A photomontage of the entire nerve was constructed using the Automate Photomerge function in Photoshop CS5. On the enlarged image on the computer screen axons were identified by the profiles of methylene blue staining of intact myelin rings encircling pale (normal) axons. The profiles were counted independently by two different people in 24 evenly spaced 40µm sided grid squares (marking each ring as it was counted; Fig. 1). The diameter of the optic nerve was around 500µm, giving a sampled area of around 20%. A total of 131,891 normal axons was counted in the 19 sampled nerves (n=7 normal and n=3,3,6 at 1, 2 and 4 weeks after injection of magnetic microspheres).

Statistical analyses

Statistical testing was performed with SPSS (SPSS Inc, Chicago, USA; version 17.0). One-way ANOVA with post hoc testing was used to assess the difference of average IOP and number of axons in the different survival groups.

RESULTS

FORTIFIED ASTROCYTES OF THE NORMAL ONH

Gross Anatomy

The optic nerve head (ONH) is a uniquely specialised segment of the optic pathway. In the rat the ONH is about 250-300µm in length (Fig 2A). Its rostral margin is the funnel shaped region where the unmyelinated optic nerve fibres (retinal ganglion cell axons, RGC) converge on the optic disc. It ends caudally over a ragged transition to the optic nerve proper, at which point oligodendrocytic myelination begins. In cross section the rat ONH has a characteristic kidney shape, some 500µm wide and 300µm dorso-ventrally (Fig. 2B). The ONH has a thick, closely apposed sheath (Fig. 2D) and lies freely within a wide CSF containing subarachnoid space, which, in the section illustrated in Fig. 2A,C is drained by an arachnoid villus. The 'hilus' of the kidney is always at the mid-ventral pole where the surface of the ONH is invaginated from outside by two large vessels, the ophthalmic vein dorsally and the ophthalmic artery ventral to this (Fig. 2D). In their passage forward from the anterior cerebral vessels, the ophthalmic vessels travel in a wide CSF space ventral to the optic nerve, which has a perfectly rounded cross section (Fig. 2E,H,I).

The rat ONH contains only three tissue components. The key to the spatial organisation of the rat ONH is a radiating array of specialised astrocytes (Fig. 2B,F,G) through which the RGC axons run at right angles and which ensheathe the endothelial cells of the microvessels. Unlike the human lamina cribrosa there is negligible connective tissue or collagenous strengthening of the perivascular spaces (Johansson, 1987). To distinguish from the larger human and primates eyes, which have a massive lamina cribrosa of connective tissue, we have preferred to use the term ONH for the entire region in the rat.

Fortified Astrocytes

The ONH astrocytes have stout end feet anchored around the mid-ventral, 'hilar' surface facing the ophthalmic vessels (Fig. 3A) As the astrocytic processes radiate out from this ventral attachment, they are separated by longitudinal channels containing the unmyelinated RGC axons (Fig. 3B). As they approach the dorsal circumferential cap, the radial processes branch repeatedly and the branches converge in an axon free - 'pre-terminal' - layer before terminating in a complex layer of fine interdigitating delicate branches at the dorsal surface (Fig. 3CD).

Electron microscopy shows that the ONH astrocytes have a unique cytoplasmic composition (Fig. 4). In marked contrast to the highly pale cytoplasm of astrocytes in virtually every other CNS location (including the retinal optic nerve fibre layer and the optic nerve), the cytoplasm of the ONH astrocytes is highly and uniformly electron dense throughout all the cell processes. An equally striking feature of the astrocytic processes is their massive cytoskeletal 'strengthening' of longitudinal massed filaments and tubules. Together with the anchoring of the astrocytes to the tough encircling sheath of the ONH, we suggest this arrangement provides the structural basis which gives the ONH its mechanical strength (Ingber, 2003) and for this reason we refer to these uniquely specialised cells as 'fortified astrocytes.' We propose that it is this strength which makes the astrocytes the primary mechanical pressure transducers in the rat ONH, vulnerable to the distorting effects of raised intraocular pressure.

Ventral surface

The stout basal end feet of the fortified astrocytes are packed into an array around the concave, mid-ventral surface of the ONH, facing the ophthalmic vein. The end feet are densely packed with swirling cables of tubules and filaments (Fig. 4A,B). Along its ventral edge the basal surface is free of axons, and is furnished with a parallel array of elongated straight, villus-like projections clothed by the uniquely thickened basal lamina (around 125µm) surrounding the entire ONH (Fig. 4A,B). Between these projections, channels of extracellular space extend deeply into the basal astrocytic cytoplasm. By means of these arrangements the entire basal surface is securely anchored in the thick encircling collagenous ONH sheath. At all surfaces where the astrocytic processes come into direct contact with each other the surfaces are richly endowed with numbers of pinocytotic pits (Fig. 4J,N).

Radial Trajectory

During their radial trajectory through the ONH the astrocytic processes branch. The larger branches consist of a central trunk with a strictly aligned core of parallel, densely packed filaments and tubules arising from the swirling mass in the basal end feet. Surrounding this core is an electron dense peripheral cytoplasmic region from which are generated very fine (tapering down to around 40µm thick) electron dense lateral processes which interweave among the unmyelinated RGC axons although leaving the deeper parts of the RGC axon bundles free of astrocytic processes, i.e. with only direct axo-axonal appositions (Fig. 4I). The radial astrocytic trunks are separated by channels containing from 1-10 layers of axons (Fig. 4A). Symmetrical desmosome-like membrane thickenings and gap junctions are found between adjoining fine astrocytic processes (Fig. 4H).

Pre-terminal Segments

As they approach their terminations at the dorsal surface of the ONH, the radial astrocytic processes branch progressively and lose their cytoskeletal cores of filaments and tubules. This narrow, pre-terminal region of the ONH (about 1-2µm deep) is devoid of axons so that

the radial processes converge into direct contact with each other in fine, parallel, electron dense arrays (Fig. 4C). This arrangement is shown to advantage in sections which pass through the lateral surfaces of the ONH where the pre-terminal processes winding up towards the dorsal surface tend to be cut in oblique section and which clearly reveal the axon free area with its interweaving array of electron dense pre-terminal processes rather resembling tiers of rice fields climbing round a mountain side (L in Fig. 4F,G). The surface of the pre-terminal segments is smooth, and pinocytotic pits are a prominent feature in situations where the astrocytic processes come into direct contact with each other (Fig. 4J,N). They are not formed on the astrocytic surfaces facing axons, nor on those apposed to the basal lamina of the ONH sheath.

In 21 out of 26 normotensive eyes small, localised patches of this pre-terminal area just under the dorsal surface of the ONH show a degree of loosening, with spaces filled with extracellular fluid separating the pre-terminal astrocytic processes (Fig. 4C,D). However, the terminal processes remained embedded in the basal lamina of the overlying dorsal sheath. These appearances are restricted to about 50µm in rostro-caudal extent. Whether this is an *in vivo* occurrence or a tissue processing artefact the tendency of the pre-terminal processes to become separated reveals that this is the only region where the tissue components have a tendency to separate in this way, and which we will identify as the curved border region along which raised IOP first starts to distort the ONH.

Dorsal Surface

At the dorsal, circumferential cap of the ONH (the former ventricular surface of the optic stalk), the fortified astrocytic processes break up into a striking interdigitating array of fine, curving, villus-like fingers (Fig. 4C,D) - Cf the straight villus-like processes of the ventral surface, Fig. 4A,B). Each finger is completely invested in a glove-like arrangement of the basal lamina, which is thickened to around 125µm (Fig. 4D) and which ensheathes the entire ONH. The fine terminal astrocytic branches have the typical electron dense cytoplasm of the ONH astrocytes and, like the pre-terminal segments, they are devoid of the cytoskeletal strengthening of filaments and tubules.

In contrast to the ONH where the dense mass of villous processes forming the surface anchoring of the electron dense astrocytic processes is differentiated into ventral and dorsal surfaces, the surface of the optic nerve is similar in all directions and consists of a simple overlay of electron pale astrocytic processes, devoid of villi, and forming the same glia-pial array as found at the surface of the rest of the CNS (Fig. 4E).

RGC axons

The majority of the axons in the rat ONH range between 0.2 and 1.0µm diameter (Fig. 4I). Occasional much larger axons (around 3µm) are found superficially in the ONH close to the lateral edges of the mid-ventral region (Figs 3B, 4A). Cross sections show that the axons are segregated by the glial processes into compartments of varying size, and run longitudinally through the ONH, in a notably strict orthogonal relation to the radial astrocytic processes.

Microvessels

The ONH microvessels form a connection between the system of posterior ciliary arteries and veins (Morrison et al., 1999; Hayreh, 2001a; Hayreh, 2001b). The microvessels travelling radially through the ONH pass through the longitudinal rows of astrocytic cell bodies. These

vessels are – as in the rest of the CNS – ensheathed by astrocytic processes from which they are separated by a basal lamina of normal thickness (around 60µm) and continuous with the thick basal lamina clothing the outer surfaces of the ONH – i.e. they are morphologically outside the CNS. The astrocyte-bounded perivascular spaces form continuous channels from the ventral to the dorsal surfaces of the ONH and contain occasional small areas of collagen. These are the rudimentary rat equivalents of the structure that constitutes the massive connective tissue plates forming the primate and human lamina cribrosa.

Long sections of the abluminal surfaces of the endothelial cells of the microvessels in the ONH are occupied by rows of contiguous pinocytotic vesicles (Fig. 4L). By contrast, in the optic nerve, there is effectively no perivascular space, the astrocytic processes lie directly apposed to the endothelial cells, separated by a uniform, narrow space of only around 100nm, and the abluminal surfaces of the endothelial cells are entirely devoid of pinocytotic pits (Fig. 4M). Microvessels in the surrounding connective tissue outside the optic nerve have pinocytotic vesicles comparable to those in the ONH. The occurrence of these pits correlates with the presence of a blood brain barrier in the optic nerve, and its absence in both the ONH and the surrounding connective tissue.

The sheath of the ONH

The entire perimeter of the ONH is tightly enveloped in a highly vascular connective tissue sheath up to 50µm thick (Fig. 2D) and occupied by densely packed and aligned collagen fibres. All the astrocytic surfaces facing this sheath are invested in the specially thickened basal lamina. Beyond this sheath the ONH lies in an encircling CSF containing space bounded by a thick dura (Fig. 2D). This space communicates caudally with the subarachnoid space around the optic nerve. Rostrally, at the retinal end, the CSF space ends blindly, by being closed off by the fusion of the dura with the sclera (Fig. 2A,C).

EFFECTS OF RAISED IOP

Intraocular pressure

The IOP in unoperated eyes was 24.59±0.42 (SEM, n=29). The effect of the injection of magnetic microspheres was to raise the IOP from the normal to 42.52±2.04 (172%, $p<0.001$) at 1 day, maintained at 43.05±2.33 (175%, $p<0.001$) at 1 week, falling over 2 and 3 weeks to 36.41±1.47 (144%, $p<0.001$) and 33.23±2.33 (132%, $p=0.0018$) of normal, and by 4 weeks was approaching normal at 28.15±4.37 (112%, p=NS; Table 1; Fig 5A).

Loss of axons in the optic nerve

The loss of axons caused by the raised IOP was measured by counting the average number of axons per grid square (based on myelin ring profiles stained with methylene blue) in a total of 24 spaced square (20% of the total cross sectional area (Fig. 1A) in the optic nerve 2mm behind the junction with the optic disc. This gave an estimate of a total of around 60,000 axons for the entire optic nerve in normotensive eyes, falling to around 38,000 at 1 week, 31,000 at 2 weeks, and 11,000 at 4 weeks (Table 1; Fig 5B). Thus, over the period from 1 to 4 weeks after injection of the magnetic microspheres, the optic nerves shows a loss of 32% of axons at 1 week, 43% at 2 weeks, and by 4 weeks 81% of axons have been lost. Comparison of the rate of axon loss with the changes in IOP shows that although the IOP over the period from 2 to 4 weeks has returned to close to normal, the axon loss over this period continues

accelerating to the point where 81% of axons has been lost by 4 weeks. This indicates that it is the initial pressure rise which is the most destructive to the axons.

Early stages of damage to the ONH (1-2 weeks)

In longitudinal sections of normal eyes, the distance from the rostral surface of the ONH to the rather ragged junction with the optic nerve is around 250µm Fig. 6A,C). In the eyes with raised IOP, the overall changes in the shape and dimensions of the ONH follow the classical stages of thinning and cupping seen clinically in glaucoma. At1 week, the retinal surface of the ONH becomes cupped and the tissue becomes progressively lost from the ONH so that the distance to the optic nerve junction is decreased (around 100µm in Fig. 6B,D). At longer survivals, the specialised fortified radial astrocytes are lost, the region between the optic nerve and the retina becomes occupied by a densely packed scar of fine astrocytic processes (see below), and there is a dramatic loss of RGC axons (Fig. 6E,F,G).

In cross sections, in 17 out of the 18 rats killed at 1 (n=9) and 2 (n=9) weeks, the earliest sign of damage, seen at 1 week, was the opening up of spaces in the region of the pre-terminal astrocytic segments just under the dorsal circumferential margin of the ONH (Fig 7A,B,C,G,H). In localised foci the astrocytic processes are completely torn away from the dorsal circumference to a depth of 75-100µm. The bleb-like spaces left in the areas vacated by the focal separation and withdrawal of the astrocytic processes are occupied by masses of tissue debris (Fig. 7H). The dense surrounding collagen sheath prevents indentation of the outline of the ONH.

Electron micrographs show that the normally aligned array of narrow, directly apposed pre-terminal segments becomes opened up, and the processes become more distorted and separated by interconnected channels of empty, fluid filled intercellular space (Fig. 8)\. This pattern of distribution is similar to that observed in some normal eyes, although the separation is both greater and shows a progressive increase and spreading in the hypertensive eyes. The terminal interdigitations of the astrocytic terminals with the thickened basal lamina coverings become withdrawn from the dorsal circumference (Figs 8, Stage 2 in 10). The complex mass of interdigitating processes is lost and the encircling basal lamina becomes smoothed out, although in places trails of redundant basal lamina remain, or empty glove-like pockets from which the astrocytic fingers have been withdrawn (Fig. 9C). With the detachment of their terminals at the dorsal surface, the thin pre-terminal sections of the astrocytes collapse into a flattened layer (s in Fig 9C). Despite this damage to their fine dorsal processes, the remainder of the astrocytes are still unaffected at this stage (Stage 2 in Fig. 10), with their stout ventral processes packed with swirling masses of cytoskeletal filaments and tubules continuing to attach them securely to the ventral basal lamina.

Later stages of damage to the ONH (4 weeks)

By 4 weeks the degenerative changes have advanced ever more ventrally into the core of the ONH (Stages 3-6 in Fig. 10). In all 10 cases the foci of damage spread and become deeper so that the entire array of terminal and pre-terminal astrocytic processes is torn away from the dorsal circumferential sheath. The remaining mutilated stems of the astrocytic processes become thickened and lose their radial array. Immunostaining of axons and astrocytes confirms that at this late stage even regions continuing to stain for GFAP show a severe loss of axons (Fig 7D,E,F). The radial astrocytes themselves are lost, the former ONH region is occupied by a shrunken, hypervascular mass of disorganised tissue (Figs 6E,7D) which can

be seen in longitudinal section to border caudally on the tract astrocytes of the optic nerve which are themselves reacting to the presence of the degenerating RGC axons (Fig 9G,H). The longitudinal section illustrated in Fig 6G shows that surviving axons are confined to the ventral-most aspect of the ONH. At all stages the thick connective tissue capsule of the ONH retains its original shape and size.

EM of axon degeneration in the ONH

As the astrocytic processes progressively retract from the dorsal surface the effect is to expose the axons in these regions. The axons lose contact with astrocytic processes and spill out from their snug astrocytic compartments into a region of extracellular space and debris (Figs 8,9A). Huge degenerating axon swellings are occupied by swirls of cytoskeletal material surrounded by a rim of mitochondria and heterogeneous clear and dense cored vesicles (Fig 9E,F). These are similar to the structures found in the human glaucomatous ONH (Quigley et al., 1981).

The ultrastructural mechanism of axon degeneration in the ONH has many unusual features different from those seen in other areas of the CNS, and in particular from the degeneration in the OpN. Electron dense degeneration does not occur in the ONH, and there is no phagocytic response by the astrocytes. The astrocytic processes in contact with the greatly swollen degeneration axon profiles show no sign of swelling or any other cytoplasmic changes (Fig. 9E,F) and contain no engulfed fragments of degenerating material. For comparison, Fig. 9 G,H illustrates the stages of phagocytosis of degenerating axons and their myelin sheaths by the astrocytes of the OpN.

No section we have seen shows any indication of invasion of the tissue by any inflammatory tissue elements such as microglia or macrophages (see also (Hernandez, 2000). We have not observed either Müller cell invasion or ingrowth of oligodendrocytes. The axonal lesion in the ONH induced by IOP is not typically an inflammatory process, although there is a pronounced increase in vascularity (see below).

Blood Vessels

In agreement with the observations in human post-mortem and primate material (Quigley and Addicks, 1980; Quigley et al., 1981), we find that the raised IOP lesion in the rat ONH is not avascular. Our material demonstrates a progressive increase in the number of profiles of microvessels in the ONH and its sheath (Fig 7D). The semithin sections of normotensive eyes had around 14 profiles per section rising to 4-5 times this number in the later lesions (13.68±2.24 in normal, 20.5±3.72, 27.4±3.78, and 43.67±8.91 at 1, 2, and 4 weeks). In places the perivascular spaces are increased (Fig 9B) with accumulations of collagen and with elastin fibres (Fig. 9D; (Pena et al., 1998; Hernandez, 2000)).

DISCUSSION

ONH astrocytes are uniquely specialised

The fibrous nature of the ONH astrocytes and their orientation transverse to the axons has been mentioned and illustrated by several authors (May and Lutjen-Drecoll, 2002; Elkington et al., 1990; Hildebrand et al., 1985; Morrison et al., 1995; Morcos and Chan-Ling, 2000). In human material the ONH astrocytes are characterised as type 1B on the basis of expression of both GFAP and NCAM (Hernandez, 2000). The astrocytes of the ONH have a number of

special properties, including a low proportion of orthogonal membrane particles in the subpial end feet (Bauerle and Wolburg, 1993) and they enclose wide perivascular spaces where the abluminal surfaces of the apposed endothelial cells are furnished with abundant pinocytotic pits. This is associated with a leaky blood brain barrier (Flage, 1977) and is in contrast to the close appositions and absence of pinocytotic pits on the microvessels of the retina and optic nerve which present a closed blood-retina / blood-brain barrier. During development the ONH astrocytes are associated with a barrier to the rostral migration of oligodendrocyte precursors (Perry and Lund, 1990; Ffrench-Constant et al., 1988) and so prevent myelination, which may be a significant factor contributing to the susceptibility of the non-myelinated axons to pressure related damage.

Combined with these observations our present data confirm that the astrocytes of the ONH are a unique glial cell type whose characteristics are summarised in Table 2.

Fortified structure of rat ONH astrocytes

In the present study we describe the unique structure and arrangement of the ONH astrocytes, with their highly electron dense cytoplasm strengthened along the radial axis by dense cytoskeletal beams of tubules and filaments. The wide bases of the cells lie attached by straight villous processes anchored deeply into the ventral surface of the thick connective tissue sheath of the ONH. The cytoskeletal elements are continued into the radial processes, which pass dorsally through the axon containing territory of the ONH, where they give rise to short very fine processes among the axons. As they approach the dorsal circumference the radial processes converge into an axon free pre-terminal layer where they branch into fine parallel segments devoid of cytoskeleton and with a tendency to separate. The terminal branches of the astrocytes end dorsally as a jungle- like mass of tiny finger like processes (also illustrated in Fig. 3 of (Hildebrand et al., 1985) devoid of filaments and tubules and shallowly inserted into the dorsal connective tissue sheath of the ONH.

ONH astrocytes are pressure transducers

We propose that the cytoskeletal tubules and filaments convey mechanical strength to the ONH astrocytes, and hence we have called them fortified astrocytes. During rises in IOP the fortified astrocytes of the rat ONH are the load bearing structures. As a result the force generated by increased IOP would be funnelled into the ONH as a longitudinal stress in the rostral to caudal direction (Fig. 2C). Initially, increasing force tend to bow the astrocytes caudally. However, the elasticity of the astrocytes will be greatly curtailed by their cytoplasmic tubules and filaments (Ingber, 2003). Once the pressure rises beyond this point a shearing force will be applied to the astrocytic attachments to the surrounding tough connective tissue sheath of the ONH.

The attachment of the dorsal processes of the ONH astrocytes is the primary target of raised IOP

Because of the asymmetric nature of the astrocytic arrangement, the resilience of the radial astrocytes is unequal along the circumference. At the ventral surface the thick bases with dense cytoskeleton and long thin villous ‘roots’ presents a strong attachment. However, the progressively finer branching of the astrocytic processes as they pass towards the dorsal circumference and the loss of cytoskeletal elements in the pre-terminal region presents the most vulnerable target to the stresses (Fig. 10). Whether or not the localised areas of separation of the pre-terminal glial processes seen in some of our histological preparations

from normotensive eyes are an *in vivo* event or an artefact of tissue preparation, they clearly reflect that this region is an area of vulnerability. The frequent pinocytotic pits on the preterminal processes may (Fig. 4J,N) represent a mechanism to counteract the tendency for fluid to accumulate in the intercellular spaces and separate the processes (Steinman et al., 1974). The variability of separation of the pre-terminal segments we observe between individual eyes (even between sides of the same individual) may reflect the individual variation seen clinically in vulnerability to raised intraocular pressure (Kass et al., 2002).

Initial responses to raised IOP

The present observations are consonant with a multi-stage cascade of events in the pathogenesis of glaucomatous damage. The initial event is the opening up of the parallel arrays of pre-terminal astrocytic processes. This loosening of the interconnections of the pre-terminal segments may be related to the early loss or altered phosphorylation of astrocytic connexion-43 (a component of the intercellular gap junctions which enable the astrocytes to form a functional syncytium). De-phosphorylation of connexin-43 has been demonstrated in cultured human ONH astrocytes as a 'mechanosensor' response to pressure (Malone et al., 2007; Johnson et al., 2000). Our observations agree with Morrison et al (Morrison et al., 2008; Morrison et al., 2005) that the dorsal ('superior') part of the ONH is the earliest location of damage caused by raised IOP. Given that the axons are distributed retinotopically in the ONH (HOYT and LUIS, 1962; Sun et al., 2009), the initial foci of axonal degeneration under the dorsal rim of the ONH provide an explanation of the selective visual field loss observed in the initial stages of glaucoma.

Increasing susceptibility to raised IOP

The detachment of the astrocytic processes along the dorsal periphery would make the remaining astrocytic ensemble less structurally stable, and therefore less able to resist continuing shearing forces. This may be a possible factor in the 'hypercompliance' described by Burgoyne et al (Burgoyne et al., 1995; Burgoyne et al., 2005) and the increasing sensitivity of glaucomatous patients to minor pressure rises (Grant and Burke, Jr., 1982; Morrison et al., 2005) which would be tolerated by an undamaged ONH where the peripheral astrocytic attachment is complete. A further major factor that could seriously aggravate the damage caused by raised IOP is the thinning of the ONH (Quigley et al., 1983; Jonas, 2010). In our material we have recorded thinning in the longitudinal plane from the 350μm in the normotensive eyes to less than 50μm. This would produce a 7-fold increase in the steepness of the pressure gradient across the remaining ONH tissue, an effect equivalent to a major further rise in IOP.

Progression to irreversibility

The variable degree of separation of the pre-terminal processes we observe in normotensive eyes is not accompanied by any signs of axon degeneration. With induced rises in IOP, however, the elasticity of the astrocytic processes is exceeded to the point where the dorsal processes are torn away from their attachment to the outer sheath. There is no indication that astrocytic processes can grow back and re-establish terminal contacts at the dorsal surface. At this point, therefore, the damage would become irreversible.

The irreversibility of the lesion is indicated by the fact that although the IOP returns to close to normal levels at 4 weeks after a single injection of magnetic microspheres, the loss of axons continues at an accelerating rate (Fig. 5A,B). This correlates with the clinical

observation that later therapeutic interventions to correct an initial rise in IOP are only partially able to prevent continuing contraction of the visual fields (Leske et al., 2003).

Mechanism of damage to RGC axons

It is unlikely that the RGC axons are themselves a direct target of the mechanical forces of raised IOP. Not only are the nerve fibres flexible but they are running longitudinally to the pressure gradient so they would not be exposed to the same stresses as the astrocytes whose processes run orthogonally across the gradient. Therefore the likeliest explanation of the loss of nerve fibres (Morrison et al., 2005; Soto et al., 2008; Hernandez et al., 2008; Quigley et al., 1981; Burgoyne et al., 2005) is that it is a secondary consequence of the damage to the surrounding fortified astrocytes. But how does this lead to destroying the axons?

Damage to the astrocytes begins at the dorsal periphery, and leaves large amounts of extracellular space, into which the nerve fibres spill (Figs 8,9A, Stage 2 in Fig. 10). This makes it less likely, therefore, that the fibres are being damaged by physical pressure, or that there is a mechanical obstruction to axoplasmic transport. Moreover, the fact that the RGC axon profiles remain sectioned at right angles in the damaged areas indicates that their course is not being distorted.

Thus, whereas the damage to the astrocytes is mechanical, we propose that the most likely link between the astrocytic events and the localised damage to RGC axons is metabolic. Recent evidence clearly shows that the energy metabolism for neuronal survival depends on astrocytes: e.g. " glucose is processed glycolytically in astrocytes, which release lactate … as the metabolic substrate to be used by neurons" (Tsacopoulos and Magistretti, 1996; Herrero-Mendez et al., 2009).

In general, 99% of the cytoplasm and mitochondria of long axon neurons is in the axons and their terminals. The mechanical damage to the ONH astrocytes leading to the withdrawal of their processes from contact with the dorsal surface of the ONH sheath (seen as the 'blebs' in Fig. 7A,B,C) will deprive RGC axons of vital energy support as they pass through this region. We propose that such withdrawal of astrocytic support at one restricted level along an axon's trajectory could have the same effect as a physical axotomy.

Comparison with human glaucoma

In the human and primate the fibroblasts and collagen of the perivascular spaces of the ONH are immensely expanded to form the connective tissue beams of the lamina cribrosa. This massive connective tissue structure is generally assumed to be the pressure transducer responsible for the axonal damage caused by raised IOP in glaucoma (Quigley et al., 1987; Quigley et al., 1988). Species such as the rat and mouse, however, have only very small and inconsistent amounts of extracellular matrix present in the perivascular spaces of the ONH. The observation that raised IOP causes comparable axon damage in these species (Samsel et al., 2010; Johnson et al., 2007; Morrison et al., 2005; Fortune et al., 2004; Jia et al., 2000; Johnson et al., 2000; Morrison et al., 2008) indicates that the injurious effects of pressure transduction can be exerted in the absence of a connective tissue lamina cribrosa.

The principal finding of our present study is that in their traverse through the rat ONH the RGC axons pass through a radial array of uniquely specialised astrocytes (also illustrated in (Morrison et al., 1995; Anderson, 1969; Sun et al., 2009)), and we propose it is the

specialised structure and arrangement of these astrocytes which makes them the target for the damage caused by raised IOP in the rat. Within the interstices of the human connective tissue lamina cribrosa the RGC axons similarly pass through an astrocytic territory (Anderson, 1969). The majority of studies of human and primate lesions, however, have concentrated on enlargement of pore diameter and thinning of the connective tissue beams of the lamina cribrosa observed in preparations from which the cellular tissue elements have been digested (Quigley and Addicks, 1981; Sawaguchi et al., 1999; Fontana et al., 1998; Maeda et al., 1999; Jonas et al., 1991) or in scanning laser ophthalmoscopy. In both approaches the astrocytes are not visualised (Maeda et al., 1999; Fontana et al., 1998). It would be interesting in future to see whether the effects of such distortions of the connective tissue lamina cribrosa are mediated not directly on the axons, but indirectly via damage to the intrinsic ONH astrocytes lining the pores.

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LEGENDS

Fig. 1

A, Low power montage of a semithin (1.5µm) resin embedded cross section of normal OpN as used to count axons showing sampling grids. All 24 squares were counted by 2 independent observers under the x40 objective. B, enlarged view. Stained methylene blue and Azur II. Scale bars, 100µm (A), 15µm (B).

Fig. 2

A, Longitudinal section across the ONH from the retina to the junction with the OpN, showing the characteristic longitudinal rows of astrocytic cell bodies. The thick sheath of the ONH (black arrows) is continuous rostrally with the sclera (s), and caudally with the dural sheath of the optic nerve (d). Asterisk, CSF containing subarachnoid space; v, ophthalmic vein; white arrow, arachnoid villus.

B, Cross section of ONH showing radial astrocytes and associated microvessels. Stout astrocytic basal processes (stained dark) are anchored on the mid-ventral surface, which is invaginated by the ophthalmic vein (v).

C, Line drawing (based on **A**) showing how the attachments of the thick sheath of the ONH (black arrows in Fig. 1A) to the sclera (scl) and the dura concentrate the force of raised IOP (arrows) into the ONH at right angles to its radial array of astrocytes. The arachnoid villus (white arrow in Fig. 1A above), acts as a drain which would lower the local CSF pressure (down arrow), thus increasing the pressure gradient across the ONH. OpN, optic nerve.

D, Cross section showing the thick sheath (s) of the ONH separated by the CSF containing subarachnoid space (*) from the dural sheath (d) of the optic nerve.

E-I, Cross sections contrasting the ONH (D,F,G) with the OpN (E,H,I). The ONH has a thick vascular outer sheath and is traversed by darkly stained 'fortified' astrocytes which are anchored by thick bases to the deep ventro-median indentation and which radiate out to terminate in thin processes attached to the overarching dorsal surface. The OpN has random arrangement of pale astrocytes, no connective tissue sheath, and the circular outline is not indented. 1.5µm thick resin sections stained with methylene blue and Azur II (A,B,D,E). Cryostats sections: GFAP (green) for astrocytes (F,H), combined with TUJ (red) for axons (G,I).

Scale bars, 100µm.

Fig. 3

A, Cross section of ONH showing stout astrocytic basal processes attached to the surface at the mid-ventral invagination (v).

B, Electron micrograph of 3 radial processes (R) arising from a stout basal process (n, nucleus). Typically, the large axons lie in this region. In the field shown, the largest axon (x) is ten times the diameter of the smallest one (arrow), indicating a thousand-fold greater volume of axoplasm.

C, Cross section from a normal ONH with a narrow rim of small intercellular spaces (white arrows) between the fine dorsal pre-terminal processes.

D, Electron micrograph showing enlarged intercellular spaces (clear) between the pre-terminal astrocytic processes, which are still attached to the normal array of interdigitating terminals on the dorsal surface (dt).

Scale bars, 50µm (A), 1µm (B), 25µm (C), 2µm (D).

Fig. 4

Electron micrographs:

A, Stout basal foot (BF) of a fortified ONH astrocyte firmly anchored into the collagenous sheath (C) by fine, straight villus projections (v) deeply invaginated into the cell cytoplasm. White arrow heads, cytoplasmic swirls of strengthening cytoskeletal filaments and tubules. R, radial processes separated by channels filled with RGC axons; f, fibroblast.

B, Enlarged view of astrocytic basal end foot (BF) with straight villi (v) invested with thickened basal lamina.

C, enlarged in D. Dorsal terminations of radial glial processes (dt) in a massive cap of complex, interdigitating curved villous processes invested with thickened basal lamina (white arrow heads)

E, Segment of the surface of the OpN. In contrast to the ONH where the surface consists of a mass of electron dense villous processes, the surface of the OpN consists of a simple overlay

of electron pale astrocytic processes (ap), devoid of villi, and forming the same glia-pial array as found at surface of the rest of the CNS. C, collagen of sheath; M, myelinated RGC axons.

F,G, The lateral surface of the ONH showing the axon free region occupied by the electron dense, directly apposed, interweaving pre-terminal processes (L). Another field at high power in G. ax, axons; collagen (c) and fibroblast (f) of sheath.

H, High power view to show astrocytic junctions: symmetrical desmosome-like (arrows) and gap junction (arrow heads).

I, Radial glial process showing aligned cytoskeletal core (f) and fine processes (arrows) arising from the periphery and interweaving among the RGC axons (x).

J, Parallel radial array of directly apposed branches of the radial astrocytic process.

K, Parallel circumferential array of fibroblasts in the dense collagenous sheath of the ONH.

L, Segment of endothelial cell surface from an ONH microvessel, showing the typical array of pinocytotic vesicles on the abluminal surface. In contrast to the OpN (panel M), the endothelial cell is separated from the astrocytic processes (out of the field) by a wide perivascular space containing a fibroblast (fbl) and collagen fibrils. Note that a permeable blood brain barrier is present in the ONH and absent in the OpN.

M, Segment of the surface of an endothelial cell (E) from an OpN microvessel. Note the absence of pinocytotic vesicles and the narrow and uniform apposition (arrows) of the astrocyte (nucleus, n), an anatomical configuration found in regions with a blood brain barrier. L, lumen.

N, Enlarged view of the closely apposed pre-terminal astrocytic processes (as in F,G) showing the rich array of pinocytotic vesicles (which may represent a local mechanism to counteract fluid accumulation and close up the spaces during reversible stage of distortion due to mild rises in IOP). See also, visible at low power in panels B and J.

Scale bars, 5µm (A), 3µm (C), 2.5µm (K), 2µm (F,G), 1µm (B,J,M), 0.5µm (D,I,L,N), 0.2µm (E,H).

Fig. 5

A: Intraocular pressures (mean and SEM) in the groups of normotensive rats (day 0, n= 29) and at rats at day 1 (n=29), and weeks 1-4 (n= 28,19,10,10) after injection of magnetic microspheres. Regression analysis for the recovery of IOP after the initial rise: R^2=0.927.

B: Total numbers (mean±SEM) of RGC axons in the groups of normotensive rats (n=7) and rats at 7, 14 and 28 days (n= 3, 3, 6) after injection of magnetic microspheres. This is calculated from counts of 24 40µm sided grid squares per section (i.e. around 20% of the total cross sectional area of the OpN) in cross sections at a level 2mm caudal to the retinal. This is based on a total count of 131,891 axons from the 19 nerves. Regression analysis for the loss of axons: R^2=0.947.

Fig. 6

Overall changes seen in longitudinal sections of the ONH after 1 and 4 weeks of raised IOP.

A, normal ONH, beginning rostrally at the optic disc (od) and expanding in diameter caudally at the transition to the OpN where the fibres become myelinated. ONH around 250µm in length.

B, After 1 week of raised IOP. At the retinal end of the ONH the optic disc (od) is depressed into a concavity. The narrow segment is greatly reduced so that the junction with the OpN is nearer the retinal end, indicating a severe loss of ONH tissue. ONH around 100µm in length.

C, D, Enlarged areas from the boxed areas in A, B across the transitions from the ONH to the onset of myelination at the OpN.

E, After 4 weeks of raised IOP, ONH tissue is difficult to recognise and it is difficult to identify fortified astrocytes. Deep cupping of the optic disc (od) at the retinal end.

F, Adjacent section to E, stained for astrocytic GFAP staining. This is mainly due to the gliotic response of OpN astrocytes to degenerating axons and myelin sheaths in the OpN. As mentioned in the text, the fortified ONH astrocytes do not show a response to the degenerating axons, but undergo degeneration themselves.

G, Adjacent section to E,F. Staining for neurofilament shows only a small cohort of RGC axons surviving on the ventral edge of the ONH as a compact sliver which becomes randomly spread as they enter the OpN (Hoyt and Luis, 1962; Horton et al., 1979) (Sun et al., 2009).

Scale bars 100µm (A,B,E,F,G), 25µm (C,D). Survival, 1 week (B), 4 weeks (E,F,G).

Fig. 7

A,B,C, A localised bleb of tissue damage on the dorsal surface (arrow) with axon loss (arrow in B) at 1 week.

D, E, F, Severe damage at 4 weeks. Massively increased vascularisation of the capsule, almost total loss of radial glial structures (see also in F), and massive loss of axons (in E).

G, Semithin section showing localised pulling away of the astrocytic terminals from the dorsal surface (arrows) and greatly increased vascularity at 1 week. The areas (asterisks, enlarged in H) pulled away from the sheath are filled with amorphous tissue debris. Similar appearances (called ‘pseudopits’) are illustrated in acellular preparations of the connective tissue lamina cribrosa in human post-mortem glaucomatous ONH by (Quigley et al., 1981) their Fig. 15).

Note that the capsular connective tissue sheath retains its normal outline, indicating that the effect of the stress is to tear the astrocytic processes away from it, beginning at the sub-capsular bleb shown in A,B,C,G,H.

Semithin sections: A,D,G,H stained with methylene blue and Azur II. Cryostat sections: B,E, TUJ immunostain for RGC axons (red). C,F, combined GFAP immunostain for astrocytes (green) and axons (red).

Scale bars 100µm (A-G), 25µm (H).

Fig. 8

The first target of damage after one week of raised IOP. The pre-terminal segments of the fortified astrocytes are the weak point. They are forced apart by large fluid filled extracellular spaces (E) empty apart from small rests of cellular debris. Their terminal segments are largely withdrawn (arrow heads) from the curly mass of interdigitating villi found at the normal dorsal surface and come to lie in piles of small, flattened processes at the interface with the sheath. High power micrographs (Fig. 6C) show loops of redundant basal lamina from which the astrocytic fingers have been withdrawn. The axons in the communicating compartments spill out (arrows) into the open tissue spaces. Large swollen degenerating axons (asterisks) are seen scattered throughout this region. Much larger degenerating axons are illustrated in Fig. 6E,F. As explained in the text, since the process of degeneration results in the cytoplasmic contents of narrow single axons being drawn into discontinuous large swollen degenerating profiles, the number of such profiles in any individual section represents only a minority of the total number of axons being lost. At this stage the central and ventral parts of the ONH are still largely unaffected. f, fibroblasts of the sheath. The areas (e) containing elastin fibres (see Fig. 6D). Montage of 83 high power electron micrographs merged in Photoshop CS4.

Scale bar, 1µm. Survival 1 week.

Fig. 9

A, Enlarged view of expanded space (E) between the pre-terminal astrocytic segments as shown in the montage in Fig. 5. Axons (arrows) spilling out into the enlarged, fluid filled extracellular spaces which contains some filamentous wisps of tissue debris.

B, Enlarged perivascular space.

C, Enlarged view of the dorsal surface, showing redundant loops of basal lamina (arrows) from which the terminal astrocytic processes have been withdrawn and come to lie in a stack (s) at the surface.

D, Elastin fibres (arrows) among the collagen fibres of the connective tissue sheath. Enlargement from Fig. 5.

E, F, Typical large degenerating fibres (d) with central cores of filaments (f) surrounded by a mass of mitochondria (m) and dense intracellular debris. E shows clearly that the

surrounding astrocytic processes (a) do not expand or change their dark cytoplasmic appearance or phagocytose the degenerating swollen axon. Cf G,H in the OpN.

G, H, Contrasting picture from the OpN where the astrocytes phagocytose the degenerating material. a: electron dense degenerating fragment completely engulfed within a membrane bounded space within the astrocytic cytoplasm; b: adjacent degenerating fragment has been degraded with total loss of membranes; c: degenerating material reduced to an amorphous, highly electron dense mass.

Scale bars, 1µm (A,C,E), 5µm (B), 0.5µm (G), 2µm (F,H) 0.25µm (D). Survival, 1 week (A,D), 4 weeks (B,C,E-H).

Fig. 10

Schematic representation of the changes occurring during damage by raised IOP.

1, Normal structure of a fortified radial astrocyte of the ONH. The enlarged basal end foot (B) is firmly anchored by long, straight villous processes (v) into the collagenous sheath at the ventral median indentation of the ONH. R, radial process branching to enclose separate territories of RGC axons. In the pre-terminal region (PreT) the finest branches come together in direct apposition to each other and excluding all axons. The terminal cap (TC) is made up of a thick layer of interdigitating curled endings invested with basal lamina.

2, The first stage of damage by raised IOP begins along the dorsal circumference. The pre-terminal processes become separated by massive spaces of tissue fluid (horizontal arrows). Their terminals are pulled away from the collagenous sheath and collapse (vertical arrow) into reduplicated layers on the dorsal surface. RGC axons spill out into the spaces; degenerating axons (grey filled circles). The loss of axons is already considerable at this stage.

3, The axon terminals are entirely pulled away (arrow) from the dorsal surface. We suggest this represents the irreversible stage of damage. The space they previously occupied is filled with cellular debris. Axon loss is severe.

4-6, Final stages of degradation leading to loss of all axons and total degeneration of the radial astrocytes.

INSET: Isobar representation of stress gradient across the ONH.

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