Article

Olfactory Ensheathing Cells Rescue Optic Nerve Fibers in a Rat Glaucoma Model

Chao Dai^{1,2}✉, Peng T. Khaw³✉, Zheng Qin Yin²✉, Daqing Li¹✉, Geoffrey Raisman¹✉, and Ying Li¹✉

¹ UCL Department of Cell and Developmental Biology; Spinal Repair Unit, Department of Brain Repair and Rehabilitation, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK

² Southwest Hospital, Southwest Eye Hospital, Third Military Medical University, Chongqing 400038, China

³ NIHR Biomedical Research Centres, Moorfields Eye Hospital and UCL Institute of Ophthalmology, Moorfields Eye Hospital 11-43 Bath Street, London EC1V 9EL, UK

Correspondence: Geoffrey Raisman, FRS, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK. e-mail: G.Raisman@ucl.ac.uk

Received: 6 June 2012

Accepted: 25 July 2012

Published: 24 August 2012

Keywords: olfactory ensheathing cells; lamina cribrosa; transplantation; optic nerve head

Citation: Dai C, Khaw PT, Yin ZQ, et al. Olfactory ensheathing cells rescue optic nerve fibers in a rat glaucoma model. *Trans Vis Sci Tech.* 2012;1(2):3, <http://tvstjournal.org/doi/full/10.1167/tvst.1.2.3>, doi: 10.1167/tvst.1.2.3

Purpose: To determine if transplantation of olfactory ensheathing cells (OECs) can reduce loss of optic nerve axons after raised intraocular pressure (IOP) in the rat.

Methods: OECs cultured from the adult olfactory mucosa were transplanted into the region of the optic disc. The IOP was raised by injection of magnetic microspheres into the anterior chamber.

Results: At 4 weeks after raising the IOP, the transplanted OECs had migrated into the dorsal area of the optic nerve head (ONH) where they surrounded the optic nerve fibers with a non-myelinated ensheathment. The mean amount of damage to the ONH astrocytic area in rats was 51.0% compared with 85.8% in those without OEC transplants ($P < 0.02$) and the mean loss of axons in the optic nerve was 51.0% compared with 80.3% in the absence of OECs ($P < 0.01$).

Conclusions: OECs transplanted into the region of the ONH of the rat can reduce the loss of axons and the damage to ONH astrocytes caused by raised IOP.

Translational Relevance: Confirmation of these preliminary experimental data, further understanding of possible mechanisms of axonal protection by OECs, and the longer-term time course of protection could provide a basis for future human clinical trials of autografted OECs, which would be available from autologous nasal epithelial biopsies.

Introduction

Ocular hypertension is the major risk factor associated with loss of vision in glaucoma.¹ However, neither medical nor surgical treatments to lower the intraocular pressure (IOP) are able to completely prevent this progression in all patients.^{2–5} The initial site of damage by the raised IOP is the narrow region where the unmyelinated axons of the retinal ganglion cells (RGCs) axons pass through the optic nerve head (ONH) before entering the myelinated region of the optic nerve and tract (as reviewed in Morrison, Johnson, and Cepurna).⁶ Here evidence is presented that in a rat model of ocular hypertension the introduction of olfactory ensheathing cells (OECs) into the ONH provided a degree of protection of the

optic nerve fibers against induced ocular hypertension.

The study on which this article is based is focused on the loss of fibers in the optic nerve because, rather than the survival of RGCs, this is the ultimate factor in the transmission of visual information to the brain. As the definitive findings of Vidal-Sanz, Salinas-Navarro, and Nadal-Nicolas,⁷ show, the death of RGCs after ocular hypertension induced by laser treatment of limbal tissues and episcleral veins, or after crush injuries, and even after total acute optic nerve transection is progressive, with a significant proportion of the axotomized RGCs surviving long after they have become unable to convey information to the brain.

Samsel, Kisiswa, Erichsen, et al.,⁸ recently described a method for producing an acute rise in IOP

by injecting magnetic microspheres into the anterior eye chamber in rats. Using this approach, it has been shown previously that during the 4 weeks after injection there is a progressive loss of astrocytic tissue in the ONH and a marked decline in the numbers of RGC axons counted in semi-thin resin sections of the optic nerve (OpN) 2 mm behind the ONH.⁹

The results of this study have shown that the rat ONH is populated by a unimodal population of specialized radially arranged astrocytes, fortified by a dense cytoskeleton and attached to the surrounding connective tissue sheath of the ONH.⁹ It is proposed that these astrocytes give the mechanical strength to the rat ONH and provide metabolic support to the axons, and that damage to these astrocytes is the primary event in raised IOP.

The olfactory system is the only part of the central nervous system where continuous growth and regeneration of nerve fibers is known to continue throughout adult life.^{9–12} The pathway for regeneration of olfactory nerve fibers is made up of a unique type of glial cells, OECs. Transplantation of cultured adult OECs has been shown to induce regeneration of severed nerve fibers in long spinal tracts and in avulsed dorsal spinal roots in experimental animals.^{13–16} In a study of transected rat OpN, transplantation of OECs into the lesion site in the OpN was shown to induce regeneration of severed RGC axons for up to 10 mm.¹⁷ This demonstrates the ability of RGC axons to respond to OECs.

Here, an exploration is undertaken of the potential for transplantation of OECs to protect the RGC axons and the surrounding astrocytes of the ONH against damage caused by raised IOP. A method was devised to introduce OECs into the ONH by injecting the cells trans-sclerally into the region of the optic disc.¹⁸ From this injection site the cells migrate into the ONH. In the current study it was shown that the transplanted OECs ensheathed the RGC axons and reduced the loss of astrocytic tissue in the ONH and the loss of axons in the OpN.

Methods

Cell Culture

The mucosal OECs were prepared as previously described.¹⁸ Briefly, adult Albino Swiss (AS) rats were decapitated under terminal anesthesia. The olfactory mucosa was dissected out and transferred to ice-cold Hanks' balanced salt solution without calcium and magnesium, supplemented with 1% penicillin-strep-

tomycin, and incubated in 1 mL of dispase II at 37°C for 45 minutes. The lamina propria was cut into small pieces, collected into 2 mL 0.25% collagenase type I in a Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 with GlutaMAX 1.0 mg/mL insulin, 0.67 mg/mL transferrin and 0.55 mg/mL selenium, 1% penicillin-streptomycin and 10% deactivated fetal calf serum (FCS), and incubated at 37°C for 5 minutes. The pieces of the lamina propria were triturated and centrifuged. The cell suspension was seeded at a density of 20,000 to 25,000 per square centimeter on 35-mm culture dishes coated with 0.1 mg/mL poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and maintained in a 5% CO₂ incubator for 10 days at 37°C, with the culture medium replaced every 3 days. Three days before transplantation the cells were transfected with a green fluorescent protein (GFP) gene harboring a lentiviral construct,^{19–21} made up to a concentration of 20×10^6 /mL in DMEM/Ham's F-12 without FCS. The cells were kept on ice during surgery.

Transplantation of OECs

Sixteen adult female AS rats (200–220 gm body weight) were deeply anesthetized with isoflurane and topical conjunctival 0.5% proparacaine hydrochloride. OECs were transplanted as previously described.¹⁸ Briefly, through a 5-mm skin incision superior to the upper lid the extraocular muscles were separated by blunt dissection. Sutures attached to the superior rectus muscle insertion were used to rotate the globe downward. Using a 15° stab knife (Fine Scientific Tools No.10315-12, Reading, UK) starting from a point 2 mm above the optic nerve attachment, a narrow incision was made through the sclera, choroid, and retina to an estimated depth of 1 mm and directed at an oblique angle toward the optic disc (Fig. 1, OEC arrow). The incision was used to insert a fine glass pipette (internal diameter approximately 70 µm) guided by hand for a depth of 1 to 2 mm (marked on the outside of the pipette), and 2 to 3 µL of the OEC suspension (approximately 50,000 cells) was injected by pressure via a 50-mL syringe, and the wound closed with skin sutures. All animals were examined to exclude intraocular inflammation. Two rats that developed cataracts were not used in this study. The autopsy observations and the histology showed that the oblique entry track of the needle through the sclera was completely closed, and there was no sign of bulging of the sclera, choroid or retinal tissue into the track.

Figure 1. Location of interventions and observations. Diagrammatic representation of the experiment. IOP arrow indicates the injection of magnetic microspheres into the anterior chamber. OEC arrow indicates the injection of OECs into the optic disc region of the retina, from where they migrate into the ONH. Tissue damage is measured in the ONH, and axons are counted in the OpN. a, anterior chamber; c, cornea; l, lens; r, retina; v, vitreous.

Induction and Measurement of Raised IOP

In 21 rats (consisting of the 14 that had received OECs 4 weeks previously and an additional seven that had not received OECs) the IOP was raised by the injection of magnetic microspheres.⁹ Briefly, rats were anesthetized as described in the preceding section. The right anterior chamber was injected with 20 μ L of 30 mg/mL suspension of 5 μ m-diameter magnetic microspheres (Aldehyde-terminated Magnetic Magic-Beads, Chi Scientific, Maynard, MA) using a 33-gauge needle (Fig. 1, IOP arrow). After injection, a small magnetic rod was used to direct the microspheres into the drainage area of the canal of Schlemm in the peripheral angle of the anterior chamber.⁸

The baseline IOP was recorded using a tonometer (Tono-Pen XL, Reichert, Inc, Depew, NY) at 1 day before and 1 day after injection of magnetic microspheres and then once a week. This occurred under local anesthesia induced by conjunctival instillation of one drop 0.5% proparacaine hydrochloride (Alcaine, Alcon Laboratories, Couvreur, Belgium). At the time of each recording, the mean of eight consecutive readings was taken as the IOP. After 4 weeks the rats were fixed by perfusion as described in the following section.

Immunohistochemistry

Four weeks after transplanting the OECs and a further 4 weeks after raising the IOP, seven rats were perfused under deep pentobarbital anesthesia with 50 mL 0.01 M phosphate buffered saline (PBS) followed by 500 mL of 4% paraformaldehyde in 0.1 M

phosphate buffer (PB) for 30 minutes. The heads were left in the fixative at 4°C overnight. The next day the eye and attached OpN were removed and immersed in 10% aqueous sucrose solution, then 20%, until the tissue sank. A continuous series of adjacent 20 μ m cryostat sections was cut in the longitudinal plane through the entire ONH and OpN (Fig. 1).

The first series of every fourth section was immunostained for rabbit anti-neurofilament heavy chains and light chains (1:500; Chemicon, Temecula, CA).²² A second series was immunostained for myelin P0 protein (1:3000 P0 antiserum, a gift from J. J. Archelos, Max-Planck Institute, Munich, Germany). After primary incubation, the sections were washed and incubated in species-specific secondary antibodies conjugated with either 1:400 Alexa-546 or Alexa-488 (Invitrogen, Paisley, UK) at room temperature for 2 hours, and counterstained for 10 minutes in the dark with SYTOX Orange (or Green) Nucleic Acid Stain (S-11,368; Invitrogen) at 5 μ M. Fluorescent images were visualized and captured using a TCS SP1 Leica confocal microscope.

EM Fixation and Resin Embedding Procedure

Under deep pentobarbital anesthesia, 14 rats (consisting of seven of the 14 that received both OEC transplants and subsequent raised IOP, and the group of seven that had raised IOP but no transplants) were transcardially perfused with 50 mL 0.01 M PBS followed by a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PB for 30 minutes. The OpNs including ONHs were dissected the next day and post-fixed in 2% aqueous osmium tetroxide for 1.5 hours. Dehydrated tissues were embedded in epoxy resin (TAAB, UK), and a nick was made in the edge of the trimmed resin block to identify the temporal side of the nerve. Serial 1.5 μ m semi-thin sections were cut transversely, and stained with 1% methylene blue and Azur II. Ultrathin sections of the ONH were cut and stained with 25% uranyl acetate in methanol for 2 minutes and Reynold's lead citrate for 15 minutes.

Cross-Sectional Area of the OpN

In 21 rats (consisting of the seven normal rats, seven rats with raised IOP alone, and seven rats with OEC transplants and raised IOP), 1.5 μ m-thick resin cross-sections of the OpN were taken at a level 2 mm from the retina and stained with 1% methylene blue and Azur II. To obtain an image quality sufficient for an accurate count of axons, a set of $\times 40$ objective

photomicrographs were prepared to cover the entire area of the OpN. The individual micrographs were montaged using the Adobe Photoshop CS5 Photo-merge function, resulting in a file size of approximately 350 MB. The area of each OpN section was obtained using the Analysis tool of the Adobe Photoshop CS5 program.

Number of Axons in the OpN

For counting axons, an overlay mask consisting of a grid of evenly spaced $24 \times 40 \mu\text{m}$ sided squares was imposed over the photomerged image.⁹ This sampled approximately 15% of the total OpN area. Each square was enlarged to the full computer screen. The myelin sheaths were identified as clear-centered methylene blue-stained rings and the counts of myelin ring profiles were the mean of two independent observers blinded to the origin of the samples. A total of 145,142 axons was counted in the 21 rats. For each rat, the total number of axons in the OpN was calculated by multiplying the individual sample count with the individual areas derived as above.

Measurement of ONH Cross-Sectional Area Occupied by Radial Astrocytes

In semi-thin cross-sections of the ONH stained with methylene blue and Azur II, the ONH astrocytes were readily identified by the high staining density and the characteristic pattern of stout ventral bases tapering dorsally into progressively finer radial processes. The outlines of the ONH, and the area occupied by the radial astrocytes were traced on screen shots of the $\times 40$ photomicrograph montages, and measured using the Analysis function of Adobe Photoshop CS5.

Statistics

Significances of group differences for IOPs and axon counts were based on one-way ANOVA (SPSS Version 20). For the IOPs and the optic nerve axon counts, exploratory data analysis (EDA) shows that the data is normally distributed. Where the Levene test statistics showed the variances of the groups were significantly different, the Welch and Brown-Forsythe Robust Tests of equality of means were applied to the ANOVA result. For post-hoc analysis, the Games-Howell test, which does not rely on homogeneity of variance, was used. For the extent of damage to the ONH, the significance of the difference between the raised IOP groups with and without transplanted OECs was based on SPSS Version 20 Independent Samples T-test. The

Pearson correlation coefficient (SPSS Version 20) was used to test the strength of the individual correlations between IOPs, amount of damage to the ONH, and axon counts of numbers of OpN axons.

The rats were maintained in a light- and temperature-controlled room and handled according to UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act of 1986, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Results

The data is based on 28 rats. Of these, 21 were prepared by electron microscopy fixation and resin embedding. These consisted of seven normal rats (group 1), seven rats with raised IOP alone (group 2), and seven rats that had OEC transplants 4 weeks before raising the IOP (group 3). A further seven rats that had OEC transplants 4 weeks before raising the IOP (group 4) were prepared for cryostat immunohistochemistry.

Raised Intraocular Pressure

The mean (normotensive) IOP in group 1 was 24.0 ± 0.4 mmHg (SEM, $n = 7$). In groups 2, 3 and 4, the IOP was measured on the day before injection of microspheres and monitored at day 1 and at weeks 1, 2, 3, and 4. After an initial rise to greater than 40 mmHg over the first 7 days, the IOP fell (Fig. 2). The mean of the five postoperative IOP measurements over the 4 weeks after injection, for each rat, was used to represent the cumulative effect of the IOP rise.²³

The mean postoperative IOPs were 37.3 ± 1.9 (SEM) in the seven rats in group 2 (raised IOP only), 33.7 ± 2.0 (SEM), in the seven rats in group 3 (raised IOP and transplanted OECs used for optic nerve axon counts), and 35.2 ± 2.0 in the seven rats in group 4 (raised IOP and transplanted OECs used for histologic analysis). All three postoperative mean IOPs in groups 2, 3, and 4 were significantly higher ($P < 0.001$) than the preoperative means and the mean IOP in the controls (group 1). Compared with group 2, there were indications of slightly greater reductions in the IOP rise in groups 3 and 4, although, with the current sample size, this was not significant ($P = 0.45$ and 0.81 , respectively).

Counts of Axons in the Optic Nerve

Axons were counted in the semi-thin resin cross-sections of the OpNs of group 1 (normal eyes), group

Figure 2. Intraocular pressures. The time course of the rise and fall in mean IOPs ($\pm$ SEM) in group 2 (raised IOP alone, triangles, $n = 7$), group 3 (IOP with OECs used for counting axons, squares, $n = 7$), and group 4 (IOP with OECs used for confocal study, circles, $n = 7$). Day 0, IOP measured the day before injection of microspheres.

2 (raised IOP only), and group 3 (transplanted OECs before raising the IOP). For each rat the total number of axons was counted in $24 \times 160 \mu\text{m}^2$ sampled squares. The cross-sectional areas of the individual OpNs were measured as in the Material and Methods section, and used to calculate the total numbers of RGC axons in each OpN.

The mean total numbers of axons in the OpNs were $76,923 \pm 1,779$ (SEM, $n = 7$) in the normotensive eyes (group 1); $15,142 \pm 3,662$ (SEM, $n = 7$), a mean loss of 80.3%, in the rats with raised IOP and no OECs (group 2); and $37,696 \pm 5,383$ (SEM, $n = 7$), a mean loss of 51.0% in the rats with raised IOP and transplanted OECs (group 3). The differences in numbers of axons between group 1 and both groups 2 and 3 were significant at $P < 0.001$. The difference between groups 2 and 3 was significant at $P < 0.01$ (Fig. 3A).

Extent of Damage to the Radial Astrocytes of the ONH

In semi-thin resin cross-sections, the entire area of the normal rat ONH (Fig. 4A) was occupied by densely methylene blue-stained astrocytes, arranged in a fan-like radial array, firmly attached ventrally to the sheath of the ONH by thick basal processes, but divided dorsally into progressively more slender processes with only delicate attachments to the

sheath. In the eyes with the raised IOP, the fine dorsal processes of the ONH astrocytes were torn away in a crescentic area underlying the surrounding dorsal sheath. Figure 4B illustrates the damage with raised pressure in the presence of transplanted OECs, and Figure 4C is an example of the more severe damage with raised pressure in the absence of OECs.

The mean area of the ONH in the seven normal rats of group 1 was 125.1 ± 4.6 (SEM) $\mu\text{m}^2 \times 10^3$, and the area of remaining normal astrocytic tissue in the seven rats of group 2 (raised IOP only) was 17.8 ± 7.4 (SEM) $\mu\text{m}^2 \times 10^3$ (a loss of 85.8%). There was a significantly greater amount of normal tissue remaining in the seven rats of group 3 (raised IOP with transplanted OECs) with an area of 59.5 ± 10.3 (SEM) $\mu\text{m}^2 \times 10^3$ (a loss of 51.0%; differences significant from normal at $P < 0.001$, and between groups 2 and 3 at $P < 0.01$) (Fig. 3B).

Distribution of Transplanted OECs

Cryostat Sections (Group 4, N = 7)

The overall distribution of transplanted OECs was examined in seven rats with raised IOPs. The OECs were identified by fluorescence of lentivirally induced GFP in cryostat sections cut in the longitudinal plane of the ONH (Fig. 4D). As described by Li, Li, Khaw et al,¹⁸ the major proportion of the transplanted cells migrate from the retinal injection site into the ONH; only occasional cells progress beyond the ONH for a short distance into the myelinated region of the OpN.

The transplanted cells were smooth-surfaced and bipolar, with a central ovoid cell body. As they entered the ONH they became elongated for up to as much as $100 \mu\text{m}$ in alignment with the RGC axons. At higher magnification the OECs appeared as double green fluorescent tracks ensheathing red fluorescent, unbranched RGC axons (Fig. 4E). The absence of P0 immunostaining showed that these ensheathments did not result in myelination, at least up to the 8-week point studied.

Semi-Thin Sections (Group 3, N = 7)

In semi-thin cross-sections of the ONH of rats with raised IOP and transplanted OECs, the dorsal crescentic areas from which the radial astrocytic processes are retracted were colonized by small scattered dark cells, some of which clearly showed typical signet-ring axon ensheathment of OECs, usually with an enclosed pale axon to one side of the nucleus (Fig. 4F). No OECs were found in the areas occupied by compact astrocytic tissues.

Figure 3. Comparison of mean axon counts. (A) In the OpN. (B) In the area of spared ONH radial astrocytes in group 1 (normal), group 2 (raised IOP only) and group 3 (raised IOP with OECs). *** $P < 0.001$; ** $P < 0.01$.

Electron Microscopy

In electron micrographs the radial processes of the ONH astrocytes were identified by their elongated shape, cytoskeletal bundles, and large and highly contrasted mitochondria with well-marked cristae (see Dai, Khaw, Yin, et al.⁹). Because of the length of the processes, the nuclei were not usually included. The nuclei and cell bodies of the transplanted OECs were found in clusters around the dorsal and lateral aspects of the ONH in the areas from which the astrocytic processes had been retracted. They lay in a collagen containing extracellular matrix bounded by one to several encircling layers of very fine fibroblastic processes. At the cellular level, the cross-sections of the OEC cell bodies were clearly distinguished from the astrocytic processes by their ovoid shape, with scant perinuclear cytoplasm, with axons invaginated and enclosed by fine processes (Fig. 5).

The OEC profiles usually ensheathed one or a small number of highly tubule-rich axons of around 1.5 μm, which was larger than the average diameter of the axons normally found in this region of the ONH. The mesaxons were very short, with no tendency for spiraling, and the ensheathment was invariably unmyelinated (Fig. 5). In addition, clusters of much smaller axonal profiles (down to 0.1 μm) were often included, and may have been sprouts from large parent axons (Fig. 5A, s). The axons associated with the OEC clusters were not all fully ensheathed; some simply lay in contact with adjacent OECs with a spectrum of interactions from simple contact via indentations of increasing depth that lead to total embedding (Fig. 5B).

Vascular Pattern

In the normal rat ONH the blood vessels ran radially across the dorso-ventral axis of the ONH in a pattern similar to the astrocytes (Fig. 4A) (also see Fig. 4 in Morrison, Johnson, Cepurna, et al.¹). As in Dai, Khaw, Yin, et al.,⁹ counts of profiles of blood vessels in the current resin-embedded cross-sections of the ONH confirmed that the raised IOP was associated with an abnormal increase in vascularity that lead to an overall increased number of microvessel profiles from a mean of 13.7 ± 0.85 (SEM, $n = 7$) in group 1 to 41.2 ± 1.66 (SEM, $n = 7$) in group 2, and 32.0 ± 3.20 (SEM, $n = 7$) in group 3. The transplanted OECs were associated with these areas of high vascularity. Electron micrographs showed that, as in the normal ONH, the vessels in the damaged ONH had rows of pinocytotic pits along the abluminal surfaces of the endothelial cells, and an even wider perivascular space.

Despite this overall increase in vascularity, the retraction of the ONH tissue from the dorsal sheath would have disrupted the local continuity of the circulation with the vessels of the dorsal sheath. This could be a significant contribution to initiating the damage to the radial astrocytes.⁹

Discussion

Key findings are that transplantation of OECs reduced the loss of optic nerve axons caused by raised IOP and the extent of damage to the radial astrocytes of the ONH.

Figure 4. Cross-sections. (A-C) ONH. (D,E) Longitudinal migration of OECs. (F) High power of ensheathment of axons by OECs. A,B,C, semi-thin cross sections of ONH: A, normal; B, with raised IOP and transplanted OECs; C, raised IOP (without OECs). In B,C, the spared radial glial structure (asterisks) of the ONH is outlined by dark red lines. The pattern of damage in B shows the typical retraction from the dorsal periphery, which, in C, comes to involve almost the entire cross-sectional area. The numbers of blood vessels (arrows in A) are 17 in A, 28 in B, and 51 in C. D,E, confocal images: Migrating OECs (green fluorescence, GFP) in the ONH (white arrow). Red, axons (neurofilament immunostaining). A small number of OECs (white arrow head) remain in the retina (RET); v, central retinal vessels; ONH, optic nerve head; OpN, optic nerve. E, high power of ONH to show elongation of OECs and ensheathment of RGC axons (white arrows). F, high power micrograph showing signet-ring ensheathment by transplanted OECs (red arrows). A,B,C,F, stained methylene blue and Azur II. Scale bars, A,B,C,D, 100 μ m; E, 20 μ m; F, 5 μ m.

Mechanism of Axon Damage

Previous electron microscopic evidence⁹ suggests that the initial damage to the axons in this model is

not due to mechanical compression of the ONH. Rather, the electron microscopic images show that withdrawal of the astrocytic processes from the dorsal periphery of the ONH leaves the axons naked in an

Figure 5. Ultrastructure of OEC ensheathment. Electron micrographs of transplanted OECs (N, nucleus) and associated large axons (X). A, full ensheathment of large axons, many tiny axon profiles (s, presumed sprouts) and concentric fibroblastic processes (arrows). B, high power view of OEC covered by basal lamina (arrowheads) to show mesaxonal arrangement (to the right of the figure), partially ensheathed axon (white asterisk), and naked axon (n) making contact with OEC. Scale bars, 1µm.

enlarged extracellular space. The axon profiles retain their uniform longitudinal orientation, i.e., their trajectories are not deflected, nor are the axons impinged upon by any connective tissue or other structures that might damage them physically.

Throughout the light period, the RGC axons are required to carry continuous heavy traffic of axon potentials over their long course from the retina to the brain. In the myelinated regions of the OpN, the energy dependent Na⁺ channels are restricted to the nodes of Ranvier. In their passage through the ONH, however, the segments of the RGC axons are unmyelinated.²⁴ In this region, they would not, therefore, have the internodal mechanism for restriction of Na⁺ channels, and the higher density of voltage gated Na⁺ channels in the unmyelinated prelaminar and laminar human optic nerve have been

shown.²⁵ This would imply that the axons have a considerably higher energy requirement^{26,27} over this part of their trajectory, and Hains and Waxman²⁸ reported a neuroprotective effect of Na⁺ channel blockade in a rat glaucoma model.

This increased energy requirement would be dependent on lactate delivered from the astrocytes,^{29,30} and may be one function of the giant mitochondria found in the radial ONH astrocytes (see also below). Therefore, in the experimental model, it is suggested that the initial effect of the raised IOP is the retraction of the astrocytic processes, and that the axon damage is secondary to loss of essential metabolic support following loss of contact with the astrocytes. A similar possibility was raised by others.^{1,5}

Migration and Survival of OECs in the ONH

Olfactory ensheathing cells transplanted into the retina migrate into the ONH.¹⁸ The system of intercellular fluid-filled spaces between the preterminal processes of the astrocytes in the region beneath the dorsal circumference of the ONH was illustrated previously (see Fig. 3 in Dai, Khaw, Yin, et al).⁹ Caudally these spaces are closed off by the densely interwoven mesh of optic nerve astrocytes. One possibility is that these spaces provide the channels through which the OECs migrate from the retina into the ONH.

Why do the transplanted OECs in the ONH survive the raised IOP? We have proposed that the cytoskeletal fortification of the radial ONH astrocytes, their tight intercellular junctions, which bind the individual cells into a rigid tissue array, their firm attachment to the dense ONH connective sheath, and their orientation at right angles to the pressure gradient make them vulnerable to raised IOP. In contrast, the OECs, which survive the raised IOP, are not cytoskeletally fortified, they travel as solitary cells, they are not attached to the ONH sheath, and they are oriented along the line of the pressure gradient, not across it.

Reduced Loss of RGC Axons in the Presence of OECs in the ONH

The current data showed that the loss of axons induced at 4 weeks after an acute rise in IOP was reduced by transplantation of OECs. Several potential, non-exclusive mechanisms for this benefit were identified.

Protection of the Axons

In the area from which the dorsal processes of the damaged radial ONH astrocytes were retracted, the naked axons were ensheathed by the transplanted OECs. As in the severed OpN,¹⁷ the transplanted OECs ensheathed the surviving RGC axons in the ONH in a strictly unmyelinated fashion. Compared with myelinated axons, this maximized the axonal surface in direct contact with glial cytoplasm, allowing for optimal transfer of energy metabolites such as lactate. The survival of these axons, therefore, may be a result of the OECs replacing the metabolic support formerly provided by the ONH astrocytes.

Protection of the Astrocytes

Results from this and a previous study⁹ indicated that the primary effect of the raised IOP was to stretch the fortified astrocytes to the point where their fine dorsal processes were torn away from their attachment to the dorsal ONH sheath. It was shown that transplanted OECs reduced the extent of this retraction of the astrocytic processes (Fig. 3B). This beneficial effect may be due to angiogenesis associated with the transplanted cells, and/or growth factors secreted by the OECs.^{31,32} Thus, as well as a direct effect of the OECs on the ensheathed axons, this would also have the effect of preserving more of the host astrocytic tissue providing the metabolic support for the axons.

Reduction of the IOP

In the current data the mean IOP in the transplanted groups 3 (33.7 ± 1.9) and 4 (35.2 ± 2.0) were slightly less than in the non-transplanted group 2 (37.3 ± 1.9). These differences did not reach significance ($P = 0.45$ and 0.81 , respectively) with the current amount of data. It seems unlikely that a difference of this magnitude could be sufficient to lead to a sparing of 30% of axons. However, more extensive data is needed to exclude the possibility that the injection/transplantation procedure might have a minor effect in reducing the IOP.

Potential Clinical Relevance

Progression

The clinical pattern of glaucoma is characterized by progressive damage to RGC axons. Previous rat data indicates that even after subsidence of an acute induced rise in IOP, axon loss continues.⁹ This correlates with the clinical observation that therapeutic interventions to correct an initially high IOP are only partially able to prevent continuing contraction

of the vision fields.^{3,5} With time, even as the IOP returns toward normal levels, results showed that once the dorsal astrocytic processes had been detached the astrocytic lesion continued to progress further ventrally, and the amount of ONH tissue as a whole was progressively reduced, so that the pressure gradient across the dwindling ONH was correspondingly steeper.

Field Deficit

Clinically, glaucoma typically progresses from initial peripheral field deficits.³³ In ocular hypertensive rodents, a selective loss of RGCs from the dorsal retina was reported.⁷ The observation made in the current study is that the initial damage to the ONH is the detachment of the fine dorsal astrocytic processes from the surrounding sheath, which is in agreement with the pattern of distribution of early damage reported by others.^{1,6} Because the RGC axons are distributed retinotopically in the ONH,^{34,35} the topographical progression of damage across the ONH suggests a potential basis for the observed progression of the visual field loss in the glaucoma.

Hypercompliance

In human and primate eyes, glaucoma is associated with hypercompliance at the ONH.³⁶ It is suggested that, in the absence of a connective tissue lamina cribrosa¹ in the rat, the equivalent of hypercompliance is destabilization of the array of fortified ONH astrocytes once their fine attachments are torn away from the dorsal periphery of the ONH.⁹ This seems to be an irreversible injury, since there has not been an indication that the astrocytes are able to regenerate these dorsal attachments. Once the dorsal astrocytic processes are detached, the whole astrocytic array becomes mechanically unstable.

Future Prospects

The observations described here along with those made by others^{9,17} establish an alternative to the current views of the mechanism and possible treatment of glaucoma. This suggests a number of future investigations of the biology of the energy dependence of the optic nerve axons on the fortified astrocytes, its potential replacement by transplanted OECs, and the kinetics of fluid transfer within the normal, glaucomatous, and transplanted ONH. The long-term time course and stability of the beneficial effects of OECs as well as the effect on functional parameters such as the STR component of the ERG³⁷ are being studied.

There will be many steps to clinical translation, but

the current biological observations provide the essential starting point that indicates the next steps to be taken. Controls will be needed for the different aspects of the surgery. In contrast to the current trans-scleral approach necessitated by the large size of the rat lens, clinical access for transplantation will almost certainly be under direct vision from the front of the eye.

In a definitive publication, Vidal-Sanz, Salinas-Navarro, and Nadal-Nicolas, et al.,⁷ document the effects of laser-induced ocular hypertension on retinal histology and the ERG. The magnitude and pattern of the acute rise and subsequent fall of the IOP in their study are similar to those in the current study with magnetic beads, although with a somewhat earlier recovery. The effects on the RGCs are documented in terms of retrograde axoplasmic flow, passive flow from the ONH, RGC specific antibodies, and neurofilament abnormalities. As in the current study, these authors conclude that the primary damage is at the level of the ONH. Comparable data on the RGCs was not available in the current study, nor was an equivalent longitudinal time series. A detailed comparison will be for a future study.

Lamina Cribrosa

Although rat ONH does not have a lamina cribrosa, comparable levels of ocular hypertension cause axonal degeneration similar to that in the laminated human and primate ONH. A mechanosensor function for the ONH astrocytes was demonstrated in tissue culture.^{38,39} In the rat ONH, the current study proposed that the fortified astrocytes were the mechanical transducers, that they were the target of the mechanical damage, that the degeneration of axons was secondary to the loss of astrocytic support, and that the re-ensheathment of the axons by transplanted OECs could protect the denuded optic nerve fibers.

Previously illustrated features of the rat ONH astrocytes suggest that the elasticity of the structure required to buffer the normal fluctuations of intraocular pressure is associated with a system of fluid-filled spaces between the fine preterminal astrocytic segments under the dorsal circumference.⁹ It is proposed that this buffer is maintained by a continuous highly energy-dependent process, evidenced by the numerous pinocytotic vesicles in the astrocytes and in the endothelial cells of the blood vessels, the giant mitochondria, and the absence of a blood-brain barrier leading to the higher permeability of the vasculature. The high pressure in the eye is main-

tained by the rigid sclera/corneal envelope, but at the point where the axons must leave this structure the deficit is made up by the less rigid ONH-fortified astrocytes whose integrity is an energy dependent function.

It seems unlikely that the human and primate ONH has abandoned this evolutionary mechanism in favor of an inert connective tissue lamina cribrosa. Astrocytic layers are interposed between the leaflets of the human lamina cribrosa.⁴⁰ The clinical relevance of the rat model will depend on whether it can be shown that comparable astrocytic damage is a factor in loss of human optic nerve fibers in glaucoma and, if so, to what extent the function of the lamina cribrosa may simply be to provide the further strengthening needed to resist stresses across the larger human and primate ONH.

The current rat data suggest that transplantation of autologous OECs in patients with progressive glaucomatous damage^{2,3} has the potential for devising novel future approaches for reducing or, perhaps, even reversing the loss of vision in glaucoma.

Acknowledgments

Funding was provided by the UK Stem Cell Foundation, Nicholls Spinal Injury Foundation, British Neurological Research Trust, Henry Smith Charity, National Natural Science Foundation of China (Grant No. 81070724/2011), Fight for Sight, Helen Hamlyn Trust, Michael Uren and the NIHR Biomedical Centre at Moorfields Eye Hospital, UCL Institute of Ophthalmology.

Stuart Law and Kerrie Venner provided superb support for the cell culture and electron microscopy, respectively.

Disclosure: **C. Dai**, None; **P.T. Khaw**, None; **Z.Q. Yin**, None; **D. Li**, None; **G. Raisman**, None; **Y. Li**, None

References

1. Morrison JC, Johnson EC, Cepurna W, Jia L. Understanding mechanisms of pressure-induced optic nerve damage. *Prog Retin Eye Res.* 2005;24: 217–240.
2. Kass MA, Heuer DK, Higginbotham EJ, et al. The Ocular Hypertension Treatment Study: a

- randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. *Arch Ophthalmol*. 2002;120:701–713.
3. Leske MC, Heijl A, Hussein M, Bengtsson B, Hyman L, Komaroff E. Factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial. *Arch Ophthalmol*. 2003;121:48–56.
 4. Kotecha A, Spratt A, Bunce C, Garway-Heath DF, Khaw PT, Viswanathan A. Optic disc and visual field changes after trabeculectomy. *Invest Ophthalm Vis Sci*. 2009;50:4693–4699.
 5. Musch DC, Gillespie BW, Lichter PR, Niziol LM, Janz NK. Visual field progression in the Collaborative Initial Glaucoma Treatment Study: the impact of treatment and other baseline factors. *Ophthalmology*. 2009;116:200–207.
 6. Morrison JC, Johnson E, Cepurna WO. Rat models for glaucoma research. *Prog Brain Res*. 2008;173:285–301.
 7. Vidal-Sanz M, Salinas-Navarro M, Nadal-Nicolas FM, et al. Understanding glaucomatous damage: anatomical and functional data from ocular hypertensive rodent retinas. *Prog Retin Eye Res*. 2012;31:1–27.
 8. Samsel PA, Kisiswa L, Erichsen JT, Cross SD, Morgan JE. A novel method for the induction of experimental glaucoma using magnetic microspheres. *Invest Ophthalm Vis Sci*. 2011;52:1671–1675.
 9. Dai C, Khaw PT, Yin ZQ, Li D, Raisman G, Li Y. Structural basis of glaucoma: the fortified astrocytes of the optic nerve head are the target of raised intraocular pressure. *Glia*. 2012;60:13–28.
 10. Graziadei PPC, Levine RR, Montgraziadei GA. Plasticity of connections of the olfactory sensory neuron: regeneration into the forebrain following bulbectomy in the neonatal mouse. *Neuroscience*. 1979;4:713–727.
 11. Moulton DG. Dynamics of cell populations in the olfactory epithelium. *Ann NY Acad Sci*. 1974;237:52–61.
 12. Schwob JE, Youngentob SL, Ring G, Iwema CL, Mezza RC. Reinnervation of the rat olfactory bulb after methyl bromide-induced lesion: timing and extent of reinnervation. *J Comp Neurol*. 1999;412:439–457.
 13. Li Y, Carlstedt T, Berthold C-H, Raisman G. Interaction of transplanted olfactory-ensheathing cells and host astrocytic processes provides a bridge for axons to regenerate across the dorsal root entry zone. *Exp Neurol*. 2004;188:300–308.
 14. Li Y, Field PM, Raisman G. Regeneration of adult rat corticospinal axons induced by transplanted olfactory ensheathing cells. *J Neurosci*. 1998;18:10514–10524.
 15. Ziegler MD, Hsu D, Takeoka A, et al. Further evidence of olfactory ensheathing glia facilitating axonal regeneration after a complete spinal cord transection. *Exp Neurol*. 2011;229:109–119.
 16. Au E, Richter MW, Vincent AJ, et al. SPARC from olfactory ensheathing cells stimulates Schwann cells to promote neurite outgrowth and enhances spinal cord repair. *J Neurosci*. 2007;27:7208–7221.
 17. Li Y, Sauv Y, Li D, Lund RD, Raisman G. Transplanted olfactory ensheathing cells promote regeneration of cut adult rat optic nerve axons. *J Neurosci*. 2003;23:7922–7930.
 18. Li Y, Li D, Khaw PT, Raisman G. Transplanted olfactory ensheathing cells incorporated into the optic nerve head ensheath retinal ganglion cell axons: possible relevance to glaucoma. *Neurosci Lett*. 2008;440:251–254.
 19. Cavalieri S, Cazzaniga S, Geuna M, et al. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood*. 2003;102:497–505.
 20. Ruitenberg MJ, Plant GW, Christensen CL, et al. Viral vector-mediated gene expression in olfactory ensheathing glia implants in the lesioned rat spinal cord. *Gene Ther*. 2002;9:135–146.
 21. Naldini L, Blomer U, Gally P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996;272:263–267.
 22. Zhang C, Szabo G, Erdelyi F, Rose JD, Sun QQ. Novel interneuronal network in the mouse posterior piriform cortex. *J Comp Neurol*. 2006;499:1000–1015.
 23. Jia L, Cepurna WO, Johnson EC, Morrison JC. Patterns of intraocular pressure elevation after aqueous humor outflow obstruction in rats. *Invest Ophthalm Vis Sci*. 2000;41:1380–1385.
 24. Hildebrand C, Remahl S, Waxman SG. Axo-glial relations in the retina-optic nerve junction of the adult rat: electron-microscopic observations. *J Neurocytol*. 1985;14:597–617.
 25. Barron MJ, Griffiths P, Turnbull DM, Bates D, Nichols P. The distributions of mitochondria and sodium channels reflect the specific energy requirements and conduction properties of the human optic nerve head. *Br J Ophthalmol*. 2004;88:286–290.
 26. Stys PK. General mechanisms of axonal damage and its prevention. *J Neurol Sci*. 2005;233:3–13.
 27. Andrews H, White K, Thomson C, et al. Increased axonal mitochondrial activity as an

- adaptation to myelin deficiency in the Shiverer mouse. *J Neurosci Res.* 2006;83:1533–1539.
28. Hains BC, Waxman SG. Neuroprotection by sodium channel blockade with phenytoin in an experimental model of glaucoma. *Invest Ophthalm Vis Sci.* 2005;46:4164–4169.
 29. Tsacopoulos M, Magistretti PJ. Metabolic coupling between glia and neurons. *J Neurosci.* 1996; 16:877–885.
 30. Herrero-Mendez A, Almeida A, Fernandez E, Maestre C, Moncada S, Bolanos JP. The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat Cell Biol.* 2009;11:747–752.
 31. Roet KC, Bossers K, Franssen EH, Ruitenberg MJ, Verhaagen J. A meta-analysis of microarray-based gene expression studies of olfactory bulb-derived olfactory ensheathing cells. *Exp Neurol.* 2011;229:10–45.
 32. Novikova LN, Lobov S, Wiberg M, Novikov LN. Efficacy of olfactory ensheathing cells to support regeneration after spinal cord injury is influenced by method of culture preparation. *Exp Neurol.* 2011;229:132–142.
 33. Quigley HA, Addicks EM. Regional differences in the structure of the lamina cribrosa and their relation to glaucomatous optic nerve damage. *Arch Ophthalmol.* 1981;99:137–143.
 34. Hoyt WF, Luis O. Visual fiber anatomy in the infrageniculate pathway of the primate. *Arch Ophthalmol.* 1962;68:94–106.
 35. Sun D, Lye-Barthel M, Masland RH, Jakobs TC. The morphology and spatial arrangement of astrocytes in the optic nerve head of the mouse. *J Comp Neurol.* 2009;516:1–19.
 36. Burgoyne CF, Downs JC, Bellezza AJ, Suh JK, Hart RT. The optic nerve head as a biomechanical structure: a new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. *Prog Retin Eye Res.* 2005;24:39–73.
 37. Alarcon-Martinez L de l V, Aviles-Trigueros M, Blanco R, Villegas-Perez MP, Vidal-Sanz M. Short and long term axotomy-induced ERG changes in albino and pigmented rats. *Mol Vis.* 2009;15:2373–2383.
 38. Salvador-Silva M, Aoi S, Parker A, Yang P, Pecan P, Hernandez MR. Responses and signaling pathways in human optic nerve head astrocytes exposed to hydrostatic pressure in vitro. *Glia.* 2004;45:364–377.
 39. Malone P, Miao H, Parker A, Juarez S, Hernandez MR. Pressure induces loss of gap junction communication and redistribution of connexin 43 in astrocytes. *Glia.* 2007;55:1085–1098.
 40. Anderson DR. Ultrastructure of human and monkey lamina cribrosa and optic nerve head. *Arch Ophthalmol.* 1969;82:801–814.