Transplanted Oligodendrocyte Precursor Cells Reduce Neurodegeneration in a Model of Glaucoma

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PURPOSE. Glaucoma is a common neurodegenerative disease for which current therapies are often insufficient; thus, new neuroprotective strategies are an important goal. Stem cells are attracting increasing attention as mediators of neuroprotection, often conferred via the trophic support of injured neurons. The purpose of our investigation was to determine whether oligodendrocyte precursor cells (OPCs), a type of neural stem cell, can protect retinal ganglion cells (RGCs) from glaucomatous damage in vivo.

METHODS. Intraocular pressure was chronically increased by trabecular laser treatment delivered unilaterally to adult rat eyes. OPCs were isolated in vitro and then transplanted intravitreally either before, or concurrent with, injury induction. Survival, migration, differentiation, and integration of grafted cells were assessed by immunohistochemistry. RGC survival was assessed by optic nerve axon quantification.

RESULTS. Transplanted OPCs were found to survive within the eye for at least 12 weeks and to localize close to the RGCs. Moreover, OPCs significantly enhanced the survival of RGCs in the glaucomatous eye, but only when concomitantly activated by inflammation. Axonal loss relative to the untreated fellow eye was 28.34% ± 11.51% in eyes that received activated OPCs, compared with 60.34% ± 8.28% in control eyes (mean ± SEM; $P = 0.05$). Amelioration of RGC death was not attributable to inflammation but relied on an interaction between inflammatory cells and OPCs. Engrafted cells also displayed multipotentiality in vivo.

CONCLUSIONS. The impressive neuroprotection conferred by OPCs in this model suggests stem cell–based therapies should be explored further as a potential treatment for glaucoma. (*Invest Ophthalmol Vis Sci.* 2009;50:4244–4253) DOI: 10.1167/iovs.08-3239

G laucoma is a common neurodegenerative disease characterized by the progressive death of retinal ganglion cells (RGCs). It shares pathophysiological features with other neurodegenerative diseases, including impairment of axonal transport,[1] oxidative stress,[2] and reactive glial changes.[3] Elevation of intraocular pressure (IOP) is the strongest risk factor,[4] and lowering of IOP is the only treatment that has been shown to reduce progressive visual loss in humans with the disease.[5] However, IOP reduction fails to arrest RGC degeneration in some patients with glaucoma, and therefore novel adjunctive treatments to protect RGC are an important goal.

Stem cells are attracting increasing attention as a potential neuroprotective therapy in degenerative CNS disease, where they commonly alleviate neurodegeneration via the supply of neurotrophic factors.[6-8] Oligodendrocyte precursor cells (OPCs) are abundant in the central nervous system (CNS), particularly in the adult where they are the most prevalent proliferative cell type.[9] In development, OPCs are responsible for the generation of oligodendrocytes, whereas in the adult they play a key role in the remyelination of axons in demyelinating pathologies.[9-11] Of interest, OPCs also appear to possess many stem cell characteristics[12,13] and have proven neuroprotective in vitro.[14]

We investigated whether OPCs could protect RGCs from glaucoma-induced death. To do so, we simulated glaucoma using an ocular hypertensive rat model.[15] OPCs were transplanted into the injured eye either before, or concomitant with, glaucoma induction and RGC survival was quantified after 4 weeks of ocular hypertension. In addition, the survival and behavior of OPCs within the glaucomatous eye were assessed.

MATERIALS AND METHODS

OPC Culture

OPCs were isolated as described previously.[16-18] Briefly, primary mixed glial cultures were generated from cortices dissected from postnatal day 0 Lewis rat pups and grown on poly-D-lysine (Sigma-Aldrich, Gillingham, UK) coated tissue culture flasks (Iwaki, Appleton Woods Laboratory Equipment and Consumables, Birmingham, UK) in DMEM (Invitrogen, Paisley, UK) containing 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). OPCs were isolated from the mixed glial culture by a series of mechanical shaking steps and then maintained in OPC expansion medium (DMEM containing 1% penicillin/streptomycin, 1% N2 [Invitrogen], 2% B27 [Invitrogen], 10 ng/mL PDGF [Sigma-Aldrich], and 20 ng/mL FGF [Sigma-Aldrich]) on poly-D-lysine coated tissue culture flasks for 1 to 2 days until used for transplantation. Microglia were cultured by using an established method[19] and assayed directly after isolation. OPCs were harvested from culture, resuspended in PBS and the number of viable cells quantified before transplantation using trypan blue exclusion. For delivery of dead OPCs, the cells were heated to between 60°C and 70°C for 10 minutes and cell death confirmed using trypan blue staining. OPC culture purity was confirmed by immunocytochemically testing for A2B5 expression,[20] using a mouse IgM anti-A2B5 antibody (1:250; Chemicon, Millipore, Watford, UK) and appropriate fluorescent secondary antibody (Invitrogen). Example photographs were captured at 100× magnification from seven separate coverslips and the percentage of DAPI+ cells that expressed A2B5 was quantified. Contamination of OPC cultures by microglial and astrocytic cells was assessed by staining cultures for ED1 and GFAP, respectively.

RNA Isolation and Semiquantitative RT-PCR

Total cellular RNA was extracted (RNeasy Mini isolation kit; Qiagen, Crawley, UK) from primary microglia or OPC cultures, both naïve and after a 24-hour exposure to zymosan A (derived from yeast cell wall protein-carbohydrate complexes; 0.1 mg/mL; Sigma-Aldrich). RNA samples (1 μg) were reverse transcribed to cDNA in 50 μL (Quantitect RT kit; Qiagen) according to the manufacturer's protocol. PCR was conducted in a 25 μL reaction volume using 1 μL cDNA with *Taq* polymerase (BioTaq; BioLine UK, Ltd., London, UK). The primers used were: rat hypoxanthine phosphoribosyl-transferase (rHPRT) forward: 5′-GCCCCAAAATGGTTAAGGTT-3′; rHPRT reverse: 5′-TCCACTTTCG-CTGATGACAC-3′; rTLR2 forward: 5′-CAGCTGGAGAACTCTGACCC-3′; rTLR2 reverse: 5′-CAAAGAGCCTGAAGTGGGAG-3′; rTNFα forward: 5′-ATGTGGAACTGGCGAGGAG-3′; and rTNFα reverse: 5′-TGGAACT-GATGAGAGGGAGC-3′.

To compensate for variable RNA and cDNA yields, the expression of HPRT was used as a control to determine the optimal number of PCR cycles for linear amplification. The amplification products were analyzed by agarose gel electrophoresis.

Animals

All animal experiments were conducted in accordance with the UK Home Office regulations for the care and use of laboratory animals, the U.K. Animals (Scientific Procedures) Act (1986), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Young adult (8 weeks) male Lewis rats (Charles River Laboratories or Harlan, UK) were used ($n = 83$). Animals had unrestricted access to food and water, and were maintained on a 12-hour light/dark cycle.

## Glaucoma Model and Intravitreal Cell Transplantation

Animals were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) plus xylazine (5 mg/kg), and local anesthetic drops were applied to the eye. Baseline IOPs were measured with a rebound tonometer (TonoLab; Tiolat Oy, Helsinki, Finland) factory calibrated for use in rats; all IOPs were recorded under light ketamine/xylazine anesthesia. Unilateral ocular hypertension was induced in the left eye ($n = 80$) by external translimbal treatment to the aqueous outflow area with a 532 nm diode laser as described previously.[15] Initial treatment was 50 to 60 spots of 50 μm diameter, 700 mW power, and 0.6 second duration. Laser treatment was repeated 1 week later if the difference in IOP between the left and right eyes was less than 10 mm Hg. After baseline IOP measurement (day 0), IOPs were recorded on days 1, 7, and 8, and weekly thereafter. The IOP integral (area under the curve of the IOP profile graph) for each animal was also calculated to estimate individual insult severity.

Intraocular transplantation of OPCs was performed either 8 weeks before, or concomitant with, glaucoma induction (Fig. 1). Cells were injected intravitreally, under direct visual observation, close to the RGC layer with a 30-gauge needle and 5 μL glass syringe (Hamilton, Reno, NV). Positioning was as described previously.[21] Stimulation of inflammatory cells, to activate OPCs, was achieved by intravitreal injection of 12.5 μg/eye zymosan A (Sigma-Aldrich), which was delivered together with the 3 μL of cell suspension. Animals were killed 4 weeks after the onset of ocular hypertension.

Experimental Groups

Transplantation of naïve OPCs was performed either at the time of glaucoma induction (acute group) or 8 weeks before (chronic group). A further group underwent transplantation of activated OPCs 8 weeks before glaucoma induction (activated group; Fig. 1A). In the acute OPC transplantation experiment, the animals received a uniocular 3 μL intravitreal injection of PBS alone ($n = 8$), live OPCs (3×10^4 cells in PBS, $n = 10$), or dead OPCs (3×10^4 cells in PBS, $n = 10$). In the chronic OPC transplantation experiment, rats received a 3 μL intra-
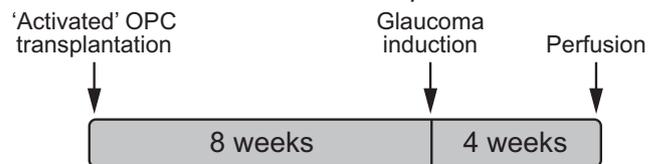
A. *Acute OPC transplant*
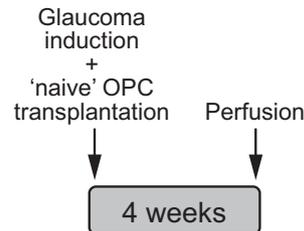
B. *Chronic OPC transplant*

C. *Chronic 'activated' OPC transplant*

FIGURE 1. Experimental outline. (**A**) Acute intravitreal transplantation of OPCs was performed at glaucoma induction and tissue was collected 4 weeks later. Animals received 3 μL vehicle (PBS), 3×10^4 dead or live OPCs in 3 μL vehicle. (**B**) For establishment of chronic grafts, OPCs were injected 8 weeks before glaucoma onset and the pathology was allowed to proceed for the following 4 weeks. Animals received 3 μL vehicle (PBS), 3×10^4 dead or live OPCs in 3 μL vehicle. (**C**) Activation of OPCs was achieved by injecting zymosan along with 3×10^4 OPCs in 3 μL PBS 8 weeks before the onset of 4 weeks' glaucoma. Control animals received vehicle (zymosan in 3 μL PBS).

vitreal injection of either PBS ($n = 10$), 3×10^4 live OPCs in PBS ($n = 9$) or 3×10^4 dead OPC in PBS ($n = 10$; Fig. 1B). For the activated OPC transplantations, rats received 3 μL of either vehicle solution (PBS containing zymosan, $n = 9$) or vehicle solution containing 3×10^4 live OPCs ($n = 9$; Fig. 1C). Three control rats did not undergo induction of glaucoma but received intravitreally activated OPC transplants 8 weeks before death.

Tissue Processing

Animals were perfused under terminal anesthesia with 0.1 M PBS followed by 4% paraformaldehyde/0.1 M PBS (PFA). For immunohistochemical analysis, tissue was postfixed by immersion in 4% PFA for 2 hours. Posterior eyecups were cryopreserved with 30% sucrose and embedded in optimal cutting temperature compound (OCT; Raymond A. Lamb UK, Eastbourne, UK) for frozen sectioning at 40 μm. Flat-mounted retinas were immunohistochemically labeled floating before mounting onto glass slides.

For assessment of RGC axonal loss, the optic nerves were immersed in 4% PFA/5% glutaraldehyde/0.1 M phosphate buffer for 7 days at 4°C, postfixed in 1% osmium tetroxide for 3 hours, dehydrated, and embedded in Araldite resin for semithin sectioning. Semithin (1 μm) transverse sections were cut from the nerve 2 to 3 mm distal to the globe, dried onto slides, and stained with 1% toluidine blue.

Immunohistochemistry

Sections were initially washed with PBS and then blocked with PBS containing 0.2% Triton (PBS-T) plus 5% normal goat serum (Invitrogen). All antibodies were diluted in this blocking solution. Sections

were incubated in primary antibodies overnight at room temperature. After thorough washing with PBS, appropriate fluorescent goat secondary antibodies (Invitrogen) were applied to the sections for 3 hours at room temperature. Slides were washed, counterstained with DAPI (Invitrogen) and coverslipped (FluorSave antifade medium; Calbiochem/Merck Chemicals, Beeston, UK).

For immunohistochemical analysis of flatmounted retinas, the retinas were washed three times for 30 minutes in PBS and then blocked in the solution specified above for 2 hours at room temperature. With constant gentle shaking, the retinas were incubated in primary antibodies for 24 hours at 4°C, washed three times for 30 minutes with PBS at room temperature, probed with appropriate fluorescent goat secondary antibodies (Invitrogen) for 24 hours at 4°C, washed again as before, flatmounted onto glass slides and coverslipped (FluorSave reagent; Calbiochem/Merck Chemicals).

The following primary antibodies were used: mouse IgG anti-βIII tubulin (1:2000; Promega, Southhampton, UK); mouse IgG1 anti-ED1 (CD68; 1:500; Chemicon, Millipore); rabbit polyclonal anti-GFAP (1:1000; DakoCytomation, Ely, UK); mouse IgG1 anti-GFAP-Cy3 conjugated (clone G-A-5; 1:500; Sigma-Aldrich); mouse IgG1 anti-Ki67 (MIB-5 clone; 1:25; DakoCytomation); rabbit polyclonal anti-Olig2 (1:1000; Chemicon); rat IgG2a anti-myelin basic protein (1:100; AbD Serotec, Kidlington, UK); and mouse IgM anti-polysialic acid-neural adhesion molecule (PSA-NCAM; 1:1000; Chemicon).

Optic Nerve Axon Quantification

Loss of RGC axons in the optic nerves of glaucomatous eyes was assessed using a modification of a previously developed semiquantitative optic nerve grading scheme.[22] However, instead of estimating damage via visual grading, we used axon counts from representative images of each zone of damage to estimate total ON damage. To do this, areas of approximately equal damage were identified under light microscopy (100× magnification), and the contribution of each zone to total ON cross-sectional area was determined. A representative photograph was captured at 630× magnification within each zone and the number of axons within each sample image was counted using the particle analysis/nucleus counter plug-in (ImageJ plug-in bundle, provided in the public domain by Wright Cell Imaging Facility, University Health Network Research, Canada; http://www.uhnresearch.ca/facilities/wcif/fdownload.html) to the image analysis software ImageJ (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD http://rsb.info.nih.gov/ij/index.html). The number of axons within each damaged zone was compared to the count obtained from a sample image of the uninjured companion eye to estimate the percentage of axonal survival. A weighted average calculation, as per the previously established method,[22] was then used to estimate the percentage of surviving axons in the total ON.

RESULTS

Characterization of Cells Cultured for Transplantation

OPC cultures were analyzed for expression of A2B5 to determine culture purity before transplantation (Fig. 2A). A2B5 is a cell surface antigen expressed by immature OPCs in culture.[23] Immunocytochemical analysis demonstrated that 93.9% ± 2.9% (mean ± SD; $n = 7$) of cells transplanted were OPCs. In addition, A2B5+ cells displayed an appropriate OPC-like morphology in culture (Fig. 2B). Immunocytochemical labeling of either ED1 or GFAP in OPC cultures demonstrated a 2.37% ± 1.9% (mean ± SD) contamination by microglia and 2.34% ± 0.9% (mean ± SD) contamination by astrocytes, respectively. Parallel staining in microglial cultures revealed 1.15% ± 1.2% (mean ± SD) contamination by OPCs and 0.84% ± 0.6% (mean ± SD) contamination by astrocytes.

Zymosan A is an agonist for toll-like receptor 2 (TLR2), through which expression of proinflammatory cytokines such
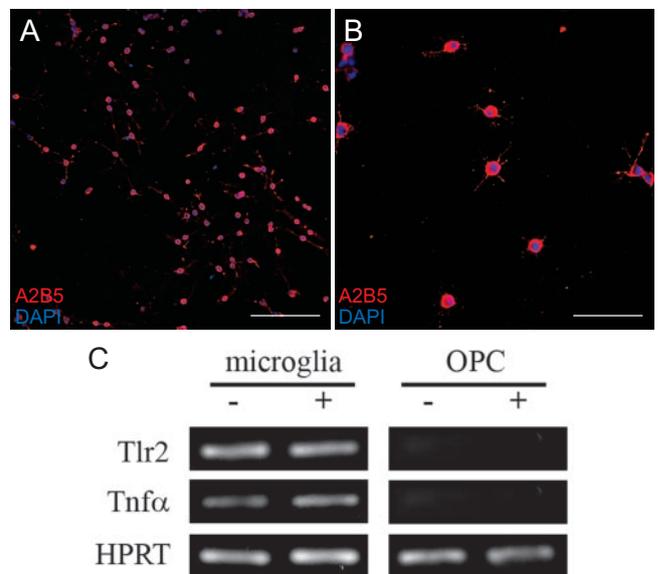
FIGURE 2. Characterization of OPCs cultured for transplantation. (A) Example of OPCs in culture immunocytochemically labeled for A2B5 expression (*red*) and counterstained with DAPI (*blue*). (B) Typical morphology of A2B5+ OPCs (*red*) in culture prior to transplantation. Counterstained with DAPI (*blue*). (C) Expression of TLR2 and TNFα by OPC and microglial cultures, both naïve (−) and after 24 hours exposure to 0.1 mg/mL zymosan A (+), as assessed by RT-PCR. HPRT was used to equalize reaction product across samples. Scale bar: (A) 100 μm; (B) 50 μm.

as tumor necrosis factor (TNF)-α can be triggered.[24–26] RT-PCR analysis demonstrated that cultured microglia expressed toll-like receptor 2 (TLR2), whereas cultured OPCs did not (Fig. 2C). Furthermore, expression of TNFα was detected in microglia cultures, but not in OPC cultures. Exposure of microglia to zymosan slightly upregulated TNFα expression but failed to stimulate OPC expression of this cytokine.

Effect of Intraocular Injections and Transplantation on Glaucoma Model

Induction of experimental glaucoma and resultant ON damage was achieved as described previously using an ocular hypertensive model.[15] IOP was elevated in all laser-treated eyes (Fig. 3). There was no difference in peak IOP or integral IOP, a measure of IOP exposure over time previously shown to correlate most closely with optic nerve damage,[15] between treatment groups within each experiment (Table 1). Peak IOP measurements were similar in the chronic OPC and activated OPC transplantation experiments, in which laser treatment commenced at 16 weeks of age, but significantly lower in the acute OPC transplantation experiment, in which glaucoma was induced at 8 weeks of age. Age has been noted as a risk-modifying factor for experimental ocular disease[27] and is well known to play a role in clinical glaucoma.

Acute Intravitreal Transplantation of Naïve OPCs Is Not Neuroprotective in Glaucoma

Quantification of optic nerve axonal survival after 4 weeks' exposure to ocular hypertension demonstrated that intravitreal transplantation of OPCs at glaucoma induction did not offer protection for RGCs from glaucomatous death (Fig. 4A). However, immunohistochemical analysis revealed that a large number of OPCs, positive for the nuclear marker Olig2, survived in the vitreous, proximal to the inner retinal surface, of the glaucomatous eye for up to 4 weeks (Figs. 4B, 4C). The number
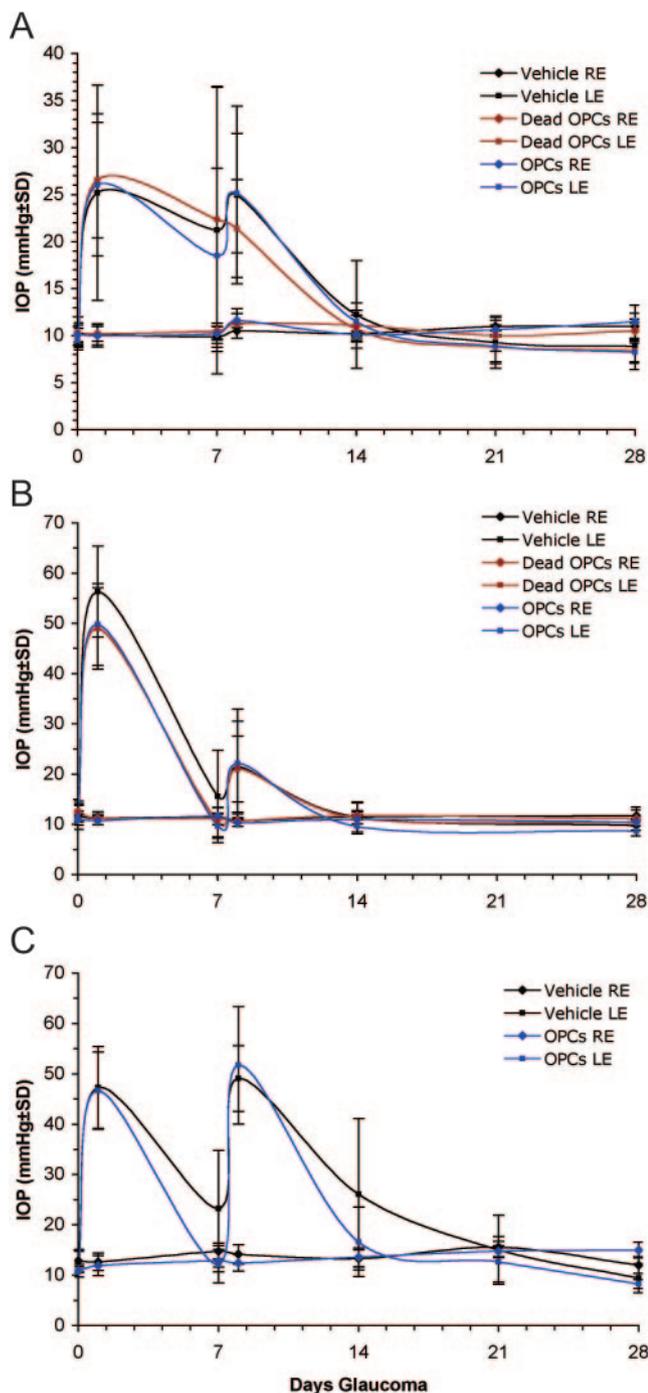
FIGURE 3. Intravitreal transplantation of OPCs did not affect intraocular pressure changes following ocular hypertension induction. (**A**) IOP profiles in animals that received acute transplantation of OPCs concomitant with glaucoma induction. (**B**) IOP profiles of rats following chronic intraocular transplantation of OPCs 8 weeks prior to glaucoma induction. (**C**) IOP profiles of rats that received intraocular transplantation of activated OPCs 8 weeks prior to glaucoma induction. LE, left eye; RE, right eye.

of Olig2$^+$ intravitreal cells was estimated at 48.3% ± 32.3% (mean ± SD), although accurate fate mapping was not possible given that the transplanted cells did not possess a definitive marker. Identification of cells expressing the macrophage/monocyte marker ED1 confirmed that intravitreal Olig2$^+$ cells were not nonspecifically labeled inflammatory cells (Fig. 4C).

Few OPCs differentiated into myelinating oligodendrocytes that expressed myelin basic protein (MBP; Fig. 4D, arrow) when transplanted acutely into the glaucomatous eye. This finding was not unexpected, given that it has been reported that an inflammatory signal is necessary for the stimulation of OPC myelination of RGC axons within the eye.[18] However, MBP immunohistochemistry did confirm oligodendroglial differentiation of engrafted cells, which were found proximal to immature OPCs labeled for Olig2. The MBP antibody also labeled patches of globular material (Fig. 4D, top left) within the vitreous that did not display an oligodendrocyte-like morphology. The identity and origin of this material is unclear; however, it may be myelin debris from degenerating, myelinated axons.

Immunohistochemical probing for expression of the neuronal marker βIII tubulin, which was used to identify inner retinal neurons, revealed that some acutely engrafted cells initiated neuronal differentiation in the glaucomatous eye (Figs. 4E–G). Quantification revealed that 10.6% ± 4.4% (mean ± SD) of intravitreal cells were βIII tubulin$^+$, although accurate quantification was not possible given that the transplanted cells did not possess a definitive marker. βIII tubulin$^+$ cells with a neuronal-like morphology (small cell body with fine processes; arrows) were identified anterior to the host retina (background βIII tubulin$^+$ axons) and juxtaposed to clusters of engrafted Olig2$^+$ OPCs (Fig. 4E). Intravitreal location of these cells, clearly separate from host retinal neurons, was confirmed in transverse posterior eyecup sections where βIII tubulin$^+$ engrafted cell bodies and neurites were observed anterior to the inner retinal surface (Fig. 4F, arrows). The neuronal-like morphology of these cells can be observed in Figure 4G (arrow). However, despite robust βIII tubulin expression, engrafted cells did not express the immature neuroblast marker PSA-NCAM (data not shown).

Acutely engrafted cells also initiated astrocyte differentiation in the glaucomatous eye (Figs. 4H, 4I). Immunohistochemical labeling for the astrocyte marker GFAP demonstrated that intravitreally engrafted cells readily differentiated into astrocytes in the injured eye. These cells also displayed an astrocytic-like morphology (Fig. 4I; arrow). The number of GFAP$^+$ intravitreal cells was estimated at 27.5% ± 26.7% (mean ± SD).

TABLE 1. Average Left Eye Peak IOPs and IOP Integrals for Each Experimental Glaucoma Group

Group	LE Peak IOP (mm Hg)	LE IOP Integral
Acute OPC transplantation		
Vehicle	33 ± 3.8*	430 ± 28
Dead OPCs	32 ± 2.5*	410 ± 15
OPCs	28 ± 2.3*	410 ± 19
Chronic OPC transplantation		
Vehicle	57 ± 2.7	520 ± 22
Dead OPCs	49 ± 2.6	470 ± 17
OPCs	50 ± 2.7	450 ± 23
Activated OPC transplantation		
Vehicle	51 ± 1.6	730 ± 56†
OPCs	54 ± 3.2	620 ± 39†

Data are expressed as the mean ± SEM. There was no significant difference in peak IOP or integral IOP between treatment groups within each experiment.

* One-way analysis of variance (ANOVA) with Tukey post hoc test (95% CI) revealed that the acute OPC transplantation group had significantly lower left eye (LE) peak IOPs than did the other experimental groups ($P < 0.05$).

† The integral IOP of the activated OPC transplantation group was significantly higher than in other experimental groups ($P < 0.05$).
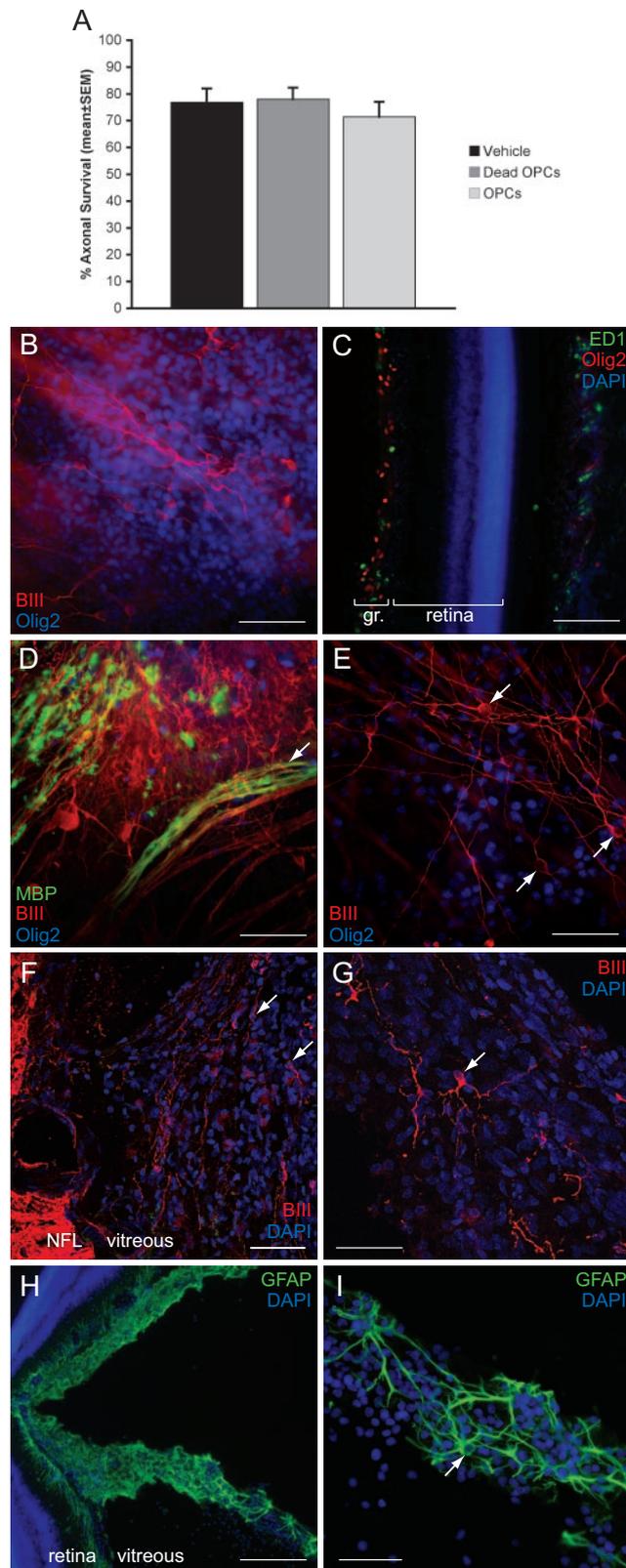
FIGURE 4. Acute intravitreal transplantation of OPCs was not neuro-protective in glaucoma, however, engrafted cells survived and initiated differentiation. (**A**) RGC axonal survival following acute intravitreal transplantation of OPCs concomitant with glaucoma induction. One-way ANOVA (Tukey post-hoc test) showed no significant difference between treatment groups. (**B**) Large clusters of OPCs survived up to 4 weeks within the vitreous, proximal to the inner retinal surface, following acute intravitreal transplantation. OPCs were immunohisto-chemically labeled for Olig2 (*blue*; nuclear) and inner retinal neurons/axons were counterlabeled for βIII tubulin in flatmounted retinas (*red*). (**C**) Immunohistochemical labeling of transverse retinal sections with the macrophage/monocyte marker ED1 (*green*) confirmed that intravitreally transplanted cells immunoreactive for Olig2 (*red*) were OPCs and not cross-reactivity of the Olig2 antibody with immune cells. Sections were counterstained with DAPI (*blue*). The engrafted cells (gr.) were observed to line large areas of the inner retinal surface but did not integrate into the neural tissue. (**D**) Few transplanted OPCs differentiated into oligodendrocytes as revealed by staining for MBP (*green*; *arrow*); inner retinal neurons/axons were counterstained for βIII tubulin (*red*) and immature OPCs were identified by Olig2 immu-noreactivity (*blue*) in flat-mounted retinas. Patches of globular material within the vitreous, possibly myelin debris from degenerating axons, also labeled for MBP. (**E**) Cells transplanted intravitreally at the onset of glaucoma induction also initiated neuronal differentiation. These cells displayed a neuronal-like morphology and expressed the neuronal marker βIII tubulin (*red*), where undifferentiated OPCs were identified by Olig2 expression (*blue*) in flat-mounted retinas. These cells were always found proximal to clusters of immature OPCs and anterior to the inner retinal surface (which can be seen in the background of this image). The clear separation of their cell bodies (*arrows*) from the inner retina precluded a retinal origin for these cells. (**F**) Immunohis-tochemical labeling of transverse retinal sections confirmed that intra-vitreally engrafted cells, identified by their positioning anterior to the inner retinal surface (DAPI; *blue*), initiated neuronal differentiation and expressed the neuronal marker βIII tubulin (*red*). These βIII tubulin$^+$ cells also displayed fine, neurite-like processes and their cell bodies (*arrows*) were clearly separate from the endogenous neural retina. βIII tubulin was also expressed by RGC axons in the nerve fiber layer (NFL). (**G**) An example of the neuronal-like morphology displayed by engrafted cells that initiated neuronal differentiation, as identified by βIII tubulin immunoreactivity (*red*) and intravitreal location. The sec-tions were counterstained with DAPI (*blue*). (**H**) Engrafted cells also differentiated toward the astrocytic lineage in the vitreous, as revealed by immunoreactivity for GFAP (*green*). Retinal sections were counter-stained with DAPI (*blue*). (**I**) An example of the astrocyte-like mor-phology (*arrow*) exhibited by intravitreally engrafted cells positive for GFAP (*green*), the nuclear counterstain was DAPI (*blue*). Scale bar: (**B**, **D–G**, **I**) 50 μm; (**C**) 100 μm; (**H**) 200 μm.

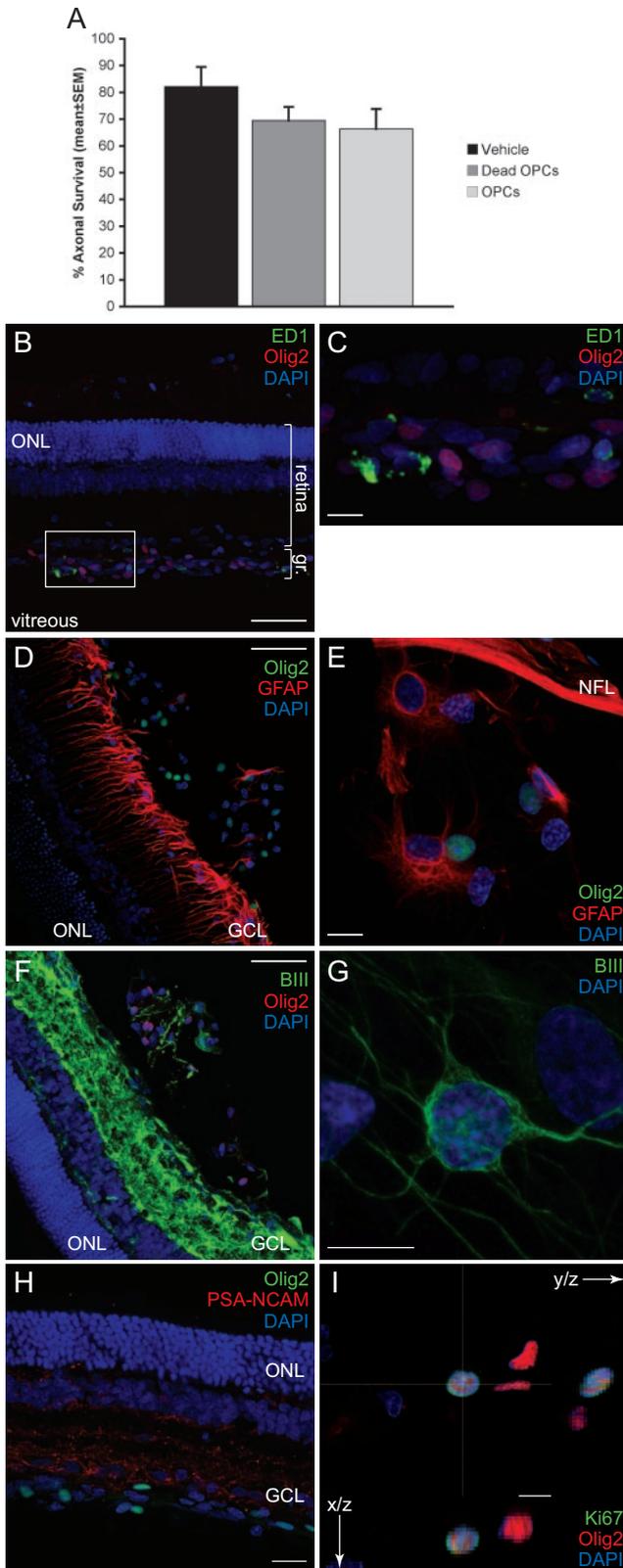## Chronic Intravitreal Transplantation of Naïve OPCs Is Not Neuroprotective in Glaucoma

Intravitreal transplantation of OPCs 8 weeks before glaucoma induction did not protect RGCs from injury-induced death, as determine by quantification of surviving axons in the optic nerve (Fig. 5A). However, it was confirmed that many en-grafted Olig2$^+$ OPCs were able to survive for the duration of the experiment, 12 weeks in total, in the vitreous adjacent to the inner retinal surface (Fig. 5B). ED1 immunohistochemical staining revealed that there was minimal infiltration of the intravitreal graft by macrophages/monocytes (Fig. 5B). ED1$^+$ cells are shown at higher magnification in Figure 5C (enlarge-ment of box in Fig. 5B).

In addition, intravitreally engrafted cells were found to express the astrocyte marker GFAP (Fig. 5D). Closer examina-tion of these cells, which were situated close to the inner retinal surface within the graft bolus and juxtaposed with Olig2$^+$ OPCs, revealed an astrocytic-like morphology (Fig. 5E).

Some chronically engrafted cells also expressed the neuro-nal marker βIII tubulin (Fig. 5F). The location of these cells within intravitreal Olig2$^+$ OPC grafts, clearly separate from the retina, is strongly suggestive of a nonhost origin. Cells within the intravitreal grafts that were immunopositive for βIII tubulin expression also displayed a neuronal-like morphology with multiple, fine processes extending from the cell body (Fig. 5G).

Despite demonstrating immunoreactivity for the neuronal marker βIII tubulin, which can be expressed both early and late in the neuronal lineage, no engrafted cells were found to

express the neuroblast marker PSA-NCAM (Fig. 5H). Faint staining of the inner retina was observed with the PSA-NCAM antibody, which was abolished when the primary antibody was omitted. Furthermore, engrafted OPCs did not express the mature neuronal marker neurofilament 160 (data not shown).
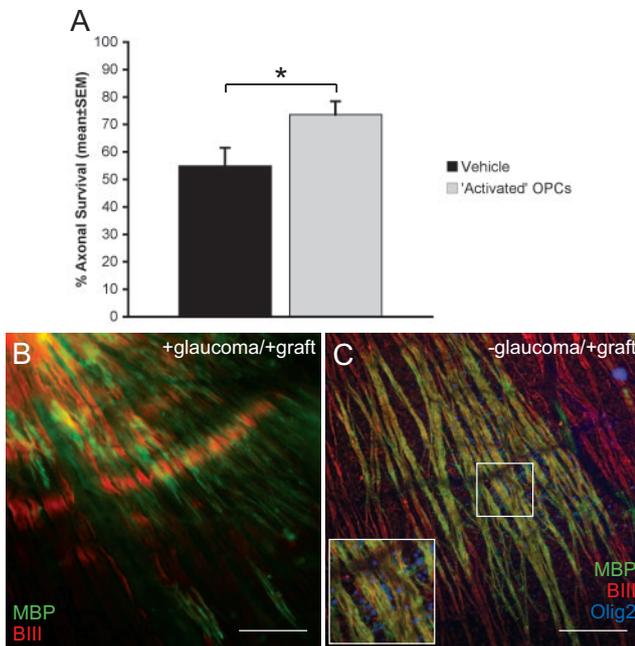
Immunohistochemical investigation of actively cycling cells with the marker Ki-67, revealed that a small number of chronically engrafted Olig2^{+} OPCs were proliferating within the vitreous of glaucomatous eyes (Fig. 5I). Confocal analysis confirmed colocalization of the cell cycle marker Ki-67 with nuclear expression of Olig2 within transplanted cells, 12 weeks after intravitreal injection. Quantification determined that $2.67\% \pm 1.1\%$ (mean $\pm$ SD; $n = 10$ sections) of Olig2^{+} cells coexpressed Ki-67.

OPC Transplantation Concomitant with Inflammatory Activation Protects Retinal Ganglion Cells from Glaucomatous Death

It has been shown that in vivo inflammatory stimulation, with zymosan, can activate transplanted OPCs over a period of 8 weeks in vivo and modify their behavior.[18] Using a similar inflammatory stimulus in the present study, optic nerve axon quantification revealed that activated OPCs offered significant neuroprotection to RGCs from a glaucomatous injury (Fig. 6A). In fact, this treatment protected approximately half of the neurons killed by a comparable injury (Table 1). Moreover, activated OPCs conferred long-term protection as they were transplanted 8 weeks prior to the onset of glaucoma. Zymosan-treated eyes were used for control comparisons in order to determine whether OPCs can protect RGCs in glaucoma as intravitreal injection of zymosan has been reported to influence RGC axon regeneration.[28]

As observed previously,[18] activation of OPCs via stimulation of inflammatory cell activity enhanced their differentiation into oligodendrocytes that expressed MBP and possibly myelinated

for 12 weeks in vivo. Immunohistochemical staining for the macrophage/monocyte marker ED1 (*green*) demonstrated that Olig2^{+} staining did not erroneously label reactive immune cells. DAPI counterstain (*blue*). (**C**) Magnified view of *boxed area* in (**B**). ED1^{+} cells (*green*) were clearly separate from Olig2^{+} OPCs (*red*). DAPI counterstain (*blue*). (**D**) Some chronically engrafted cells were immunoreactive for the glial marker GFAP (*red*), while double-staining for Olig2 (*green*) confirmed that these cells were contained within intravitreal grafts of OPCs. Müller cells within the inner retina were also immunoreactive for GFAP. DAPI counterstain (*blue*). (**E**) An example of the astrocytic-like morphology displayed by GFAP[+] (*red*) cells within the chronic Olig2^{+} (*green*) intravitreal OPC graft, adjacent to the retinal nerve fiber layer (NFL). DAPI counterstain (*blue*). (**F**) Cells transplanted 8 weeks before glaucoma induction were also observed to generate neuronal-like cells immunoreactive for the neuronal marker βIII tubulin (*green*). The inner retinal neurons were also positive for βIII tubulin expression. Intravitreal OPC grafts were identified with Olig2 (*red*) immuno-histochemistry and retinal sections were counterstained with DAPI (*blue*). (**G**) An example of the neuronal-like morphology displayed by βIII tubulin[+] (*green*) cells within the chronic intravitreal OPC graft. The cell nuclei were counterstained with DAPI (*blue*). (**H**) Intravit-really engrafted OPCs, adjacent to the RGC layer (GCL), did not demonstrate immunoreactivity for the neuroblast marker PSA-NCAM (*red*). Faint staining of the inner retina was observed for this antigen. Chronic intravitreal OPC grafts, anterior to the GCL, were identified by staining for Olig2 (*green*) and sections were counterstained with DAPI (*blue*). (**I**) Some chronically engrafted OPCs, positive for Olig2 (*red*), expressed the cell cycle marker Ki67 (*green*). Nuclear colocalization of Olig2 and Ki67, with the nuclear counterstain DAPI (*blue*), was confirmed visually via *x/z* and *y/z* projection of an optically sectioned confocal *z*-stack. ONL, outer nuclear layer; GCL, ganglion cell layer. Scale bar: (**B**, **D**, **F**) 50 μm; (**C**, **H**) 20 μm; (**E**, **G**, **I**) 10 μm.

Figure 5. Chronic intravitreal transplantation of OPCs was not neuroprotective in glaucoma, however, engrafted cells survived, proliferated and initiated differentiation. (**A**) RGC axonal survival after intravitreal transplantation of OPCs 8 weeks prior to induction of glaucoma. One-way ANOVA (Tukey post hoc test) showed no significant difference between treatment groups. (**B**) Olig2 immunoreactivity (*red*) revealed OPCs transplanted 8 weeks before glaucoma induction distributed across the inner surface of the retina (graft, gr.) and survived

A

B   +glaucoma/+graft

C   -glaucoma/+graft

MBP
BIII

MBP
BIII
Olig2

FIGURE 6.   Chronic intravitreal transplantation of activated OPCs was neuroprotective in glaucoma. (A) RGC axonal survival after intravitreal transplantation of OPCs and zymosan, which stimulates immune cell activity, 8 weeks prior to glaucoma induction. Significantly (*$P < 0.05$) more RGC axons survived in glaucomatous eyes that received activated OPCs, compared to those that received vehicle alone (unpaired, two-way Student's *t*-test). (B) Activated OPCs were capable of differentiating into mature oligodendrocytes, immunopositive for myelin basic protein (MBP; *green*), which extended processes along RGC axons in the glaucomatous eye (labeled for βIII tubulin in the flat-mounted retina; *red*). (C) Activated OPCs were also capable of differentiating into mature oligodendrocytes that expressed MBP (*green*) and aligned with RGC axons (βIII tubulin; *red*) in the non-glaucomatous eye. Immunohistochemical labeling of Olig2 (*blue*; box magnified in the *inset*) confirmed that OPCs survived for 12 weeks in the eye following concomitant injection with the inflammatory stimulator zymosan. Scale bar, 100 μm.

RGCs within the eye (Figs. 6B, 6C). A similar pattern of MBP staining has been demonstrated, as confirmed by electron microscopy, to be indicative of myelination by OPCs in the retina using this transplantation model.[18] Injury did not appear to influence the extent of this behavior, as levels were comparable in glaucomatous (Fig. 6B) and nonglaucomatous retinas (Fig. 6C). Furthermore, immunohistochemical labeling of engrafted cells for Olig2 expression (Fig. 6C, inset) confirmed that activated OPCs readily survived within the hypertensive eye for extended durations.

DISCUSSION

Transplanted OPCs Can Protect Neurons from Degeneration In Vivo

In the present study, we found that intravitreal transplantation of OPCs protected RGCs from glaucoma-induced death in vivo, but only after the engrafted cells had been activated by concomitant stimulation of inflammatory cells. Impressively, a single injection of OPCs was found to protect neurons from an insult delivered 8 weeks later. Analysis of data from control groups revealed that inflammation alone did not mitigate RGC loss, as OPCs were found to protect significantly more axons than treatment with zymosan-containing vehicle alone. Thus, we can infer that engrafted OPCs were responsible for reducing RGC loss, rather than neuroprotection arising as a side

effect of inflammatory processes. This is a key point, as cytokine-activated astrocytes can support injured neurons[29] and ocular inflammation, including that triggered by intravitreal delivery of zymosan, can potentiate axonal regeneration in the optic nerve.[28,30-32] However, inflammatory stimulation was necessary to elicit OPC-mediated neuroprotection as we also found that naïve OPCs injected into the eye did not alleviate glaucomatous neurodegeneration. Together, these results indicate that neuroprotection was mediated by the engrafted OPCs and it was triggered by a signal, or signals, transmitted by reactive immune cells. It seems unlikely that OPCs responded directly to zymosan given they appear to lack TLR2, the innate receptor responsible for detecting zymosan, and failed to respond to zymosan exposure in vitro. This conclusion supports other reports that stimulation of inflammatory cells can influence OPC behavior in vivo.[18,33-36] In contrast to OPCs, microglia expressed TLR2 and its downstream effector TNFα, as reported previously.[24,37] Given the plethora of factors secreted by activated immune cells, it is likely that OPC activation was communicated via a diffusible signal. However, it is also possible that the signal was contact-mediated, as invading inflammatory cells were observed close to engrafted cells. The factors that promote activation of OPCs by inflammatory cells are yet to be fully elucidated, but may involve reactivation of an earlier developmental phenotype.[38] Identification of this signaling cascade may have important implications in the treatment of demyelinating diseases, such as multiple sclerosis, as inflammation appears to play a key role in triggering remyelination by endogenous OPCs.[33,39-41] Such a discovery may also facilitate exploitation of OPCs for future stem cell based therapies in neurodegenerative diseases.

As reported previously,[18] inflammation was also found to enhance expression of MBP by OPCs in vivo and possibly OPC-mediated myelination of RGC axons, which are normally unmyelinated within the retina. Indeed, without inflammatory signals, OPCs differentiated poorly into mature oligodendrocytes within the eye. We observed less OPC differentiation into MBP-expressing cells within the retina than reported earlier. It is unclear why this difference in myelin production was found, however it may be due to the use of differing breeds of rats, as strain differences in inflammatory response and protective autoimmunity have been documented.[42-47] This relative lack of oligodendrocytic differentiation suggests that the neuroprotection conferred by OPCs was transmitted by a mechanism other than myelin-mediated support of axons. In addition, OPCs did not migrate into the neural tissue and were not located in direct contact with inner retinal neurons, instead remaining within the vitreous, separate from the retina. These results intimate that OPC mitigation of glaucomatous RGC death was mediated via the supply of diffusible trophic factors. This conclusion is supported by previous reports that OPCs can enhance neuronal survival in vitro via the provision of neurotrophic factors such as IGF-1 and GDNF.[14,48] Indeed, many investigators have now reported that a variety of stem cell types can alleviate neurodegeneration in an assortment of neuropathologies in vivo. This outcome may be achieved in several ways, including inherent provision of neurotrophic support factors[6,8,14,48-50] and by immunomodulatory mechanisms.[7,51-53]

Transplanted OPCs Persist Chronically In Vivo and Confer Long-Term Neuroprotection in Disease

OPCs injected into the vitreous of both the uninjured and glaucomatous eye were found to survive well in all experiments. Moreover, engrafted OPCs were observed to disperse across the inner retinal surface, placing them in the ideal

location for mediating the observed neuroprotection. The number of engrafted cells was less in chronic grafts, where OPCs had been in vivo for 12 weeks, compared with acute grafts, in vivo for only 4 weeks, however very little immune rejection of the transplanted cells was observed. Long-term survival of healthy engrafted OPCs may have been facilitated by their observed low-level in vivo proliferation, which may have maintained their intravitreal population. No difference in behavior between short- and long-term grafts was observed and neuroprotection was observed only after transplantation concomitant with stimulation of inflammatory cells.

Transplanted Precursor Cells Can Proliferate and Differentiate into Multiple Neural Lineages In Vivo

Interestingly, engrafted cells were found to initiate neuronal and glial differentiation within the glaucomatous eye, both acutely and chronically. As expected, OPCs possessed the ability to differentiate into MBP-expressing oligodendrocytes within the retina.[18] Furthermore, transplanted cells also demonstrated a propensity to differentiate into GFAP[+] astrocytic-like cells within the eye. OPCs are known to differentiate into astrocytes,[54] especially in the presence of serum[55,56] or BMP.[57] Astrocytic changes have been thought to play an important role in the development of glaucomatous optic neuropathy, and the presence of reactive astrocytes at the optic nerve head has been associated with disease progression in animal models.[58] On this basis, the logic of transplanting cells with the potential to produce reactive astrocytes into the eye could be questioned. However, the detrimental effects mediated by native astrocytes at the optic nerve head in glaucoma, including effects on local homeostasis and the integrity of neural and connective tissue, appear to be highly localized.[59] Transplanted cells in our study did not localize to the optic nerve head, and thus it is not surprising that they had a different effect.

Engrafted cells were also found to express βIII tubulin within the injured eye and displayed a neuronal-like morphology, suggestive of neuronal differentiation. As the engrafted cells were not definitively traceable, precise OPC tracking in vivo was not possible. However, given the clear intravitreal location of their cell bodies and processes, and their consistent juxtaposition to undifferentiated OPCs, it seems likely that these neuronal-like cells originated from the transplanted cells. Despite being of glial origin, OPCs have been reported previously to generate neurons in vitro, in vivo and after transplantation.[12,54,60–62] Moreover, the differences between oligodendroglia and neurons are becoming blurred with the observation that OPCs receive input from excitatory synapses[63] and the recent discovery of OPC action potential generation within the brain.[64] Together with these previous findings, our results support the view that OPCs are not restricted progenitor cells, as originally thought, but multipotential stem cells.[13]

The abundance of both undifferentiated OPCs and graft-derived astrocytes within the vitreous of glaucomatous eyes suggests that either cell type may be responsible for the observed amelioration of neuronal loss. Indeed, OPCs are reported to protect neurons via the provision of trophic support[14,48,65] and astrocytes are well known to provide neuronal energy support and trophic factors (for a review, see Ref. 29). Astrocytes have also been implicated in promoting the production of neurons from OPCs,[60] which in these experiments may be a role played by host astrocytes on the inner retinal surface or perhaps graft-derived intravitreal astrocytes.

CONCLUSIONS

We have demonstrated that transplantation of OPCs can ameliorate RGC death in vivo and that this neuroprotective capacity is dependent upon inflammatory cell activation of OPCs. Moreover, this reduction in neuronal loss did not appear to be contact mediated, or conferred via myelination of naked axons, but was most likely due to the release of diffusible neurotrophic factors by activated OPCs. Such long-term alleviation of RGC death in glaucoma by an experimental intervention is rarely seen, which hints at a novel approach to the development of a neuroprotective strategy in glaucoma.

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