Use of an Adult Rat Retinal Explant Model for Screening of Potential Retinal Ganglion Cell Neuroprotective Therapies

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PURPOSE. To validate an established adult organotypic retinal explant culture system for use as an efficient medium-throughput screening tool to investigate novel retinal ganglion cell (RGC) neuroprotective therapies.

METHODS. Optimal culture conditions for detecting RGC neuroprotection in rat retinal explants were identified. Retinal explants were treated with various recognized, or purported, neuroprotective agents and cultured for either 4 or 7 days ex vivo. The number of cells surviving in the RGC layer (RGCL) was quantified using histologic and immunohistochemical techniques, and statistical analyses were applied to detect neuroprotective effects.

RESULTS. The ability to replicate previously reported in vivo RGC neuroprotection in retinal explants was verified by demonstrating that caspase inhibition, brain-derived neurotrophic factor treatment, and stem cell transplantation all reduced RGCL cell loss in this model. Further screening of potential neuroprotective pharmacologic agents demonstrated that betaxolol, losartan, tafluprost, and simvastatin all alleviated RGCL cell loss in retinal explants, supporting previous reports. However, treatment with brimonidine did not protect RGCL neurons from death in retinal explant cultures. Explants cultured for 4 days ex vivo proved most sensitive for detecting neuroprotection.

CONCLUSIONS. The current adult rat retinal explant culture model offers advantages over other models for screening potential neuroprotective drugs, including maintenance of neu-

rons in situ, control of environmental conditions, and dissociation from other factors such as intraocular pressure. Verification that neuroprotection by previously identified RGC-protective therapies could be replicated in adult retinal explant cultures suggests that this model could be used for efficient medium-throughput screening of novel neuroprotective therapies for retinal neurodegenerative disease. (*Invest Ophthalmol Vis Sci.* 2011;52:3309–3320) DOI:10.1167/iovs.10-6873

The development of adjunctive neuroprotective therapies for degenerative retinal diseases such as glaucoma is a high priority, given these diseases are common causes of blindness and current therapies are often inadequate. Efforts to identify novel neuroprotective compounds for retinal therapy are limited by an experimental model gap that exists between high-throughput screening methods (e.g., dissociated cell culture), which are rapid but limited in their ability to reproduce in vivo conditions, and preclinical animal models, which have greater fidelity but lower efficiency and experimental control.

Retinal explant cultures have been used widely to examine a variety of biological processes, including retinal development,¹ CNS regeneration,^{2–4} and neurodegeneration.^{5,6} However, one of the difficulties in interpreting findings from the field is the wide variety of culture methods used, which can cause subtle variations in tissue behavior. This prompted us recently to develop an adult rodent retinal explant system using a serum-free, chemically defined medium as a tool for studying retinal effects in an easily controlled environment.⁷ We believe that this organotypic, ex vivo preparation has advantages over other experimental in vitro systems because it maintains mature neurons in situ and in contact with their normal cellular environment, thereby facilitating physiological interactions. Although this organotypic system does not exactly recapitulate in vivo homeostasis, it does permit direct retinal manipulation, allow greater control of retinal environment, and provide greater efficiency compared with animal models. We investigated whether the adult retinal explant system could be used to screen therapies for retinal ganglion cell (RGC) protection. Other in vitro systems, such as dissociated retinal cell cultures, have been used to study RGC neuroprotection; however, their predictive ability for in vivo efficacy appears limited, perhaps because of their lack of normal cellular interactions. The RGC-5 cell line has also been used extensively to investigate RGC-specific neuroprotective interventions, although results obtained using these cells should be interpreted in light of recent RGC-5 recharacterization as a neuronal precursor cell line with some RGC-like features.⁸ A clear advantage of the explant model over isolated cell lines, which by necessity are not mature neurons, is that mature RGCs are studied in situ.

The purpose of this investigation was to determine whether adult organotypic retinal explant cultures can be used as a

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medium-throughput screening tool to identify novel RGC neuroprotectants for further investigation using preclinical disease models. Furthermore, we attempted to clarify the parameters of the system for optimal detection of neuroprotective effects to facilitate future use of the model for screening neuroprotective therapies.

METHODS

Animals

Adult (8- to 10-week-old) male Sprague-Dawley rats were used in accordance with UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act (1986), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Explant Cultures

Adult rat retinal explant cultures were prepared as detailed previously, with a maximum of four explants generated from each eye and cultured with the inner retinal surface facing up.⁷ Explants were cultured at the air/medium interface and were not immersed in culture medium. Retinal explant cultures were maintained in humidified incubators with 5% CO₂ at 35°C or at 37°C. Where specifically indicated, a multigas incubator was used to culture retinal explants in low oxygen conditions in which the injection of nitrogen gas achieved a steady state of 3% O₂. Half the retinal explant culture medium was changed at 1 day ex vivo (1DEV) and every second day thereafter. Explants were fixed at various time points, as indicated.

Coculture of GFP⁺-mesenchymal stem cells (MSCs) with retinal explants was undertaken as detailed previously⁹ using an established cell line.¹⁰ Briefly, a 2-μL droplet of cell suspension (containing 1500 cells) was transferred onto the RGC surface of each explant 2 hours after retinal explantation at 0DEV. An equivalent volume of explant medium (without cells) was transferred onto the surface of control explants.

Laser-Induced Ocular Hypertension

Unilateral ocular hypertension (OHT) was induced in the left eye (*n* = 8) by external translimbal treatment to the aqueous outflow area with a 532-nm diode laser, as described previously.^{10,11} A single treatment of 50 to 60 spots of 50-μm diameter, 700-mW power, and 0.6-second duration was applied to each eye. After baseline intraocular pressure (IOP) measurement (day 0) using a rebound tonometer (TonoLab; TiolatOy, Helsinki, Finland), IOPs were recorded on days 1 and 7 to

confirm OHT. The average peak IOP for OHT eyes was 45.3 ± 7.1 mm Hg (mean ± SD) compared with 10.4 ± 0.7 mm Hg in contralateral eyes (*P* < 0.001), similar to that reported previously. Half the OHT rats (four rats), along with naive controls (two rats), were used to make explants at both 7 days (average peak IOP, 46.0 ± 10.1 mm Hg OHT eyes vs. 10.0 ± 0.0 mm Hg contralateral eyes; *P* < 0.01) and 14 days after laser treatment (average peak IOP, 44.5 ± 3.9 mm Hg OHT eyes vs. 10.8 ± 0.5 mm Hg contralateral eyes; *P* < 0.001).

Optic Nerve Crush Procedure

The rat optic nerve was subjected to crush injury in vivo, as previously described.¹² Briefly, the optic nerve was exposed intraorbitally and subjected to a 10-second crush injury 1 to 2 mm behind the left eye (two rats). The contralateral nerve was not injured. Retinal explants were made from the injured retinas 1 week after optic nerve crush (ONC), and two naive rats were used to make uninjured, control retinal explants for comparison.

Pharmacologic Agents

Retinal explants were cultured with drugs, with the exception of tafluprost, dissolved in the culture medium at the concentrations indicated in Table 1. Concentrations used were previously efficacious in vitro; references and the names of the companies from which the drugs were purchased are included in Table 1. Half the retinal explant culture medium, containing fresh drug, was changed at 1DEV and every second day thereafter. Furthermore, a 3-μL droplet of culture medium containing drug was transferred onto each explant surface 2 hours after explantation at 0DEV and every day thereafter at 24-hour intervals. Control explants received a 3-μL droplet of culture medium containing equivalent vehicle. Tafluprost was purchased as a commercial, preservative-free preparation (15 μg/mL solution, equivalent to 33.15 μM) and was applied directly onto the explant surface (3-μL droplet of commercial solution); it was not added to the culture medium. Control tafluprost explants received equivalent volumes of explant medium. The caspase inhibitor used, Z-VAD-FMK, is a cell-permeable pan-caspase inhibitor. Forskolin was used in conjunction with brain-derived neurotrophic factor (BDNF) to potentiate in vitro BDNF signaling by increasing trafficking of TrkB receptors to the cell surface.^{14,15}

Histology

Retinal explants were processed for histologic analysis as detailed previously.⁷ Transverse retinal explant sections were cut at 14 μm on a cryostat. Immunohistochemical staining of sections was performed

TABLE 1. Details of Pharmacologic Agents Used

Agent	Action	Company	Vehicle	Concentration
Z-VAD-FMK	Pan-caspase inhibitor	Promega, Southampton, UK	0.5% DMSO	100 μM ¹³
BDNF (+ forskolin)	Neurotrophin (cAMP release)	Sigma-Aldrich UK, Gillingham, UK	PBS (forskolin DMSO)	BDNF 100 ng/mL (forskolin 10 μM) ^{14,15}
Betaxolol, hydrochloride	β1-Adrenoceptor antagonist	Sigma-Aldrich UK	PBS	100 μM ¹⁶
Brimonidine (UK 14304)	α2-Adrenoceptor agonist	Sigma-Aldrich UK	0.1% DMSO	100 μM ¹⁶
Losartan, K salt	Angiotensin-II receptor antagonist	Sigma-Aldrich UK	PBS	1 μM ¹⁷
Tafluprost, preservative-free solution (15 μg/mL)	Synthetic prostaglandin-F _{2α} analog	Saflutan; Merck Sharp & Dohme Ltd., Hoddesdon, UK	N/A	3 μL of 33.15 μM solution daily on explant surface
Simvastatin, Na salt	Hypolipidemic drug (statin), HMG-CoA reductase inhibitor	Merck Chemicals Ltd., Nottingham, UK	0.01% DMSO	10 μM ¹⁸

Concentrations listed are final concentrations in explant medium, for both drug and vehicle. Tafluprost was not diluted in the culture medium but was applied directly to the explant surface daily. DMSO, dimethyl sulfoxide; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; N/A, not applicable.

as described previously.⁷ Primary antibodies used were as follows: chicken anti-GFP (1:5000; Millipore UK, Watford, UK), rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; DAKO UK Ltd., Ely, UK), mouse anti- β III tubulin (clone 5G8; 1:1000; Promega, Southampton, UK), mouse anti-neuronal nuclei (NeuN; clone A60; 1:500; Millipore UK), rabbit anti-calbindin (1:5000; Merck Chemicals Ltd.), and mouse anti-Islet-1 homeobox (39.4D5 concentrate; 1:500; hybridoma developed by T. Jessell and S. Brenner-Morton, concentrate from the Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA). Fluorescent secondary antibodies (Invitrogen Inc., Paisley, UK) were all used at 1:1000 dilution, and nuclei were counterstained with DAPI (1:10 000; Invitrogen Inc.). Primary antibodies were omitted for negative control staining. Sections were imaged using either a laser scanning confocal microscope (TCS-SPE; Leica Microsystems UK, Milton Keynes, UK) or an epifluorescence microscope (DM6000; Leica Microsystems UK).

For immunohistochemical staining of whole-mounted explants, after fixation and isolation from cell culture inserts, explants were washed in PBS and then incubated for 1 hour at room temperature in blocking solution, which consisted of PBS containing 5% normal goat serum (Invitrogen Inc.) and 0.2% Triton X-100 (Sigma-Aldrich UK). Explants were then incubated in mouse monoclonal anti- β III tubulin primary antibody (clone 5G8; 1:1000; Promega) for 20 to 22 hours at 4°C, washed thoroughly, and incubated in fluorescent secondary antibody for 20 to 22 hours at 4°C. Explants were washed, whole-mounted on slides and imaged using a laser scanning confocal microscope (TCS-SPE; Leica Microsystems UK).

Histologic Analysis

To quantify RGC survival in explants, transverse retinal explant sections were imaged using a 20 $\times$ objective on an epifluorescence microscope (DM6000; Leica Microsystems UK). Five fields per explant, each from separate sections (total of five sections analyzed per explant) sampled evenly across the tissue, were imaged. A masked investigator quantified the number of either Islet-1 or NeuN immunoreactive cells, or DAPI-positive nuclei, in the RGC layer (RGCL) of each image. Values were averaged for each explant to calculate the number of cells per millimeter of RGCL and were expressed as mean $\pm$ SEM. A two-tailed unpaired Student's *t*-test (95% confidence interval [CI]) was used when comparison between two groups was needed. One-way ANOVA (95% CI) with Bonferroni multiple comparisons post hoc test (computed only if overall *P* < 0.05) was used when comparison of three or more treatment groups was required (GraphPad Prism; GraphPad Software Inc., La Jolla, Ca).

Retinal morphometric analysis was performed on DAPI-stained transverse explant section images, collected as described, by a masked investigator. Total retinal thickness and the thickness of each laminar retinal layer (the three nuclear layers and the two plexiform layers) were measured for each image, and data for each explant were averaged. The retinal layers measured are indicated (see Fig. 2F). Data are expressed as mean $\pm$ SEM. One-way ANOVA (95% CI) with Bonferroni multiple comparisons post hoc test (computed only if overall *P* < 0.05) was used for statistical analysis of morphometric data (GraphPad Prism; GraphPad Software Inc.).

RESULTS

RGCL Changes in Retinal Explant Cultures over Time Ex Vivo

Histologic analysis of transverse sections was used to assess RGCL cell survival in retinal explants over time in culture. In all experiments, three markers were used to quantify changes in cell density within the RGCL: Islet-1 or NeuN immunohistochemistry, in conjunction with DAPI labeling of nuclei. This quantification was used as a proxy for RGC survival. Separate examples of explant sections immunolabeled for Islet-1 (Fig. 1A) or NeuN (Fig. 1B), along with an example of DAPI labeling (Fig. 1C), are shown.

FIGURE 1. RGCL changes in retinal explant cultures over time ex vivo. Three approaches were used to assess RGCL changes in transverse sections of retinal explants. Immunohistochemistry was used to label RGCs for either Islet-1⁺ (A) or NeuN⁺ (B) expression, and the number of positive cells (arrows, positive labeling) in the ganglion cell (GC) layer was counted. The number of DAPI⁺ nuclei (C) and calbindin⁺ (D) cells in the GC layer were also quantified (arrows, positive labeling). Examples of negative control goat anti-mouse (E) and goat anti-rabbit (F) secondary antibody labeling are also shown (note: these images were acquired using a laser intensity 3 to 4 times higher than that used in (A–D) because no background fluorescence was visible under normal excitation intensity). Representative images shown are all from explants cultured for 4DEV; an identical pattern of staining was observed at all time points. Quantification over time (G; *n* = 4 explants per time point) revealed a fairly steady loss of RGC marker expression in cultures up to approximately 7 days ex vivo, after which the rate of loss appeared to plateau. Furthermore, loss of calbindin⁺ neurons (mostly displaced amacrine cells) from the RGCL was comparatively flat over time ex vivo. All further comparative experiments were quantified at 4DEV and 7DEV. Values are mean $\pm$ SEM. INL, inner nuclear layer. Scale bar, 50 μ m (A–F).

Islet-1 typically labeled fewer cells within the RGCL than NeuN. The number of positive cells within the RGCL for each marker was quantified at various time points over 2 weeks ex vivo to determine the rate of RGC loss (Fig. 1G). Quantification revealed a steady decline in RGCL cell number, as assessed using all three labels, up to approximately 7DEV, after which the rate of cell loss slowed. In addition, the number of calbindin⁺ neurons in the RGCL was quantified to gauge changes in displaced amacrine cell number (Fig. 1G; representative image Fig. 1D). A slight decrease

in calbindin⁺ neurons within the RGCL of explants was noted over time; however, most loss occurred within the first few days in vitro, after which the number was fairly constant. Calbindin is also expressed by some RGCs.^{19,20} Double labeling of explant sections (0DEV) for calbindin and β III-tubulin expression estimated $13.5\% \pm 3.6\%$ (mean $\pm$ SEM) of calbindin⁺ RGCL neurons were also β III-tubulin⁺ and, thus, RGCs. This small number of RGCs was unlikely to have affected the displaced amacrine cell quantification substantially. Survival of RGCs in all subsequent experiments was assessed at both 4DEV and 7DEV.

Effect of Reduced Oxygen Tension on RGC Survival and Explant Morphology

RGCL cell density in cultures maintained in 3% O₂ was compared with that in cultures maintained at normal atmospheric conditions (i.e., ~21% O₂).

In both cases, temperature was set to 37°C, whereas previously established culture conditions specified 35°C.⁷ Thus, an additional group was cultured in normal conditions to assess whether the higher temperature alone would impact explant survival. Quantification of Islet-1⁺ and NeuN⁺ cells, and of DAPI⁺ nuclei, in the RGCL of explants at 4DEV demonstrated that culture in low oxygen greatly reduced neuronal survival (Fig. 2A; see Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6873/-DCSupplemental>, for mean $\pm$ SEM values). In contrast, no significant difference in RGCL neuronal survival was found between explants cultured at 35°C and 37°C (atmospheric oxygen) at 4DEV. Cell counts from freshly isolated retinal explants (0DEV) are shown for comparison. At 7DEV, only Islet-1 quantification demonstrated fewer neurons in the low-oxygen explants compared with controls; no difference was observed in NeuN or DAPI quan-

FIGURE 2. Effect of reduced oxygen tension on neuronal survival and explant morphology. RGC survival in explants cultured in 3% O₂ (37°C) was compared with that in control cultures (37°C, air) at 4DEV (**A**; $n = 4$ explants/group) and 7DEV (**B**; $n = 4$ explants/group). Explant culture in reduced oxygen was found to significantly impair RGC survival. Given retinal explants are normally kept at 35°C,⁷ RGC survival at 37°C/air was also compared with that in explants cultured in normal conditions at 35°C/air. An increase in culture temperature did not greatly impact explant health. Data from freshly isolated explants (0DEV) are included for comparison. Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ using one-way ANOVA with Bonferroni post hoc comparison. Immunohistochemical labeling of β III tubulin in whole-mounted retinal explants at 7DEV (**C-E**; $n = 4$ explants/group) demonstrated explant culture in reduced oxygen (**E**) severely impaired RGC survival and axonal health compared with control cultures (**D**). Specifically, RGC density was greatly reduced, RGC axons appeared disrupted, and axonal swellings were common. Explants cultured at 37°C (**D**) showed no obvious difference in RGC morphology compared with those kept in normal conditions at 35°C (**C**). In contrast to negative RGC effects, DAPI staining of 7DEV explant sections indicated that reduced oxygen culture conditions (**I**) alleviated retinal thinning over time *ex vivo* compared with control retinas (**H**). No difference in retinal thickness was observed in cultures at 37°C (**H**) compared with those in normal conditions at 35°C (**G**). An example of a DAPI-stained transverse section of freshly isolated explant at 0DEV (**F**) is included for comparison. Morphometric analysis of DAPI-stained explant sections revealed that in 3% O₂, explants were significantly thicker, compared with controls, at both 4DEV and 7DEV (**J**; $n = 4$ explants/group). The ONL was also significantly thicker in explants cultured in 3% O₂, compared with controls, at both 4DEV and 7DEV (**K**; $n = 4$ explants/group). Culture of explants in 3% O₂ did not significantly affect the thickness of other retinal layers compared with controls (data not shown). Data from freshly isolated explants (0DEV) are included for comparison. Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ for groups indicated. # $P < 0.05$, ### $P < 0.01$ compared with 0DEV data. Statistical analysis one-way ANOVA with Bonferroni post hoc comparison. GC, ganglion cell layer; IP, inner plexiform layer; IN, inner nuclear layer; ON, outer nuclear layer; OP, outer plexiform layer. Scale bar, 50 μ m (**C-I**).

7DEV (**J**; $n = 4$ explants/group). The ONL was also significantly thicker in explants cultured in 3% O₂, compared with controls, at both 4DEV and 7DEV (**K**; $n = 4$ explants/group). Culture of explants in 3% O₂ did not significantly affect the thickness of other retinal layers compared with controls (data not shown). Data from freshly isolated explants (0DEV) are included for comparison. Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ for groups indicated. # $P < 0.05$, ### $P < 0.01$ compared with 0DEV data. Statistical analysis one-way ANOVA with Bonferroni post hoc comparison. GC, ganglion cell layer; IP, inner plexiform layer; IN, inner nuclear layer; ON, outer nuclear layer; OP, outer plexiform layer. Scale bar, 50 μ m (**C-I**).

tification (Fig. 2B; see Supplementary Table S1 for mean $\pm$ SEM values). A slight, but significant, decrease in the number of Islet-1⁺ cells in the RGCL of explants cultured at 37°C, compared with 35°C, was noted at 7DEV. This difference was not found at 4DEV.

A small number of whole-mounted 7DEV explants were immunolabeled for β III-tubulin, which is expressed by RGCs. β III-tubulin immunohistochemistry labels RGC soma, dendrites, and axons, thereby facilitating qualitative observation of RGC morphology in flat-mounted tissue. This analysis revealed that RGCs in explants cultured in low oxygen exhibited noticeably degenerated axons (Fig. 2E). Specifically, axons appeared disrupted, and axonal swellings were common. Furthermore, RGC density was much lower in explants cultured in low oxygen than in those cultured under normal conditions (Fig. 2D). In contrast, no differences were observed between explants cultured at 37°C (Fig. 2D) compared with those cultured under normal conditions (35°C; Fig. 2C), and RGC morphology in these explants appeared normal.

In contrast to poor RGC survival in explants cultured in low oxygen, examination of DAPI-labeled explant sections suggested that low-oxygen culture enhanced preservation of the gross morphology of other retinal layers (Figs. 2F–I). Explants maintained in low oxygen (Fig. 2I) were noticeably thicker than those cultured in normal atmospheric oxygen (Fig. 2H); in particular, the outer nuclear layer (ONL) appeared thicker. No obvious difference was observed between explants cultured at 37°C (Fig. 2H) and those cultured at 35°C (Fig. 2G). An image of a section from a freshly isolated retinal explant (0DEV) is shown for comparison (Fig. 2F). Morphometric analysis of explant sections demonstrated that the total retinal thickness of explants cultured in low oxygen levels was significantly greater than that of explants cultured in normal oxygen levels (Fig. 2J; see Supplementary Table S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6873/-DCSupplemental>, for mean $\pm$ SEM values). No difference in total retinal thickness was found between explants cultured at 37°C and those maintained at 35°C. In addition, although retinal explants cultured in normal oxygen levels (at both 35°C and 37°C) were significantly thinner than freshly isolated explants (0DEV), those maintained in low oxygen were not. Further morphometric

analysis revealed that the ONL was much thicker in explants cultured in low oxygen, compared with controls, at both 4DEV and 7DEV (Fig. 2K; see Supplementary Table S2 for mean $\pm$ SEM values). No significant morphometric differences were detected for the other retinal layers examined (see Supplementary Table S2 for mean $\pm$ SEM values). However, it was noted that the ONL in low-oxygen explants at 4DEV was thicker than that of freshly isolated explants (0DEV), whereas that of control explants was marginally thinner. This might have been due to tissue relaxation or swelling in culture conditions. By 7DEV, there was no difference in ONL thickness between low-oxygen explants and 0DEV explants; however, the ONL thickness of 7DEV control explants was significantly thinner than that of 0DEV explants.

Inhibition of Apoptosis or Delivery of Neurotrophic Support Enhanced RGC Survival in Retinal Explants

Application of a pan-caspase inhibitor (Z-VAD-FMK) to the explants significantly reduced RGCL cell loss at both 4DEV (Fig. 3A) and 7DEV (Fig. 3B) compared with vehicle-treated controls (see Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6873/-DCSupplemental>, for mean $\pm$ SEM values). Neuroprotection was detected using all three RGCL labels (Islet-1, NeuN, and DAPI) at 7DEV but was only demonstrated in the NeuN⁺ and DAPI populations at 4DEV. The neuroprotective effect of BDNF was also examined. No BDNF neuroprotection was detected at 4DEV (Fig. 3C; see Supplementary Table S1 for mean $\pm$ SEM values), but significantly more Islet-1⁺ RGCL cells survived in 7DEV explants than in vehicle controls (Fig. 3D; see Supplementary Table S1 for mean $\pm$ SEM values).

Coculture of Mesenchymal Stem Cells with Retinal Explants Strongly Enhanced RGC Survival in Explants

GFP⁺ MSCs were cocultured on the inner retinal surfaces of explants for 4DEV (Fig. 4A) or 7DEV (Fig. 4B), and RGCL cell survival was quantified (see Supplementary Table S1, <http://www.iovs.org/>

FIGURE 3. Inhibition of apoptosis or delivery of neurotrophic support enhances RGC survival in retinal explants. Culture of explants with a pan-caspase inhibitor significantly reduced RGC death at both 4DEV (A; $n = 6$ explants/group) and 7DEV (B; $n = 6$ explants/group), compared with controls (vehicle). In addition, culture of explants with BDNF plus forskolin (F) was found to improve RGC survival, as assessed by Islet-1 immunohistochemistry, at 7DEV (D; $n = 8$ explants/group) but did not affect RGC survival when assessed at 4DEV (C; $n = 8$ explants/group). Values are mean $\pm$ SEM. $^{**}P < 0.01$ using two-tailed Student's *t*-test.

FIGURE 4. Coculture of MSCs with retinal explants strongly enhanced RGC survival in explants. GFP⁺-MSCs were cocultured on the inner retinal surface of explants for 4DEV (A; $n = 4$ vehicle, $n = 3$ MSCs) and 7DEV (B; $n = 4$ explants/group). At both time points, GFP⁺-MSC coculture significantly enhanced RGC survival, as assessed immunohistochemically and histologically (MSC nuclei were excluded from these counts by their colocalization with GFP). (A, B) Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ using two-tailed Student's *t*-test. Robust, significant MSC-mediated RGC neuroprotection was also observed in explants in which RGCs were injured prior to culture by 7 days' OHT (OHT; C), 14 days' OHT (D), and 7 days after ONC (E), compared with injured retinas treated with vehicle alone (OHT/ONC+MSCs vs. OHT/ONC+Vehicle). In addition, neither 7 days' OHT nor 14 days' OHT potentiated RGC death ex vivo (OHT+Vehicle vs. Naive+Vehicle; C, D). In comparison, ONC 7 days before explantation tended to enhance RGC loss where a significant difference in survival was detected using NeuN immunohistochemical analysis (Naive+Vehicle vs. ONC+Vehicle; E). (C-E) All explants were cultured for 7DEV. All values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ using one-way ANOVA with Bonferroni post hoc comparison.

lookup/suppl/doi:10.1167/iovs.10-6873/-DCSupplemental, for mean $\pm$ SEM values). At both time points examined, coculture of explants with MSCs conferred robust neuroprotection. Examples of Islet-1, NeuN, and DAPI labeling of explants cocultured with GFP⁺ MSCs are shown (Figs. 5A, 5B). Moreover, MSC coculture appeared to preserve RGC projections at both time points, as shown by improved nerve fiber and inner plexiform layer preservation, compared with controls (Fig. 5C). In addition, though Müller cells expressed GFAP in all explants, this expression appeared upregulated in MSC cocultured explants compared with controls, particularly after 7DEV (Fig. 5D). Müller cell morphology was also well preserved in MSC-treated explants.

We also investigated whether MSC coculture could protect RGCs after they were injured in vivo prior to explantation. Three

types of in vivo injury were used: 7 days in vivo OHT (Fig. 4C), 14 days in vivo OHT (Fig. 4D), and ONC 7 days prior (Fig. 4E) (see Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6873/-DCSupplemental>, for mean $\pm$ SEM values). Explants were made from retinas after injury and were cultured for 7DEV before RGCL cell survival was assessed. Prior RGC injury by either 7 days' in vivo OHT (Fig. 4C) or 14 days' in vivo OHT (Fig. 4D) did not accelerate RGCL cell loss in explant cultures (OHT+Vehicle vs. Naive+Vehicle groups). Furthermore, MSC coculture with explants after both 7 days in vivo OHT (Fig. 4C) and 14 days in vivo OHT (Fig. 4D) significantly reduced RGCL cell loss compared with vehicle-treated OHT explants (OHT+MSCs vs. OHT+Vehicle groups).

MSC coculture also conferred significant protection to RGCL cells in explants made from retinas subjected to in vivo

FIGURE 5. Representative immunohistochemical images of retinal explants cocultured with GFP⁺ MSCs. Retinal explants were cultured, for 4DEV or 7DEV, untreated (control), or cocultured with GFP⁺ MSCs (green) on the inner retinal surface. Transverse explant sections were immunohistochemically labeled for Islet-1 (A, red), NeuN (B, red), β III-tubulin (β 3T; C, red), or glial fibrillary acidic protein (GFAP; D, red). Nuclei were counterstained with DAPI (blue). Cocultured MSCs did not migrate into the retinal tissue, but were associated with increased Islet-1⁺ and NeuN⁺ cell density within the RGCL at both 4DEV and 7DEV (quantification presented in Figs. 4A, 4B). In addition, MSC coculture resulted in a thicker retinal explant overall, a thicker inner plexiform layer (IPL, white vertical lines), and a well-preserved nerve fiber layer (NFL, arrows) at both time points (C). GFAP immunoreactivity (D, red) in astrocytes within the NFL and Müller cells throughout the retina was present in all explants but appeared upregulated, particularly in Müller cells (arrowheads), with MSC coculture at 7DEV. INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer; β 3T, β III-tubulin. Scale bar, 100 μ m.

ONC 7 days prior (Fig. 4E; ONC+MSCs vs. ONC+Vehicle groups). In contrast to cell survival in explants from OHT retinas (Figs. 4C, 4D), a trend toward greater neuronal death was observed in explants made from retinas injured by previous ONC (Fig. 4E; ONC+Vehicle vs. Naive+Vehicle groups). However, a significant difference in RGCL cell density for these two groups was found only in the NeuN⁺ population.

Screening RGC Neuroprotection by Pharmacologic Agents Using Retinal Explant Cultures

Finally, we used the retinal explant model to screen a series of pharmaceutical compounds for RGC neuroprotection in a whole-tissue system (Fig. 6; see Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6873/-DCSupplemental>, for mean $\pm$ SEM values). Treatment of retinal explants with betaxolol (100 μ M) improved RGCL cell survival, as assessed by Islet-1, NeuN, and DAPI quantification, compared with controls, after 4DEV (Fig. 6A) but not at 7DEV (Fig. 6B). In contrast, brimonidine (100 μ M) did not reduce RGCL cell death at

either 4DEV (Fig. 6C) or 7DEV (Fig. 6D). Exposure of explants to losartan (1 μ M) offered modest neuroprotection after 4DEV, with a significant difference in NeuN⁺ quantification found compared with controls (Fig. 6E). No effect was found for losartan at 7DEV (Fig. 6F). Application of tafluprost reduced both neuronal and total cell loss from the RGCL at 4DEV (Fig. 6G) compared with controls, but no effect was found after 1 week (Fig. 6H). Finally, simvastatin (10 μ M) treatment alleviated the loss of NeuN⁺ neurons at 4DEV (Fig. 6I), but not at 7DEV (Fig. 6J), compared with controls. Total RGCL cell survival was also greater in simvastatin explants at 4DEV.

DISCUSSION

Before addressing the usefulness of the explant model for screening RGC neuroprotective therapies, it was necessary to define RGC degeneration over time ex vivo. We predicted that to detect RGC neuroprotection optimally, active cell loss must be ongoing rather than have reached a maximum plateau. Three parameters were used to assess RGCL cell survival in

A. 4DEV **B. 7DEV** **C. 4DEV** **D. 7DEV** **E. 4DEV** **F. 7DEV** **G. 4DEV** **H. 7DEV** **I. 4DEV** **J. 7DEV**

FIGURE 6. Screening RGC neuroprotection by pharmacologic agents using retinal explant cultures. Retinal explants were cultured for either 4DEV or 7DEV and were treated with a variety of drugs. Explants were continuously exposed to the drugs during culture. Betaxolol (A; $n = 6$ /group), losartan (E; $n = 6$ /group), tafluprost (G; $n = 6$ /group), and simvastatin (I; $n = 6$ /group) were all found to reduce RGC loss in 4DEV explants. In contrast, brimonidine (C, D; $n = 6$ /group, C; $n = 5$ vehicle, $n = 6$ brimonidine, D) did not affect RGC loss in retinal explants. Pharmacologic neuroprotection did not prevent RGC death, as no neuroprotective effects by betaxolol (B; $n = 6$ /group), losartan (F; $n = 6$ /group), tafluprost (H; $n = 6$ /group), or simvastatin (J; $n = 6$ /group) were detected at 7DEV, but instead delayed neurodegeneration thereby providing a temporal window in which to observe direct effects on RGC survival. All values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, two-tailed Student's t -test.

explant sections, as a proxy for RGC survival. Quantification of the number of cells in the RGCL of explant sections over 2 weeks *ex vivo* demonstrated that the rate of cell loss was most rapid from 0DEV up to approximately 5DEV to 7DEV, after which the rate of loss slowed. To determine the stage at which cultures were reliably sensitive for detecting neuroprotection, we compared two time points in subsequent experiments. By 4DEV, sufficient cell death had occurred to permit the detection of neuroprotection and cell death was steadily ongoing (injured cells were available to protect). By 7DEV, the rate of RGCL cell loss had slowed (fewer injured cells to protect) however a large number of cells had died, thereby providing a larger potential difference to detect preservation. We also observed that the rate of cell death was somewhat user and/or laboratory dependent, with slightly different survival rates detected by individual experimenters. Thus, it is advisable for end-users to characterize the time course of RGC death in this system before use. It is also important to note that RGC survival can vary between experiments conducted at different times, even within the same laboratory, probably because of the slight variability in culture conditions or tissue viability after dissection. Thus, it is critical that experimental and control explants be cultured in parallel (with explants from each group distributed evenly across culture plates) to ensure identical culture conditions for meaningful comparison of results. Interestingly, RGC injury before explant culture had little effect on subsequent RGC survival *ex vivo*. It could be expected that injury before explantation would initiate cell death pathways *in vivo* and, thus, accelerate RGC loss in explant cultures. In fact, no effect of previous OHT on explant RGC survival was found, and only a mild trend to increased cell death was noted in explants after previous ONC injury.

It should be noted that the histologic assessments used in this model are unlikely to represent a pure measure of RGC survival. Choice of a selective RGC marker to assess survival after injury requires careful consideration because available RGC markers are imperfect. Specifically regarding the assessments used in this study, quantification of RGCL nuclei by DAPI overestimates RGC survival because of the inclusion of glial, vascular, and immune cells. It was for this reason that more specific immunohistochemical markers were also assessed. Immunohistochemical methods, rather than retrograde RGC labeling, were chosen because the latter technique would have necessitated intracranial surgery *in vivo* before explantation. An ideal antigen for RGC quantification would be RGC specific, with nuclear expression (for accurate quantification) unaffected by cellular injury. Both Islet-1 and NeuN are nuclear antigens expressed by RGCs, although both markers are also expressed by some displaced amacrine cells.^{21,22} However, we examined displaced amacrine cell loss in retinal explants over time *ex vivo* and found it to be modest (Fig. 1D). Thus, we concluded that the loss of immunohistochemically positive displaced amacrine cells would not significantly influence assessment of RGC degeneration. In addition to imperfect specificity, neuronal protein markers may be downregulated after neuronal injury but before cell death,^{23–26} which could underestimate the numbers of surviving cells. Despite these caveats, all three cell labels used here have been used previously to quantify RGCs *in situ*.^{27–31} Although other antigens are more specifically expressed by RGCs (e.g., β III-tubulin, Thy1, γ -secretase), their additional labeling of RGC processes produces a staining pattern too disorganized for accurate quantification. In addition, immunohistochemistry for Brn3, another RGC-specific marker, was tested but failed to produce a clear and consistent signal in explants, possibly due to gene downregulation after explantation. Overall, however, it is important to note that the same assumptions applied to quantification in all explants, including parallel controls. Therefore, quantification caveats

should be comparable across groups and controlled for with respect to individual treatments.

Tissue/cell culture experiments are normally conducted at atmospheric oxygen tension (i.e., $\sim 21\%$ O₂), despite most cells experiencing lower oxygen concentrations *in vivo*. Interestingly, maintenance of cultures in reduced oxygen can enhance neuronal survival *in vitro*, presumably through reduced oxygen toxicity.^{32–34} Thus, we examined whether the maintenance of retinal explants in reduced oxygen improved explant longevity and enhanced neuronal survival. Analysis of RGC survival and morphology clearly demonstrated that low oxygen tension exacerbated RGC degeneration in explant cultures. In contrast, the survival of photoreceptors, as assessed by ONL morphometric analysis, appeared potentiated in these cultures. This could, however, be partially attributed to tissue relaxation or swelling in culture conditions. This may indicate a difference between RGC and photoreceptor sensitivity to oxygen toxicity, and/or their respective oxygen demands. These differential features may reflect the conditions to which each cell type is typically exposed *in vivo*, where oxygen tension is lower in the outer retina compared with that of the vascularized inner retina.³⁵

The purpose of the initial treatment experiments was to verify that RGC neuroprotection previously demonstrated *in vivo* could be replicated *in vitro* using the retinal explant model. The first compounds tested were a pan-caspase inhibitor and BDNF. BDNF has been well established as an important prosurvival factor in RGCs, and dysregulation of BDNF signaling has been strongly implicated in RGC degenerative diseases such as glaucoma.^{36,37} It has also been demonstrated that apoptosis plays a key role in glaucomatous RGC death and that the blockade of apoptosis can enhance RGC survival after injury.^{38,39} In addition, we recently demonstrated that the intravitreal transplantation of MSCs strongly protects RGCs *in vivo*¹⁰ and, therefore, examined whether this effect could also be replicated *ex vivo*. Application of either the caspase inhibitor or BDNF enhanced RGC survival in adult retinal explant cultures, thereby replicating results previously observed in other systems. Intriguingly, the neuroprotective effects of both caspase inhibition and BDNF in retinal explants were modest. There could be several reasons the protective effects observed were perhaps less than expected. Prosurvival signaling by BDNF in RGCs appears to exhibit variability depending on which cellular compartment (soma or axon) initiates the signaling cascade, with retrograde BDNF apparently more potent than endogenous retinal signaling.⁴⁰ Thus somatic application in explants may be less effective. Moreover, the efficacy of BDNF neuroprotection is notoriously short-lived, perhaps because of changes in receptor expression after chronic ligand exposure or in disease states.^{23,37,41} A larger reduction in RGC death by caspase inhibition might also have been expected. It is unclear why only a moderate, though strongly significant, neuroprotective effect was observed after caspase inhibition in retinal explants. It is possible that the inhibitor dose chosen was inadequate for robust signaling blockade in explants, or perhaps the axotomy injury was sufficient to overcome blockade of cell death pathways. In contrast, coculture of MSCs with retinal explants was found to confer strong RGC protection, comparative to that observed previously *in vivo*, which lasted for a relatively long time (for at least 7DEV). Almost all other pharmacologic neuroprotection was detected only at 4DEV. Indeed, MSC coculture was by far the most effective neuroprotective treatment tested in this study. Furthermore, injury to RGCs before explantation for culture did not impact the strong MSC-mediated RGC neuroprotection *in vitro*. These results, taken with the BDNF and caspase inhibitor observations, suggest that organotypic adult retinal explants can provide a system in which to model *in vivo* retinal neuroprotection.

Given that retinal cells are maintained in situ and within an environment more similar to that in vivo, compared with other in vitro systems, it is possible that the retinal explant model will provide a useful tool for screening novel neuroprotectants. However, the accuracy of its predictive ability remains to be established.

Based on these results we used the adult retinal explant system to investigate RGC neuroprotection by a number of pharmacologic compounds, some previously reported to be efficacious alongside more novel compounds. The main purpose of these experiments was to demonstrate the potential of the adult retinal explant system rather than to explore the therapeutic potential of any single agent in great detail. One advantage of an organotypic model over in vivo preparations is its ability to isolate direct retinal effects from systemic effects. For example, it appears that several ophthalmic drugs routinely used to control IOP in glaucoma may also protect RGCs by acting on the retina directly. Here, use of the explant system would permit the study of direct RGC protective effects independently of effects on IOP, which may indirectly protect RGCs. In the present study, we examined the direct retinal neuroprotective effects of several clinical ocular hypotensive drugs on RGC survival in retinal explants. All had been previously reported to protect RGCs from death using in vivo glaucoma models, results from which are potentially complicated by IOP effects. An additional advantage of the explant model over dissociated retinal cultures may be sensitivity to neuroprotective drugs that work through indirect mechanisms involving other retinal cell types (e.g., glia).

Betaxolol is a selective β 1-adrenoceptor antagonist reported to alleviate RGC death both in vitro and in vivo.^{42–44} There is some experimental evidence that betaxolol treatment may elicit better visual field outcomes in glaucoma patients, compared with other IOP lowering β -blockers (e.g., timolol), despite lower reductions in IOP.⁴³ The interpretation of these human studies remain controversial because the possibility remains that the apparent neuroprotective effect of betaxolol could actually occur through an effect on the IOP profile not captured by the study design. Nevertheless, such studies have been used to support the idea that betaxolol may modulate RGC survival by both IOP-dependent and IOP-independent mechanisms. Betaxolol has been postulated to confer neuroprotection by blocking sodium and calcium channel activity.^{43,44} In the present study, betaxolol was found to reduce RGC loss robustly in retinal explants. Clearly, this is a direct cellular effect given that explants are isolated from IOP, and it supports the conclusion that betaxolol not only lowers IOP but could potentially have a direct neuroprotective effect if delivered to RGCs at an appropriate concentration.

In contrast, no direct neuroprotective effect was found after treatment of the explants with the α 2-adrenoceptor agonist brimonidine. This drug has also been reported to protect RGCs from death in vitro and in vivo,^{45–48} possibly by modulating Ca^{2+} influx or intracellular signaling pathways.^{45,46,49} However, a lack of effect on Ca^{2+} -channel activity has also been reported,¹⁶ and neuroprotective efficacy has not been confirmed in an evidence-based review of relevant clinical trials.⁵⁰ Lack of neuroprotection by brimonidine in retinal explants would appear consistent with these later data.

Losartan is a selective, competitive angiotensin-II receptor (type 1) antagonist reported to lower IOP in glaucoma patients,⁵¹ and drugs from this class (in particular candesartan) can reduce retinal degeneration in rat models of diabetic retinopathy,⁵² ischemia-reperfusion injury,⁵³ and glaucoma.⁵⁴ This neuroprotective effect may be mediated by modulating retinal production of reactive oxygen species.⁵³ In retinal explant cultures, we detected a modest losartan-mediated protective effect on RGCs, suggesting direct neuroprotection indepen-

dent of IOP reduction. This effect was not as strong as that observed after betaxolol application.

Tafluprost is a preservative-free synthetic prostaglandin- $F_{2\alpha}$ receptor analog. Prostanoid agonists have been found to protect RGCs from injury in vivo,^{55–57} perhaps by suppressing apoptosis through the inhibition of caspase-3 activation.⁵⁸ In support of previously published work, application of tafluprost to retinal explants enhanced RGC survival significantly, suggesting a direct neuroprotective ability concomitant with ocular hypotensive effects.

Simvastatin is an HMG-CoA reductase inhibitor belonging to the statin class of drugs that were traditionally developed as hypolipidemic drugs to control hypercholesterolemia in cardiovascular disease. These drugs have no ocular hypotensive effect and are not used for glaucoma therapy. Since their original clinical application several off-target effects have been discovered, including a potential neuroprotective capacity.^{59,60} Indeed, in vivo statin treatment can protect RGCs after axotomy and ischemia/reperfusion.^{61–63} The neuroprotective mechanism of statins appears to be multimodal (see Ref. 59 for a review). However, importantly for RGC neuroprotection, they are reported to modulate Bcl-2, BAX, BDNF, and heat shock protein expression, and also activation of Akt, Wnt, and ERK signaling pathways,^{59,62–66} which are all known mediators of RGC survival. In support of previous studies, simvastatin treatment of adult retinal explant cultures offered significant RGC neuroprotection, suggesting the ability of this drug to alleviate RGC death in glaucoma/OHT should be further investigated.

Of all pharmacologic agents tested, none prevented RGC death completely but instead only delayed degeneration. This is perhaps unsurprising given that RGCs are axotomized upon explantation. This observation suggests there is a temporal window in which to detect neuroprotective explant effects. In the present study this time point was approximately 4DEV. However, variation in explant survival was noted between laboratories and, to a lesser degree, between experiments. Therefore, the system requires optimization by end-users for the detection of neuroprotection. Furthermore, some variability in RGC marker ability to reveal differences in survival was observed, with NeuN appearing more consistent and sensitive in the present study. This finding, in conjunction with other considerations discussed, highlights the need to choose the RGC marker/assessment used with care. We also demonstrated that no previous RGC injury is needed to generate an ex vivo system in which to detect RGC protection. Replication of previously established in vivo neuroprotective treatments, such as caspase inhibition and MSC transplantation, suggests that the organotypic adult retinal explant model is a useful medium-throughput system in which to screen novel neuroprotective therapies. Treatments identified in this way could then be tested in preclinical models to verify efficacy in vivo. For example, the identification of robust RGC protection by MSC and simvastatin treatment in the present study suggests these novel therapies should be investigated further as potential adjunctive glaucoma therapies.

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