

Interleukin-6 Protects Retinal Ganglion Cells from Pressure-Induced Death

Rebecca M. Sappington,¹ Matilda Chan,² and David J. Calkins¹

PURPOSE. The response of retinal ganglion cells (RGCs) to ocular pressure in glaucoma likely involves signals from astrocytes and microglia. How glia-derived factors influence RGC survival at ambient and elevated pressure and whether the inflammatory cytokine interleukin-6 (IL-6) is a contributing factor were investigated.

METHODS. Primary cultures of retinal astrocytes, microglia, and RGCs were prepared using immunomagnetic separation. Comparisons were made of RGC survival at ambient and elevated pressure (+70 mm Hg) and with pressure-conditioned medium from glia with, and depleted of, IL-6.

RESULTS. Pressure elevated for 24 to 48 hours reduced RGC density, increased TUNEL labeling, and upregulated several apoptotic genes, including the early immediate genes *c-jun* and *jun-B*. Pressure-conditioned medium from astrocytes reduced RGC survival another 38%, while microglia medium returned RGC survival to ambient levels. These effects were unrelated to IL-6 in microglia medium. Neither astrocyte- nor microglia-conditioned medium affected ambient RGC survival unless depleted of IL-6, which induced a 63% and a 18% decrease in RGCs, respectively. Recombinant IL-6 equivalent to levels in glia-conditioned medium doubled RGC survival at elevated pressure.

CONCLUSIONS. For RGCs at ambient pressure, IL-6 secreted from astrocytes and microglia under pressure is adequate to abate other proapoptotic signals from these glia. For RGCs challenged by elevated pressure, decreased IL-6 in astrocyte medium is insufficient to counteract these signals. Increased IL-6 in microglia medium counters not only proapoptotic signals from these cells but also the pressure-induced apoptotic cascade intrinsic to RGCs. (*Invest Ophthalmol Vis Sci.* 2006;47:2932–2942) DOI:10.1167/iovs.05-1407

Glaucoma is typically associated with sensitivity to intraocular pressure; in fact, elevated pressure is a significant risk factor.^{1–3} Identifying extracellular signals that modulate retinal ganglion cell (RGC) survival in glaucoma and determining whether these signals depend on pressure are essential for delineating the mechanisms of the disease and for defining

novel targets for its treatment. Astrocyte glia and microglia represent a source of these signals. In the glaucomatous optic nerve, tumor necrosis factor- α (TNF- α) expression by astrocytes and microglia increases with disease severity,^{4,5} whereas TNF- α can influence RGC survival in vitro.^{6–8} Similarly, in neovascular glaucoma, increased concentrations of IL-6 in the aqueous humor correlate with severity of the disease.⁹ In addition, treatment with IL-6 and its soluble receptor inhibits retinal excitotoxicity.¹⁰ Finally, IL-6 is known to modulate neuronal survival and glial function during development^{11,12} and in other diseases of the central nervous system.^{13–24} As an antiapoptotic factor, IL-6 activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway that leads to expression of many stress-related factors, including heat shock proteins 70 and 90.^{25–28} In addition to IL-6 itself, proteins related to IL-6, such as ciliary neurotrophic factor (CNTF) and neuropoietin, may also influence the development and survival of retinal cells.^{29,30} Thus, glia-derived IL-6 could modulate RGC survival in glaucoma, particularly in the retina, where astrocytes and microglia are in proximity to RGC somas and axons. We tested this possibility using an in vitro preparation to investigate how astrocytes and microglia subjected to elevated pressure affect the survival of RGCs and whether IL-6 is a contributing component. We found that signals secreted from astrocytes and microglia affect RGC survival differently and that IL-6 can serve as a neuroprotectant for RGCs challenged by pressure.

MATERIALS AND METHODS

Animals

This study was conducted in accordance with regulations set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal protocols were approved by the Institutional Animal Care and Use Committees of the University of Rochester Medical Center and the Vanderbilt University Medical Center.

Cell Separation and Culture

Eyes ($n \geq 24$ /preparation) from postnatal day 2 to 4 Sprague-Dawley rats were enucleated, and the retina of each was dissected free and stored on ice in Dulbecco modified Eagle medium plus 5% glucose (DMEM/Glu; Gibco, Carlsbad, CA). Tissue was dissociated by centrifugation at 70g, triturated by pipetting, and incubated for 15 minutes at 37°C in 1 mg/mL papain (Worthington, Lakewood, NJ) and 0.005% DNase I in Earle balanced salt solution. Viability was assessed by trypan blue exclusion and was found to be greater than 98%.

To generate primary cultures of astrocytes, microglia, and RGCs, we followed a protocol for immunomagnetic separation previously established as an excellent means for generating purified RGC cultures.^{6,7,31–34} The cell suspension was centrifuged at 250g and resuspended in DMEM/Glu with antibodies against cell type-specific markers. First, to remove astrocytes, we used a monoclonal antihuman astrocyte antibody (4 μ g/mL; catalog no. MAB5314; Chemicon, Temecula, CA). The suspension was incubated on ice for 15 minutes in antimouse immunoglobulin M (IgM) secondary antibody conjugated to magnetic microbeads and loaded into a pre-equilibrated column in the presence of a magnetic field (Miltenyi Biotech, Auburn, CA). Next, to remove microglia, we applied to the suspension a monoclonal anti-rat

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RT 1a/OX18 antibody (5 $\mu\text{g}/\text{mL}$; Chemicon) and followed the same procedure using microbeads conjugated with antimouse IgG. Finally, to isolate RGCs, we followed published studies and used a monoclonal anti-Thy1.1 (CD90) antibody (5 $\mu\text{g}/\text{mL}$; BD PharMingen, San Diego, CA), again applying microbeads conjugated with antimouse IgG.^{6,7,31-33,35,36} Here we demonstrate the purity of the cultures prepared with these antibodies.

Cells positively selected by anti-OX18 or anti-astrocyte antibodies were plated at a density of 5×10^4 (1 mL/well) on two-chamber glass slides (Nalge-Nunc, Rochester, NY) and grown in a 50:50 mixture of Dulbecco modified Eagle medium and F12 medium (DMEM/F12; Gibco) medium plus 2 mM L-glutamine, 15 mM HEPES buffer, 0.1% gentamicin, 10% fetal bovine serum, and 1% G₅ supplement (insulin 500 $\mu\text{g}/\text{mL}$, transferrin 5 mg/mL, selenite 520 ng/mL, biotin 100 $\mu\text{g}/\text{mL}$, hydrocortisone 360 ng/mL, FGF 500 ng/mL, and epidermal growth factor (EGF; 100 ng/mL; Gibco). Cultures were grown to approximately 80% confluence (10–14 days) in a standard incubator with 5% CO₂ before our timed experiments.

Cells positively selected by the anti-Thy1.1 antibody were plated at a density of approximately 2×10^4 cells in each well of two-chamber glass slides or 3×10^3 cells in each well of eight-chamber glass slides (Labtek 2; Nalge-Nunc) coated with laminin (0.01 mg/mL; Sigma, St. Louis, MO) and poly-D-lysine (0.01 mg/mL; Sigma) and grown in serum-free, B27-supplemented medium (NeuroBasal; Gibco), as previously described.^{6,7,31-33,35,36} The growth medium also contained 2 mM glutamine, 0.1% genomycin, 1% N₂ supplement (insulin 500 $\mu\text{g}/\text{mL}$; transferrin 10 mg/mL; progesterone 630 ng/mL; putrescine 1.6 mg/mL and selenite 520 ng/mL; Gibco), 50 ng/mL brain-derived neurotrophic factor (Invitrogen, Carlsbad, CA), 20 ng/mL ciliary neurotrophic factor (Invitrogen), 10 ng/mL bFGF (Invitrogen), and 100 μM inosine (Sigma). Before our timed experiments, RGCs were maintained with the medium described above in a standard incubator with 5% CO₂ until homeostasis was reached, as determined by neurite outgrowth and a stable level of viability (4–6 days).

Hydrostatic Pressure Experiments

We constructed a pressure chamber of polycarbonate (Lexan; GE Plastics, Pittsfield, MA), equipped with a regulator and a gauge mounted directly to the lid. For pressure experiments, the entire chamber was placed in a 37°C oven, and an air mixture of 95% air and 5% CO₂ was pumped into the chamber at a constant pressure maintained by the regulator. For ambient pressure experiments, cells were kept in a standard incubator. Elevated intraocular pressures in glaucoma patients typically range between 20 and 35 mm Hg and are accompanied by RGC loss over a period of years. To determine the short-term effects of elevated pressure on RGCs, we exposed our cultures to a much higher pressure based on the literature for in vitro models (33–60 mm Hg)^{6,37-43} and acute in vivo models (100–140 mm Hg).^{41,44-47} We evaluated RGC death in our pressure chamber over time and at pressures between 30 and 70 mm Hg and determined that 70 mm Hg produced the most reliable and measurable loss of RGCs in a relatively short time (24–48 hours). For lesser pressures and longer times (>4–6 days), the attrition in culture made experimental manipulation unrealistic. Thus, we conducted all pressure experiments at +70 mm Hg for exposures of 8 to 72 hours, subsequent to the short period of stabilization.

In Situ Apoptosis Assay and Quantification

We assessed apoptosis of RGCs using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Chemicon, Temecula, CA). In this assay, fluorescein-tagged oligonucleotides specifically hybridize to the free 3'-OH DNA termini that occur at breaks in DNA cleaved by endonucleases activated during late-stage apoptosis.^{48,49} Cultures were fixed in 100% acetone for 10 minutes, air dried, incubated for 1 hour at 37°C in the TUNEL solution, counterstained with a 1:100 dilution of DAPI nuclear stain (Molecular Probes, Eugene, OR) in d.d.H₂O for 5 minutes at room temperature, and coverslipped. We photographed 10 random fields in each well of the

culture plate to obtain a minimum of 30 fields for each experimental condition. To obtain cell density measurements, we counted the total number of DAPI-positive cells in each field and divided by the area of the field. For quantification of apoptotic RGCs, we calculated the number of TUNEL-positive cells to the total number of cells in a given field (Image Pro Plus, version 4.5.1; Medium Cybernetics, San Diego, CA).

Cytotoxicity Assay

As previously described,⁵⁰⁻⁵² we used a lactate dehydrogenase (LDH) assay to determine the extent of necrotic cell death in RGC cultures after exposure to elevated pressure (Promega, Madison, WI). The LDH assay was performed according to the manufacturer's specifications. To measure LDH release in response to various experimental conditions, equal volumes of culture medium and tetrazolium salt solution were incubated for 30 minutes at room temperature, and optical density was measured by a microplate reader (Molecular Devices, Sunnyvale, CA). To measure maximal LDH release for each culture, cells were lysed before LDH assay. The percentage of cytotoxicity was calculated for each culture as the ratio of LDH release under experimental conditions to maximum possible release multiplied by 100. Fresh medium was used as a control for background noise created by phenol red and spontaneous LDH release. For statistical comparison between treatment groups, we analyzed the percentage of cytotoxicity as the mean percentage across a minimum of three experiments.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from cultured cells with Trizol (Invitrogen), according to manufacturer's instructions. The RNA was treated with DNase (Invitrogen), to remove contaminating DNA and with RNase Out to inhibit the degradation of total RNA by RNase (Invitrogen). Isolated RNA was incubated with oligo dT primer (1 μM) in a solution containing 100 mM dNTP and H₂O at 65°C for 5 minutes before the first-strand reaction, which was performed with reverse transcriptase for 1 hour at 50°C and for 1 hour at 55°C (Invitrogen). Second-strand synthesis was performed with DNA polymerase (Invitrogen) with a primer that contained (5'-3') a 20-mer heel sequence absent from the mammalian database, a stretch of five random nucleotides, and a defined pentameric sequence at the 3' end (Invitrogen). Gene-specific PCR was conducted in a 25- μL reaction (5 μL RT product, $\times 10$ polymerase buffer, polymerase [Invitrogen], and 1 μM forward and reverse primers) for 30 cycles. Primer sequences for *actin* (5'-TCC TGG GTA TGG AAT CCT GTG G-3'; 5'-CTT GAG TCA AAA GCG CCA AAA C-3'), *bax* (5'-TTCTTG GTGGACGCATCC TG-3'; CCCAC-CAGCTCTGAA CAGCAGATCATG-3'), *bcl-2* (5-AGGTAT GCACCCAGAGT-GATGC-3; 5-CATGTGTGTGGA GAGCGCAAC-3), *cjun* (5'-CTACGC-CAACCT CAGCAACTTCAA-3'; 5'-CTGTTTAAGCTGTGC CACCTGTTC-3'), and *jun-B* (5'-GGTCTACAC CAACCTCAGCAGTTA-3'; 5'-TTCT-CAGCCTTG AGTGTCTTACC-3') were designed against rat (*cjun*, *jun-B*) or mouse (*actin*, *bax*, *bcl-2*) mRNA sequences that span an intron of gene and produced PCR products of 514 bp, 137 bp, 240 bp, 433 bp, and 381 bp, respectively (Integrated DNA Technologies, Coralville, IA). These PCR products were separated on an agarose gel stained with ethidium bromide and digitally imaged on a gel reader (Alpha Innotech, San Leandro, CA). To evaluate contamination of RNA samples by genomic DNA, PCR was conducted on RNA samples from each culture.

Conditioned Medium Experiments, Neutralization of IL-6, and Phorbol Ester Experiments

Conditioned medium from retinal glial cells was added to the culture medium of RGCs to produce a 10% dilution. As a control, for RGCs maintained at ambient or elevated pressure in the absence of conditioned medium, RGC growth medium was added in a volume equal to that of the conditioned medium. For depletion of IL-6 from the glia-derived medium, we added 0.09 $\mu\text{g}/\text{mL}$ anti-rat IL-6 antibody (ND₅₀ = 0.03–0.09 $\mu\text{g}/\text{mL}$; R&D Systems, Minneapolis, MN) to the conditioned medium 1 hour before treatment of the RGCs. This antibody is widely used and is known to reliably inhibit bioactivity of IL-6 in vitro and in vivo.⁵³⁻⁵⁶ To verify our

ability to detect secretion of IL-6, we exposed cells to the phorbol ester, phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO). PMA is known to induce cytokine production in glial cells and in macrophages.⁵⁷⁻⁶² A 1 mM stock of PMA in dimethyl sulfoxide (DMSO; Sigma) solvent was added to 1 mL culture medium to yield a final concentration of 1 μ M. After 10 to 14 days in culture, cells were exposed to PMA in a standard incubator for 7 hours. To control for solvent effects, control cells were treated with an equal volume of DMSO.

Enzyme-Linked Immunoabsorbent Assay

The medium was immediately removed from cultures and centrifuged at 300g to remove any intact cells and again at 15,000g to remove any cellular debris. ELISAs were conducted with an anti-rat IL-6 kit (R&D Systems) containing precoated 96-well plates and were performed according to manufacturer's recommendations. An equal volume of sample and diluent was added to each well and was incubated for 2 hours at room temperature. Each sample well was run in duplicate, thus providing six measurements per *n* of three. The plate was washed and incubated in the conjugate antibody solution for another 2 hours at room temperature. Washes were repeated, and substrate solution was added to each well and incubated for 30 minutes at room temperature. The reaction was quenched, and the optical density of each well was measured using a microplate reader (Molecular Devices). To determine the sensitivity of the IL-6 ELISA kit, we calculated the minimal concentration of cytokine necessary for detection. Based on the experimental OD values, we performed a back calculation of cytokine concentration for the zero standards in 16 separate assays. These concentrations were averaged, and the SD was multiplied by 2 to obtain the minimum reliable detection of 3.4626 pg/mL.

Immunocytochemistry

Immunocytochemistry (ICC) against cell type-specific markers was performed on primary RGC, astrocyte, and microglia cultures. Cultures were fixed in 10% formalin for 10 minutes and washed in phosphate-buffered saline (PBS). To quench autofluorescence, samples were treated with 0.1% sodium borohydride for 30 minutes at room temperature, then washed in PBS and incubated in a blocking solution containing 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for 2 hours at room temperature. Primary antibodies used were mouse anti-rat Thy1.1 (5 μ g/mL; BD PharMingen), rabbit anti-bovine glial acidic fibrillary protein (GFAP; 10 μ g/mL; DAKO, Carpinteria, CA), mouse anti-human fibroblast (3 μ g/mL; DAKO), rabbit anti-human CD68 (4 μ g/mL; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-rat RT1a/OX18 IgG (1 μ g/mL; Chemicon), and mouse anti-human cyclin d3 (2.5 μ g/mL; BD PharMingen). Samples were incubated overnight at 4°C in a solution containing primary antibody, 3% NGS, and 0.1% Triton X-100 in PBS, washed four times in PBS, and incubated for 2 hours at room temperature in a secondary antibody solution containing 1:200 secondary antibody (goat anti-mouse IgG, goat anti-rabbit IgG, goat anti-mouse IgM; Molecular Probes), 1% NGS, and 0.1%

Triton X-100 in PBS. Samples were again washed in PBS and either coverslipped or counterstained with DAPI. Controls for ICC experiments were conducted with no primary antibody and with various IgG isotypes.

Statistical Analysis

All preparations, experiments, and measurements were performed at least in triplicate. We applied an unpaired Student *t* test, where *P* < 0.05 was significant, to compare the means of groups of different treatments (e.g., ambient versus elevated pressure) and one-way analysis of variance (ANOVA) to compare means across multiple groups of the same treatment (e.g., within ambient pressure experiments). For analyzing our PCR results, we collected densitometry readings from each reaction product and normalized to the expression level of actin under the same conditions. We calculated a χ^2 statistic to determine the departure from the null of the ratio at high pressure to that at ambient pressure. All data are reported as mean \pm SEM.

RESULTS

Homogeneity of Primary Retinal Cultures

Immunomagnetic separation is widely used for purifying RGCs and for depleting microglia.^{6,7,31-33,35,36} To confirm the purity of our RGC cultures, we tested the expression of Thy-1 and the absence of markers for other retinal cells (Fig. 1). RGCs highly expressed Thy-1 and extended an axonlike process and multiple dendritic processes (Fig. 1). This morphology is similar to that described in other recent studies.⁷ RGC cultures did not express a variety of glial cell markers, including glial fibrillary acidic protein (GFAP; astrocytes), CD68 (microglia and macrophages), and cyclin d3 (Müller cells⁶³; Fig. 1). We also tested for fibroblast infiltration using immunocytochemistry and PCR and found none (data not shown).

The use of immunomagnetic separation for the positive-selection of retinal astrocytes and microglia for cell culture is novel. To isolate astrocytes, we used an antibody that recognizes a 200-kDa surface protein isolated from human brain astrocytes (catalog no. MAB5314; Chemicon). Western blot analysis confirmed recognition of the same protein in retinal extracts from postnatal and adult rats (Fig. 2A). In the adult rat retina, immunolabeling with this antibody colocalizes in the nerve fiber layer with GFAP (Fig. 2B). In culture, the cells isolated with the anti-astrocyte antibody were three-dimensional and phase-bright and exhibited the same morphology typical of astrocytes isolated for culture by other means (Fig. 3A).⁶⁴ Approximately 98% of these cells were immunopositive for GFAP and negative for microglia, fibroblast, and Müller cell markers (Fig. 3B); the remainder could not be labeled and probably were not viable.

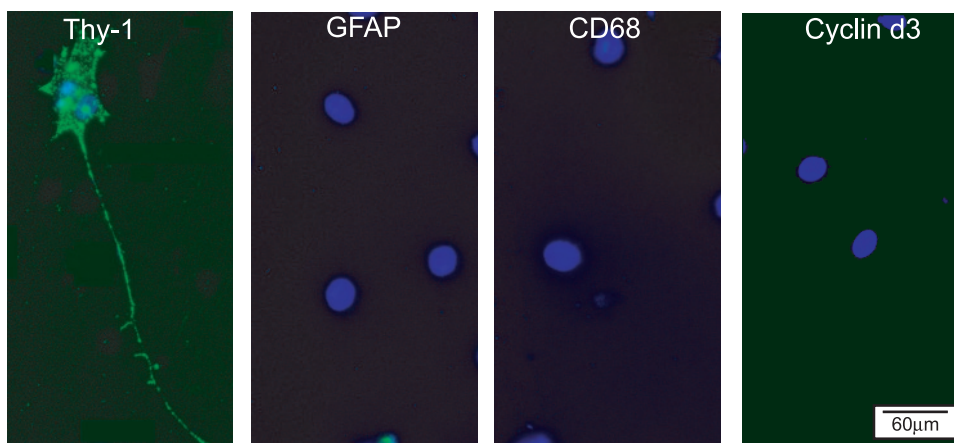


FIGURE 1. Homogeneity of RGC primary cultures. RGC cultures counterstained with the nuclear dye DAPI (blue) express Thy1.1 (green) but not the astrocyte marker GFAP (green), the microglia marker CD68 (green), or the Müller cell marker cyclin d3 (green). Scale applies to each panel.

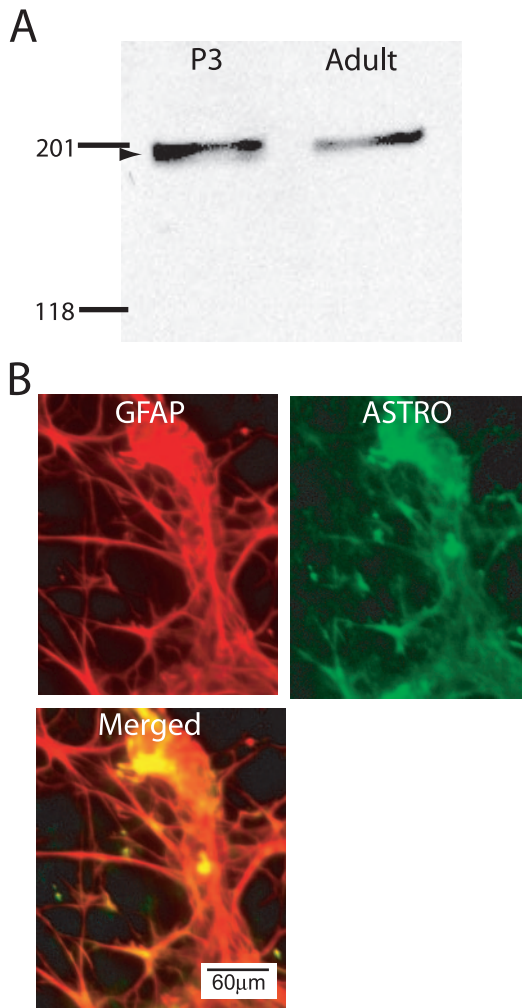


FIGURE 2. Anti-human astrocyte antibody against an unknown surface protein colocalizes with GFAP in the nerve fiber layer of rat retina. (A) Western blot of anti-human astrocyte (IgM) antibody against protein extract from postnatal day 3 (P3) and adult rat retina demonstrating a single band at 200 kDa (arrowhead). (B) High-magnification light micrograph of rat retina immunolabeled with GFAP (red) and antiastrocyte (green) antibodies. The merged image demonstrates colocalization to astrocytes of the nerve fiber layer. Scale applies to each panel.

For isolation of microglia, we used a monoclonal antibody against OX18, a major histocompatibility class I antigen that is expressed by microglia in retina and brain of rats.^{65–68} These cells were three dimensional and phase bright and exhibited either an ameboid or a ramified shape, which is consistent with the morphology of microglia maintained in vitro by other means (Fig. 3C).⁶⁹ Approximately 97% of these cells exhibited positive label for the microglia markers, CD68 and OX18, but were negative for Müller cell, astrocyte, and fibroblast markers (Fig. 3D). Again, the remainder of the cells could not be labeled and probably were not viable.

Pressure-Induced Apoptosis of RGCs In Vitro

The death of RGCs in certain models of glaucoma is known to be apoptotic.^{3,6,70–73} In our experimental system, exposure to 70 mm Hg pressure for 48 hours led to clear morphologic changes in RGCs, including the retraction of cellular processes, a more rounded appearance, and decreased density (Fig. 4). These morphologic changes were accompanied by increased TUNEL staining (Fig. 4). Quantitatively, in the postplating period necessary to reach homeostasis, RGCs stabilized at den-

sities of 550 to 650 cells/mm². We maintained cultures at ambient pressure for an additional 48 hours without significant change in density ($P = 0.60$; Fig. 5A), and the variability for this period was small (552 ± 22 cells/mm²; Fig. 5A). Exposure to either 6 or 12 hours of elevated pressure induced little or no change in survival ($P = 0.98$ and $P = 0.69$, respectively; Fig. 5A). However, after 24 hours of elevated pressure, the density of RGCs decreased by 27% compared with their ambient levels at the same time point ($P < 0.01$; Fig. 5A). Similarly, exposure for 48 hours induced a 37% decrease in RGC density from ambient levels for the same time ($P < 0.01$; Fig. 5A). Because the densities of RGCs did not change appreciably for 6- and 12-hour exposures, we focused additional measurements on the longer exposures. At 24 hours of elevated pressure, the fraction of TUNEL-positive RGCs increased by 65% from the fraction at ambient pressure for the same time ($P < 0.01$); at 48 hours of pressure, the fraction nearly doubled from the ambient level ($P < 0.01$; Fig. 5B). Thus, exposure to elevated pressure for 24 hours or more induces a decrease in cell density that is accompanied by a reciprocal increase in TUNEL-positive cells. The small concentration of cytosolic LDH in the medium did not change after 24 hours of elevated pressure ($P = 0.17$; Fig. 5C), indicating no change in the degree of necrotic death.

RGC death in glaucoma models and with various other insults is coupled to Bax-dependent mitochondrial pathways.⁷⁴ As such, we examined changes in expression of mRNA encoding both Bax and another BH3 domain protein, Bcl-2. At ambient pressure, RGCs expressed trace levels of *bax*, indicative of the small degree of cell death occurring in culture (Fig. 6A). Expression of *bax* mRNA increased significantly after 24 hours of elevated pressure ($P < 0.01$; Fig. 6A). Expression of the antiapoptotic gene *bcl-2* also rose significantly after 24 hours of pressure ($P = 0.03$; Fig. 6A). At 48 hours, these trends continued, with significant increases in expression at elevated pressure for both *bax* ($P = 0.04$) and *bcl-2* ($P \ll 0.001$). Interestingly, expression of *bax* and *bcl-2*, when normalized to actin, decreased dramatically between 24 and 48 hours ($P = 0.05$; Fig. 6A). Induction of immediate early genes from the Jun and Fos families often precedes transcription of apoptosis-related genes, including *bax* and *bcl-2*.^{75–77} Thus, we examined expression of *c-jun* and *jun-B* in RGCs maintained at ambient or elevated pressure for 24 hours. After 24 hours at ambient pressure, RGCs did not express appreciable levels of *c-jun* or *jun-B* (Fig. 6B). However, after 24 hours of elevated pressure, expression of both *c-jun* ($P = 0.02$) and *jun-B* ($P < 0.01$) rose significantly (Fig. 6B).

Pressure-Induced Secretion of IL-6 by Retinal Glia

Previous studies^{4–6,39} describe pressure-induced changes in the release of TNF- α and the production of nitric oxide by retinal and optic nerve glia. We sought to examine the impact of elevated pressure on the release of IL-6 by astrocytes and microglia. To verify that retinal microglia and astrocytes maintained in culture can secrete measurable levels of IL-6, we treated the cultures with 1 μ M of the phorbol ester PMA and used ELISA to measure the concentration of IL-6 released into the medium. For astrocytes, IL-6 release rose from 111 pg/mL to 6054 pg/mL with PMA treatment, a 54-fold increase ($P < 0.01$; Fig. 7A). For microglia, IL-6 rose from 247 pg/mL to 4917 pg/mL with PMA treatment, a 20-fold increase ($P < 0.01$; Fig. 7B). Therefore, retinal astrocytes and microglia in isolation are capable of secreting very high levels of IL-6.

In a companion study, we maintained astrocytes and microglia at elevated pressure over a range of exposure times and found that 24 hours of exposure induced the maximal change in IL-6 release by astrocytes and microglia relative to release at ambient pressure (data not shown). Consistent with this finding, elevated

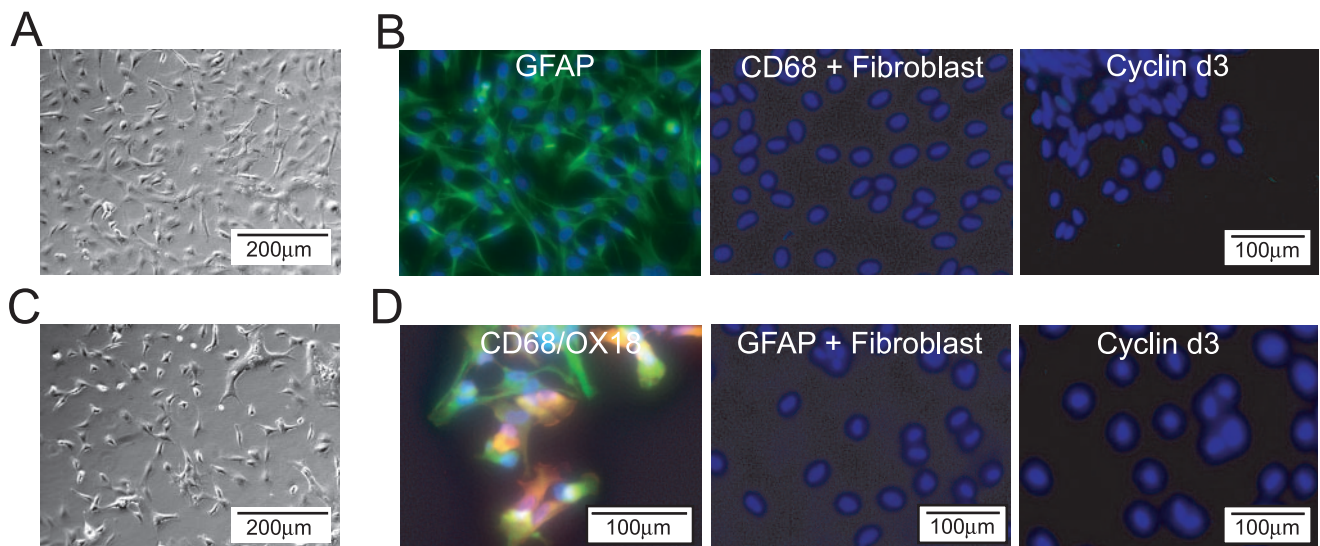


FIGURE 3. Homogeneity of retinal glia primary cultures. (A) Phase-contrast micrograph of astrocytes in primary culture grown to 90% confluence for 14 days. (B) Astrocyte cultures counterstained with the nuclear dye DAPI (blue) express GFAP (green), as expected, but not CD68 (green), fibroblast antigen (red), or cyclin d3 (green). Scale applies to each panel. (C) Phase-contrast micrograph of primary microglia culture grown to 60% confluence for 10 days. (D) Microglia cultures counterstained with DAPI (blue) express CD68 (green) and OX18 (red), as expected, but not GFAP (green), fibroblast antigen (red), or cyclin d3 (green). Scale applies to each panel.

pressure in this study induced a fourfold reduction in IL-6 release by astrocytes ($P < 0.01$) but a twofold increase in release by microglia ($P < 0.01$; Fig. 7B). Therefore, exposure to elevated pressure induces opposite effects on IL-6 release by astrocytes and microglia, resulting in an 1100 pg/mL difference in the concentration of secreted IL-6. This large difference in IL-6 concentration at 24 hours suggests that astrocyte-derived and microglia-derived signals may exert different effects on RGC survival at elevated pressure. We tested this possibility next.

Effect of Glia-Derived IL-6 on RGC Survival

Our analysis of pressure-induced RGC death indicates a nearly twofold increase in TUNEL-labeled cells between 24 and 48 hours of pressure and an 18% decrease in the density

of RGCs, indicating an increased rate of apoptosis. Thus, we focused on RGCs exposed to elevated pressure for 48 hours to determine how signals released by retinal glia might influence survival. We exposed astrocytes and microglia to ambient or 70 mm Hg pressure for 24 hours and removed the medium. We then treated RGC cultures for 48 hours at either ambient or elevated pressure with a 10% dilution of medium collected from the glia. To ensure that medium obtained from glia under normal conditions did not alter RGC survival, we measured the cell density of RGCs treated with medium from astrocytes and microglia maintained at ambient pressure. At ambient pressure, treatment with neither astrocyte nor microglia medium altered the density of RGCs (Fig. 8A). Similarly, neither astrocyte nor microglia

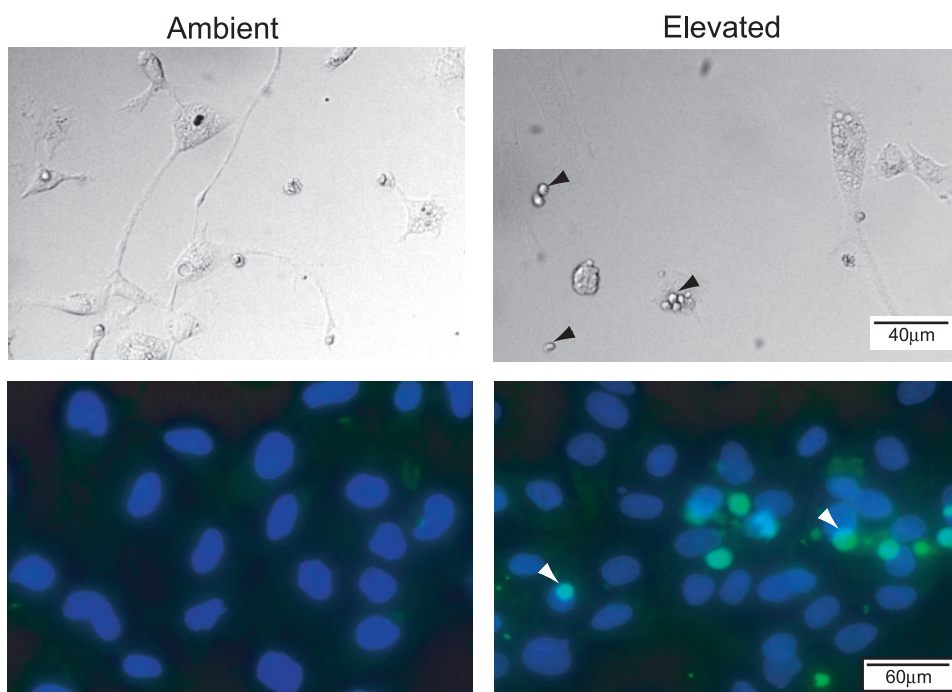


FIGURE 4. Elevated pressure induces apoptosis of RGCs. *Top:* Differential interference contrast (DIC) micrographs of RGC primary cultures exposed to either ambient (*left*) or elevated (*right*) pressure for 24 hours. Pressure promotes changes in morphology, including retraction of cellular processes and formation of vacuoles and apoptotic bodies (*arrowheads*). Scale applies to both micrographs. *Bottom:* TUNEL-labeling (green) of DAPI-stained RGCs (blue) exposed to ambient (*left*) or elevated (*right*) pressure for 24 hours. Label increases with elevated pressure, almost exclusively in condensed nuclei (*arrowheads*). Scale applies to both panels.

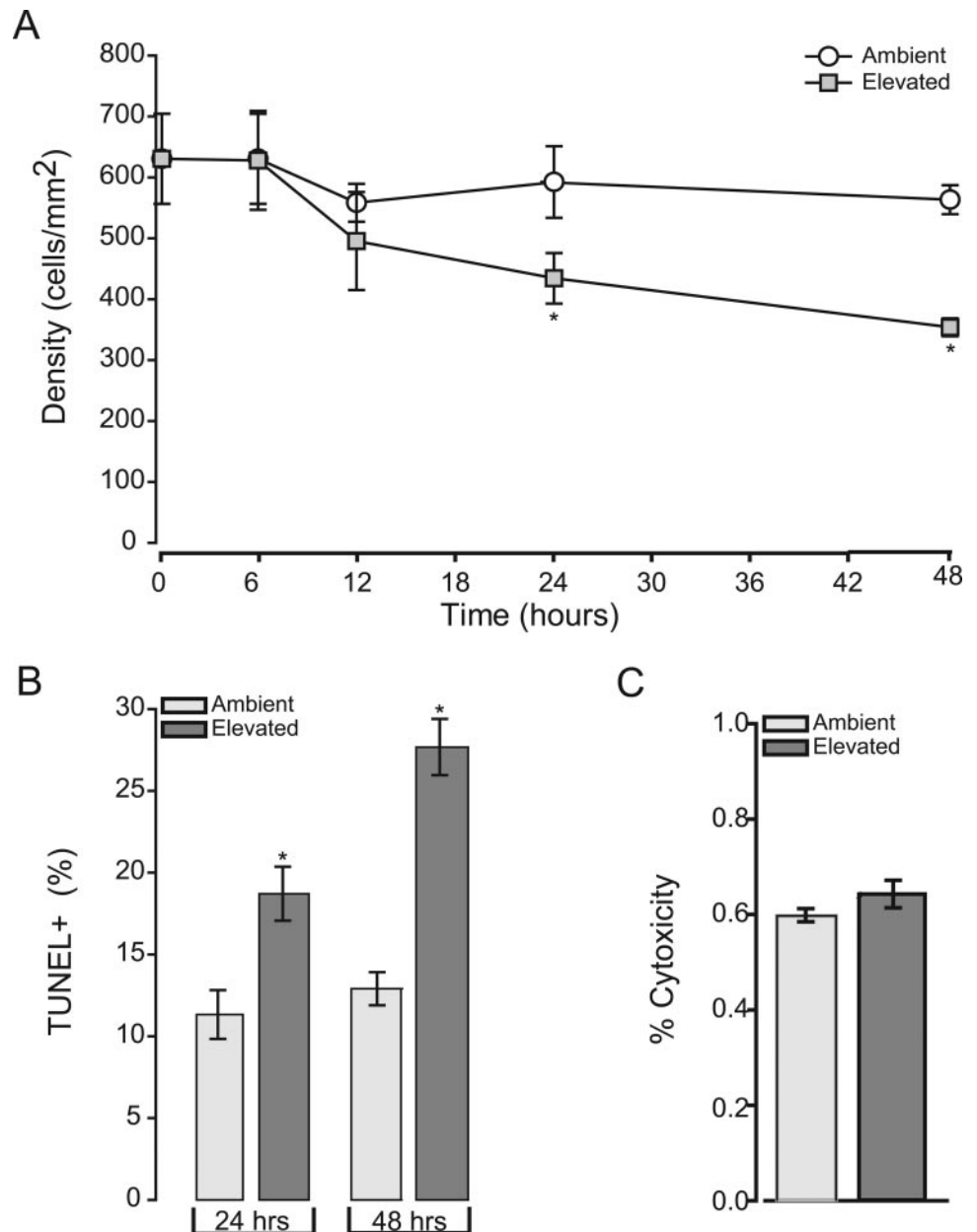


FIGURE 5. Quantification of RGC apoptosis. (A) Density of RGCs maintained at either ambient or elevated pressure for 0 to 48 hours, determined by the number of DAPI-stained nuclei per unit area. At ambient pressure, RGC density does not change significantly between 6 and 48 hours (*open symbols*). At elevated pressure, RGC density is reduced by 27% after 24 hours and 37% after 48 hours (*closed symbols*). (B) The percentage of TUNEL-positive RGCs increases by 65% after 24 hours of elevated pressure and nearly twofold after 48 hours. The percentage of TUNEL-positive RGCs at ambient pressure does not increase significantly between 24 and 48 hours. (C) Percentage of cytotoxicity of RGC cultures, as determined by LDH release, does not change with 24-hour exposure to elevated pressure. * $P \leq 0.05$ represents statistical difference from ambient pressure for the same time point.

medium altered the pressure-induced decrease in RGC density (Fig. 8A). Given that media obtained from astrocytes and microglia at ambient pressure did not alter the survival of RGCs, all further comparisons were made between untreated RGCs and those treated with pressure-conditioned glia medium. For RGCs maintained at ambient pressure, treatment with the pressure-conditioned medium from either astrocytes or microglia did not alter the overall cell density ($P = 0.62$, astrocytes; $P = 0.25$, microglia) or the percentage of TUNEL-positive cells ($P = 0.24$, astrocytes; $P = 0.36$, microglia; Figs. 8B, 8C). However, when depleted of IL-6 by neutralizing antibodies, astrocyte- and microglia-conditioned media decreased the density of RGCs at ambient pressure by 63% ($P < 0.01$) and 18% ($P = 0.01$), respectively (Figs. 8B, 8C). These decreases in cell density were accompanied by 48% and 27% increases in the percentage of TUNEL-positive RGCs, respectively (Fig. 8B, 8C).

For RGCs at elevated pressure, astrocyte-conditioned medium further decreased cell density by 38% ($P < 0.01$) and increased the percentage of TUNEL-positive cells by 23%

($P = 0.05$; Fig. 8B). In contrast, treatment with microglia-conditioned medium induced a 33% increase in RGC density ($P < 0.01$) and a corresponding 38% decrease in the fraction of TUNEL-positive RGCs ($P < 0.01$; Fig. 8C). Depletion of IL-6 did not alter the effects of astrocyte-derived medium on cell density ($P = 0.71$) or on the percentage of TUNEL-positive RGCs ($P = 0.68$; Fig. 8B). However, depletion of IL-6 from the microglia medium further decreased the cell density of RGCs exposed to elevated pressure by 32% ($P < 0.01$), which was equivalent to the density of RGCs exposed to pressure alone ($P = 0.34$; Fig. 8C). This decrease in cell density was accompanied by a 31% increase in the number of TUNEL-positive cells ($P = 0.05$; Fig. 8C). Thus, the removal of IL-6 reversed the protective effect of microglia medium for RGCs exposed to pressure. These data suggest that pressure-induced release of IL-6 by astrocytes and microglia has the potential to influence the survival of RGCs. However, only IL-6 release by microglia increased the survival of RGCs exposed to elevated pressure.

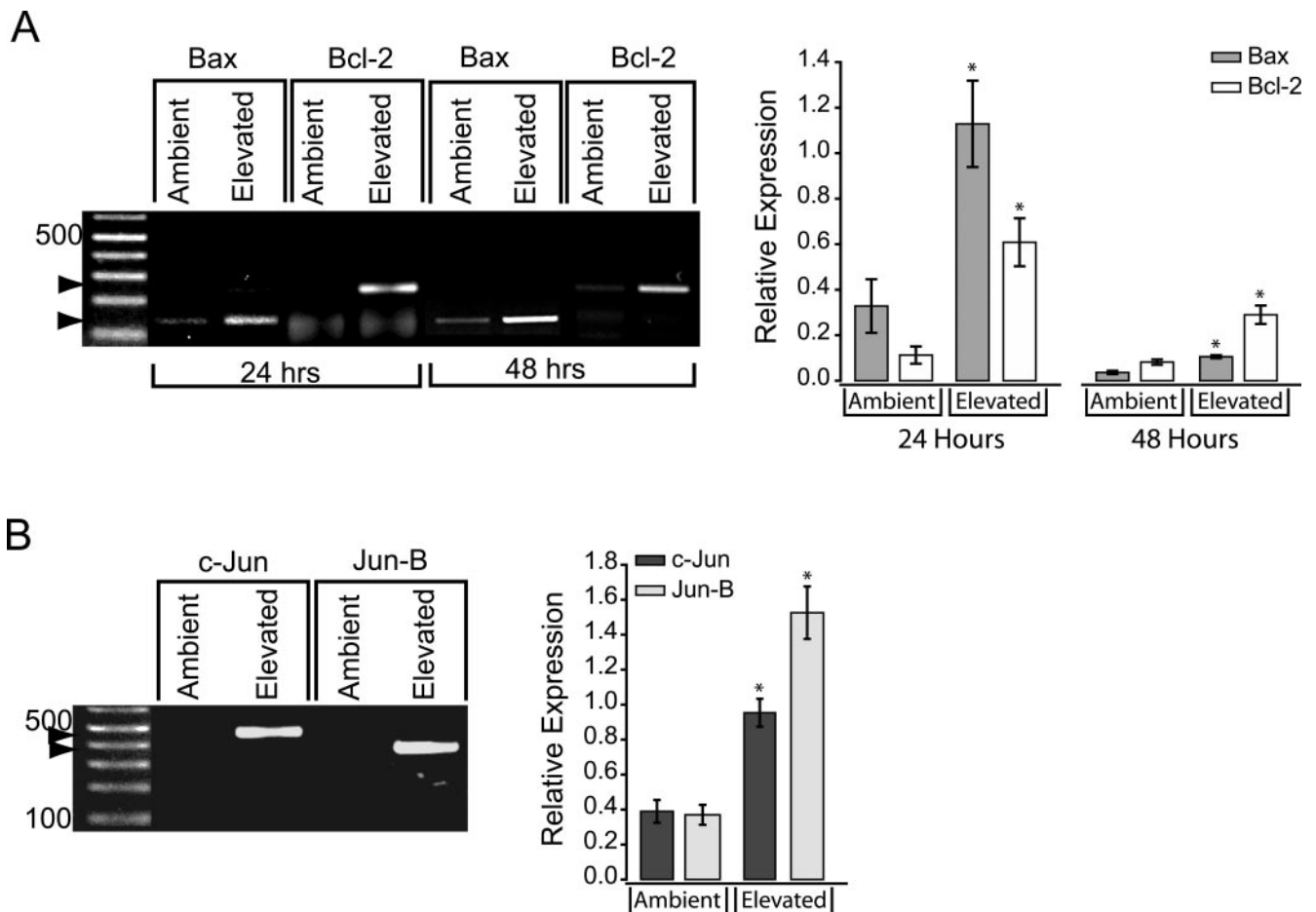


FIGURE 6. Elevated pressure increases mRNA expression of apoptosis-related genes in RGCs. (A) Expression of *bax* and *bcl-2* mRNA in RGCs (left) increases with exposure to 24 and 48 hours of elevated pressure compared with ambient pressure. However, *bax* and *bcl-2* expression decreases between 24 and 48 hours of elevated pressure. Arrowheads indicate expected size of PCR products. Densitometry measurements for *bax* and *bcl-2* expression in RGCs (right) exposed to ambient or elevated pressure are presented as a ratio of mRNA expression for β -actin. (B) Expression of immediate early genes *c-jun* and *jun-B* in RGCs (left) increases with exposure to 24 hours of elevated pressure. Arrowheads indicate expected size of PCR products. Densitometry measurements for *c-jun* and *Jun-B* expression in RGCs (right) exposed to ambient or elevated pressure are presented as a ratio of mRNA expression for β -actin. * $P \leq 0.05$ represents statistical difference from ambient pressure.

Effect of Recombinant IL-6 on RGC Survival

We next tested the sufficiency of IL-6 to inhibit pressure-induced death of RGCs. We treated RGC cultures with recombinant rat IL-6 (rIL-6) at concentrations identical to those in the astrocyte-conditioned medium (10 pg/mL) and the microglia-conditioned medium (120 pg/mL) and exposed the cultures to ambient or elevated pressure for 48 hours. At ambient pressure, neither 10 pg/mL nor 120 pg/mL of rIL-6 altered the density of RGCs ($P = 0.20$ and $P = 0.31$, respectively; Fig. 8D). Similarly, neither concentration of rIL-6 altered the percentage of TUNEL-positive RGCs ($P = 0.98$ and $P = 0.36$, respectively; Fig. 8D). Consistent with our previous experiments, exposure to 48 hours of elevated pressure decreased the density of RGCs by 35% ($P \ll 0.01$) and increased the percentage of TUNEL-positive cells by twofold ($P \ll 0.01$; Fig. 8D). Treatment with 10 pg/mL rIL-6 induced a 92% increase in the density of RGCs ($P \ll 0.01$) and a corresponding 58% decrease in the percentage of TUNEL-positive RGCs ($P \ll 0.01$; Fig. 8D). This increase in the density of RGCs exceeded that of ambient levels by 20% ($P = 0.01$; Fig. 8D). Similarly, treatment with 120 pg/mL rIL-6 increased the density of RGCs by 84% ($P \ll 0.01$) and decreased the percentage of TUNEL-positive RGCs by 50%

($P \ll 0.01$; Fig. 8D). These levels of RGC density and the percentage of TUNEL-positive cells were equivalent to those measured at ambient pressure ($P = 0.35$ and $P = 0.59$, respectively; Fig. 8D). Thus, treatment with 10 to 120 pg/mL IL-6 alone has the ability to inhibit the pressure-induced death of RGCs.

DISCUSSION

In this study, we examined the impact of glia-derived IL-6 on the pressure-induced death of RGCs in vitro. We also described a novel technique for producing primary cultures of purified astrocytes and microglia from rat retina (Figs. 2, 3). We confirmed that exposure to 70 mm Hg hydrostatic pressure induces apoptosis in RGCs (Figs. 4, 5) and found that this pressure-induced apoptosis involves the induction of immediate early genes (Fig. 6). Exposure to 70 mm Hg hydrostatic pressure for 24 hours decreased IL-6 release by retinal astrocytes but increased release by retinal microglia (Fig. 7). Our conditioned medium experiments indicated that astrocyte-derived factors exacerbate pressure-induced death of RGCs, whereas microglia-derived factors counter it (Figs. 8A-C). Finally, IL-6 contributes to the protective effect

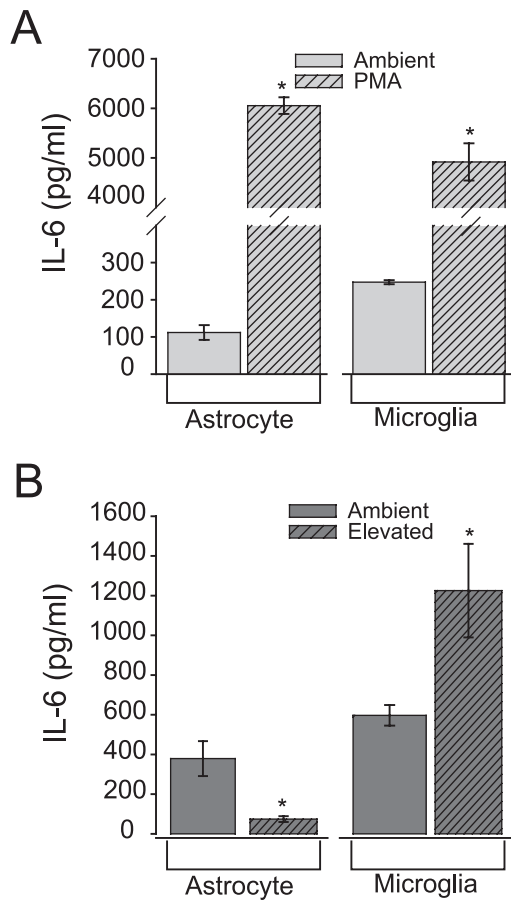


FIGURE 7. Elevated pressure decreases astrocyte IL-6 release but increases release from microglia. (A) ELISA-measured concentration of secreted IL-6 increases 54-fold in astrocytes and 20-fold in microglia from ambient levels after 10 to 14 days in culture and treatment for 7 hours with 1 μ M PMA (B). Exposure to elevated pressure for 24 hours reduces IL-6 secretion by astrocytes but increases secretion by microglia compared with ambient levels. * $P \leq 0.05$ represents statistical difference.

of microglia-derived factors on RGC survival, and treatment with recombinant IL-6 is sufficient to mimic this protective effect (Fig. 8D).

Consistent with previous findings,^{3,4,6,39,70-73} we determined that pressure induces RGC death in vitro through an apoptotic mechanism that is at least partially mitochondria dependent. We observed morphologic changes (e.g., retraction of processes) consistent with a preapoptotic state in cultured cells,⁷⁸ time-dependent decreases in cell density and corresponding increases in DNA cleavage (TUNEL reactivity), and increases in gene expression of the apoptosis-related proteins Bax and Bcl-2 (Figs. 4-6). Upregulation of *bax* and *bcl-2* by RGCs indicated an active regulation of cell survival during exposure to elevated pressure. Although *bax* and *bcl-2* expression increased compared with ambient pressure, expression of both genes relative to actin decreased dramatically between 24 and 48 hours of elevated pressure (Fig. 6). This decrease in expression is consistent with our observation of a twofold increase in RGC TUNEL reactivity at 48 hours, which marks the end stage of apoptosis (Fig. 5B). The changes in *bax* and *bcl-2* expression suggest that elevated pressure also induces the activation of upstream factors that would regulate gene transcription. Activation of immediate early genes is typically considered one of the first steps in stimuli-dependent gene transcription and is associated with the transcription of *bax* and

bcl-2 before the initiation of apoptosis.⁷⁵⁻⁷⁷ Previous studies^{79,80} demonstrate a role for disease-related activation of *c-jun* and *c-fos* in in vivo models of glaucoma. Here we found that *c-jun* is induced by elevated pressure in RGCs in vitro; we also describe the novel finding that *jun-B* is induced in the same temporal pattern (Fig. 6).

The idea that glia in the optic nerve head influences the survival of RGCs in glaucoma is widely accepted.^{4-6,39,81} However, the contribution of retinal glia to the pathologic processes associated with glaucoma is less clear. Consistent with studies using astrocytes isolated from optic nerve head,⁶ we determined that factors produced by retinal astrocytes at elevated pressure decrease the survival of RGCs under pressure (Fig. 8A). In contrast, we also presented the novel finding that microglia-derived factors attenuate pressure-induced apoptosis of RGCs. Interestingly, neither astrocyte nor microglia medium altered the survival of RGCs at ambient pressure (Figs. 8A, 8B). In addition, medium from astrocytes and microglia maintained at ambient pressure did not alter the survival of RGCs at either ambient or elevated pressure. These findings suggest that elevated pressure induces the release of signals from astrocytes and microglia that can directly modulate the survival of RGCs but that RGCs are only sensitive to these factors in the presence of an apoptotic challenge. Furthermore, exposure to elevated pressure is necessary to initiate the release of these signals by astrocytes and microglia.

Previous studies^{4,5,39} indicate that glia-derived nitric oxide and TNF- α can promote RGC death in glaucoma. In contrast, IL-6 and the IL-6-like cytokine CNTF, also implicated in glaucoma, can act to promote the survival of RGCs.^{29,82-84} IL-6 is known to act as an antiapoptotic factor and a neurodestructive factor that can induce glial scarring and angiogenesis.⁸⁵ Here we examined IL-6 derived from retinal glia as a potential modulator of RGC survival in response to elevated pressure. We determined that IL-6 is secreted by astrocytes and microglia in vitro at ambient pressure and that elevated pressure decreases astrocyte IL-6 but increases microglia IL-6 (Fig. 7B). We treated RGCs with conditioned medium from retinal astrocytes and microglia at a 10% dilution, yielding an exposure of 10 pg/mL IL-6 for astrocyte medium and 120 pg/mL IL-6 for microglia medium. Depletion of IL-6 from both conditioned media decreased the survival of RGCs maintained at ambient pressure (Figs. 8A-C), suggesting that IL-6 released by astrocytes and microglia acts to counterbalance proapoptotic factors, most likely TNF- α and NO.^{4,5,39} For RGCs at elevated pressure, depletion of IL-6 from astrocyte medium did not alter RGC survival (Figs. 8A, 8B). In contrast, depletion of IL-6 from microglia medium reversed the enhancement of RGC survival observed with intact microglia medium, suggesting that IL-6 is a key component of microglial response to elevated pressure (Figs. 8A, 8C).

Finally, we demonstrated the sufficiency of IL-6 to inhibit pressure-induced death of RGCs by treating RGCs with recombinant IL-6 at concentrations equivalent to those measured in astrocyte- and microglia-conditioned medium. We showed that 10 pg/mL and 120 pg/mL IL-6 can protect RGCs from pressure-induced death (Fig. 8D). These data suggest that exposure to elevated pressure induces the release of IL-6 by astrocytes and microglia at concentrations that can protect RGCs. However, in the case of astrocytes, the protective effect of IL-6 can be overwhelmed by the simultaneous release of proapoptotic factors. As such, an examination of the usefulness of IL-6 as a neuroprotectant must take into consideration not only the effects of IL-6 concentration itself but also the presence of additional factors with counteractive effects. Further investigations should examine the impact of IL-6 signaling in vivo in which responses can differ

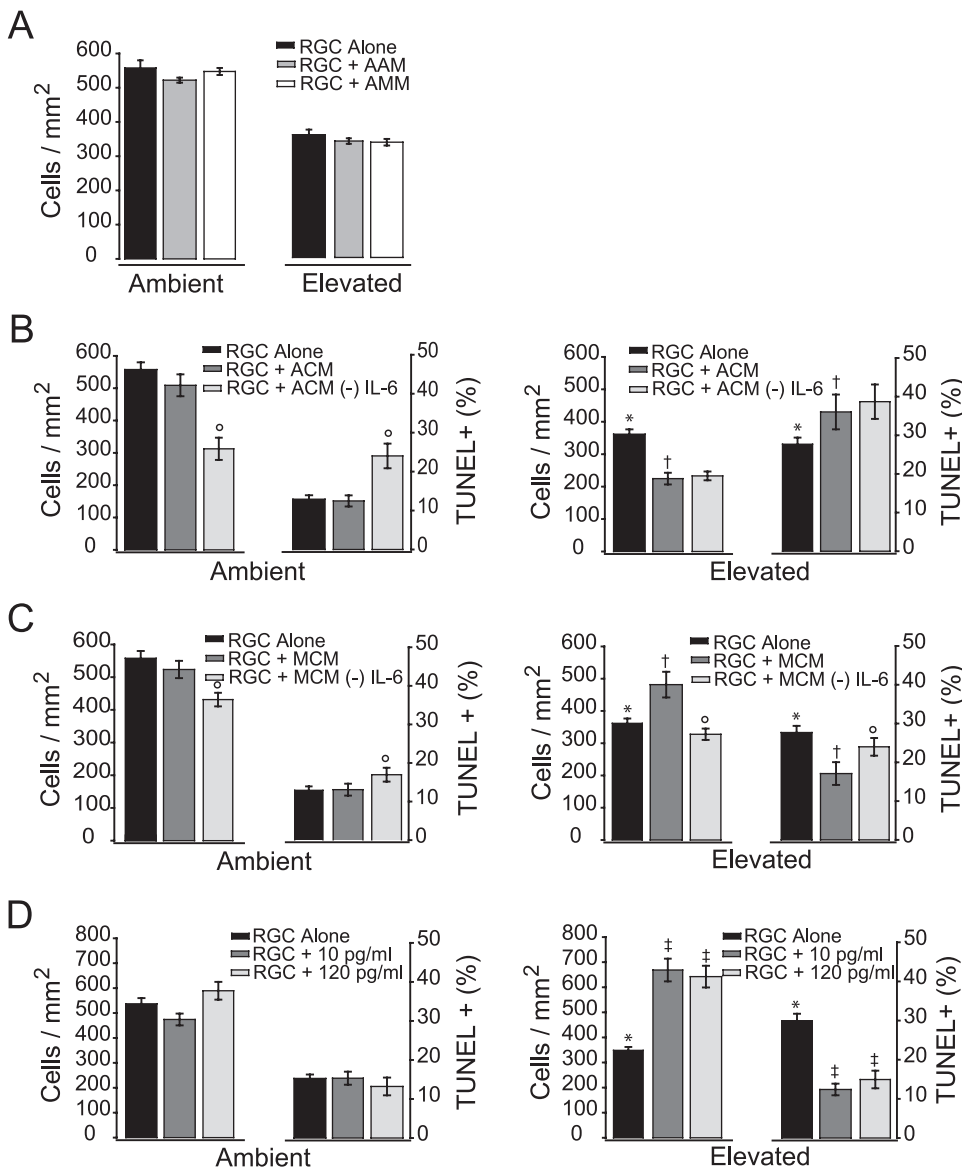


FIGURE 8. Microglia and astrocyte signals modulate RGC survival. (A) Density of RGCs maintained at ambient or elevated pressure for 48 hours. Exposure is for RGCs alone with 10% ambient astrocyte medium (+AAM) or with ambient microglia medium (+AMM). (B) Density and TUNEL-labeling of RGCs maintained at ambient pressure (left) or elevated pressure (right) for 48 hours. Conditions include: RGCs alone with 10% astrocyte conditioned medium (+ACM) or again with ACM and neutralizing antibodies against IL-6 (-IL-6). (C) Same conditions described for (B) but using 10% microglia-conditioned medium (MCM). Data for RGCs alone are actual repeats of the experiment. (D) Survival of RGCs at either ambient (left) or elevated (right) pressure for 48 hours with the addition of recombinant rat IL-6 (10 pg/mL or 120 pg/mL). Data for RGCs alone again are new. *Significant difference for RGCs alone between ambient and elevated pressure ($P \leq 0.05$). †Significance between RGCs alone and RGC+CM at same pressure condition ($P \leq 0.05$). ‡Significance between RGC+CM and RGC+CM (-IL-6) ($P \leq 0.05$). §Significance between RGCs alone and RGC+recombinant IL-6 (10 or 120 pg/mL; $P \leq 0.05$).

from those in vitro.^{83,84} IL-6-activated effectors that are known to be antiapoptotic, such as the JAK and STAT families of transcription factors,^{25–28} should also be evaluated as potential comodulators of RGC survival.

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