Changes in Gene Expression in Experimental Glaucoma and Optic Nerve Transection: The Equilibrium between Protective and Detrimental Mechanisms

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PURPOSE. The authors studied retinal gene expression changes in rats after experimental intraocular pressure elevation and optic nerve transection to elucidate molecular mechanisms of retinal ganglion cell (RGC) death.

METHODS. Translimbal laser photoagulation was used to induce unilateral IOP elevation in 41 albino Wistar rats. In 38 additional animals, unilateral transection of the optic nerve was performed. Retinas were harvested 1 day, 3 days, 1 week, 2 weeks, 4 weeks, and 8 weeks after each treatment, and total RNA was isolated. Pooled RNA from each time point was analyzed with rat genome arrays. Array results were confirmed by real-time PCR, and localization studies were performed using in situ hybridization for select genes.

RESULTS. Genes that were upregulated in glaucoma, but not after transection, included Cyclin D2, Stat1, Stat3, c-fos, Junb, Anxa1, Anxa 3, and CCAAT/enhancer binding protein (Cebp-delta). In glaucoma and transection models, the upregulation of c-Jun, Activating transcription factor 3, Heat shock protein 27, and Timp1 were observed. Comparisons among microarray databases were performed between our data and reports of in situ hybridization studies were performed using in situ hybridization for select genes.

CONCLUSIONS. Gene expression changes specific to experimental glaucoma injury were identified. The present analysis supports the importance of neuroinflammation and the participation of the tumor necrosis factor alpha signaling pathway in glaucoma injury. The alterations observed include processes that are both protective of and detrimental to the survival of RGCs. (Invest Ophthalmol Vis Sci. 2007;48:5539–5548) DOI: 10.1167/iovs.07-0542

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laucoma, the second leading cause of blindness worldwide, involves the progressive degeneration of retinal ganglion cells (RGCs) and alterations of the connective tissues of the optic disc leading to a characteristic excavation. Experimental intraocular pressure (IOP) elevation in animals, including mouse, rat, and rabbit, causes loss of RGCs that simulates aspects of human glaucoma. RGC death in human and experimental glaucoma is attributable to apoptosis. The mechanisms that are being investigated as participants in the complex process of RGC death in glaucoma include impaired neurotrophic support, excitotoxicity, oxidative stress, and neuroinflammation. Recent investigations have found that competing processes supporting RGC survival and promoting RGC death occur simultaneously in the glaucomatous retina.

Microarray-based studies of transcriptomic changes in animal disease models can provide important insight into the mechanisms and pathways involved in human disease. However, interpretation of such microarray research is complicated by several features. First, a large amount of data is generated whose variability requires maximal numbers of animals and careful interpretation so that biologically important changes can be separated from incidental changes. Second, it is difficult to separate primary from secondary expression changes. Third, when whole retinal expression is studied, the contribution of many cell types is included, some of which (such as RGCs) represent a small minority of the total. Finally, as in all experimental animal model research, there may be important differences between the disease in humans and the disease in animals.

Despite these limitations, gene expression studies of animal models of glaucoma also have a number of advantages. Studies of gene expression in human glaucoma eyes can only be performed on postmortem tissue in established disease and at single points in time for each individual. Experimental models permit study of events at multiple time points, including those occurring at the initiation of IOP-induced insult. Although the study of whole retina gives results representing a mixture of gene expression from the entire complement of neurons, glia, and vasculature, this shows the overall response of the retina to the experimental disease process. By comparison, studies of dissociated, cultured RGCs can indicate gene expression unique to RGC, but only under conditions in which their axons have been transected and they are separated from their synaptic partners and surrounding glia.

Microarray technology has recently been applied to animal glaucoma models, demonstrating the upregulation of genes associated with retinal glial activation (astrocytes, Müller glia, and microglia), suggesting that inflammatory reaction occurs in IOP-related retinal injury. Surprisingly few genes were down-regulated in these studies. To expand on these earlier studies, we used whole genome microarrays to study gene expression changes in two rat models, experimental glaucoma and optic nerve transection, from 1 day to 8 weeks after injury. This allowed the comparison of two processes, each leading to RGC death but involving different initial insults. We reasoned that this would allow separation of expression changes and path-

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ways common to RGC death in general compared with those specific to experimental glaucoma. Similarly, time-course analysis of gene expression change should aid in the differentiation of primary from secondary effects.

**METHODS**

**Animals**

Male Wistar rats, each weighing between 300 and 350 g, were treated under protocols adherent to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Johns Hopkins School of Medicine Animal Care Committee. They were housed under a 14-hour light/10-hour dark cycle and fed standard chow and water ad libitum.

**Experimental Glaucoma and Optic Nerve Transection**

For the induction of experimental glaucoma, animals were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg), and the eyes were anesthetized with 0.5% proparacaine hydrochloride ophthalmic drops. A 552-nm diode laser was used to scar the trabecular meshwork and to decrease fluid outflow, as previously described. Rats were initially treated with 45 to 55 spots at 50-μm size, 0.6-W power, and 0.6-second duration. Each rat in the 1-day (1D), 3-day (3D), and 1-week (1W) treatment groups received a single laser treatment. Several animals that failed to achieve any increase in IOP were not included here. Rats followed up for 2, 4, and 8 weeks (2W, 4W, and 8W) were retreated 1 week later with 35 to 45 spots of the same size, power, and duration. Topical 5% erythromycin ointment was applied at the end of each procedure.

During the induction of experimental glaucoma, IOP was measured (TonoLab; Colonial Medical Supply, Franconia, NH) while the rats were under sedation. IOP was measured immediately before laser treatment, 1 day after treatment, and before kill in those animals whose kill time was less than 2 weeks after initial treatment. In rats that survived for 2, 4, and 8 weeks, the IOP was also measured once weekly. IOP in the laser-treated eyes averaged 15 to 27 mm Hg above that in the fellow eyes in the six groups of animals at various times after injury. A measure of the exposure to increased IOP in these eyes was the area under the IOP-time curve for which IOP exceeded control in each eye. Mean positive integral values (in mm Hg, days) were 50, 128, and 121 for the 1W, 2W, 4W, and 8W groups of rats, respectively.

Rats that in the 1W, 2W, 4W, and 8W groups of rats, respectively.

**RNA Extraction and Microarray Analysis**

Total RNA was prepared, using reagent (TRizol; Invitrogen, Carlsbad, CA), from rat retinas that were harvested 1D, 3D, 1W, 2W, 4W, and 8W after laser treatment or optic nerve transection. RNA was also prepared from the retinas of untreated control animals. All RNA samples were further purified with RNeasy reagent (Qiagen, Valencia, CA), from rat retinas that were harvested 1D, 3D, 1W, 2W, 4W, and 8W after laser treatment or optic nerve transection. RNA was also prepared from the retinas of untreated control animals. All RNA samples were further purified with RNeasy reagent (Qiagen, Valencia, CA), according to the protocol provided by the manufacturer. The quantity and quality of total RNA was determined (Bioanalyser; Agilent, Santa Clara, CA). Two micrograms of total RNA from each individual retina in each experimental group was pooled for microarray studies, which were performed using array chips (SensorChip 230 2.0; Affymetrix, Santa Clara, CA). All hybridizations were performed at the Johns Hopkins Microarray Core Facility.

**Quantitative RT-PCR**

One microgram of total RNA from each sample was used as template for reverse transcription with DNA polymerase (Superscript II; Invitrogen). Real-time PCR (Sybrgreen; Bio-Rad, Hercules, CA) was performed on a thermocycler (Myiq; Bio-Rad). Relative starting quantities of template cDNA were normalized to those of GAPDH. Sequences of primers for PCR amplification of candidate genes are shown in Appendix 1, quantitative (Q)PCR primers table; all Appendices are online at http://www.iovs.org/cgi/content/full/48/12/5539/DC1. Paired t tests were used to compare gene expression levels measured by QCRs between experimental eyes and fellow eyes, whereas t tests were used to compare PCR values between glaucoma and transaction groups at each time point after injury. For data that showed unequal SD between groups, nonparametric Wilcoxon rank sign and rank sum tests were performed. Because of the six time points for each condition, the P = 0.01 was accepted as significant after correction for multiple comparisons by the Bonferroni method.

**In Situ Hybridization**

Rats were intracardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Eyecups were prepared from enucleated rat eyes by removing the anterior segment, cryoprotecting the remaining tissue in increasing concentrations of sucrose, and embedding the tissue in a sucrose/OCT mixture on dry ice. Retinal sections of 15-μm thickness were cut and placed on Superfrost slides such that sections from each experimental eye and its corresponding fellow eye were in parallel on the same slide. Antisense digoxigenin-labeled riboprobes were reverse transcribed by T7, T3, or SP6 RNA polymerase from linearized plasmid templates, which carried the cloned cDNA fragments of Sneg (nucleotide 18-433) or Nefl (nucleotide 320-1219). Nonradioactive in situ hybridization was performed with sections that were air-dried, post-fixed with 4% PFA, treated with proteinase K (20 μg/mL; Invitrogen), and acetylated with acetic anhydride in 0.1 M triethanolamine (Sigma). Sections were incubated with riboprobes at 55°C for 16 hours, washed with 2× sodium chloride/sodium citrate buffer and 50% formamide at 65°C, and treated with RNase A (20 μg/mL; Invitrogen) to remove unbound probes. Sections were then incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:5000; Roche, Nutley, NJ) for 16 hours and washed with 0.1% Tween 20 in PBS. Color reactions of alkaline phosphatase were developed to the desired degree.

**RESULTS**

**Study Design and Overview**

To explore the molecular events that underlie RGC death, we used whole genome microarray technology to profile retinal gene expression changes in two rat models of RGC injury, translimbal laser photocoagulation-induced IOP elevation (glaucoma) and optic nerve transection (axotomy). We used the array analysis as a primary screen for expression changes and then followed up the more significant and interesting putative changes with real-time quantitative QPCR analysis. The time points examined were 1D, 3D, 1W, 2W, 4W, and 8W. For the microarrays, RNA samples from four or five similar treated animals were pooled for each array. For the QPCR studies, RNA samples from each of 4 to 11 animals at each time point, and for each model, were measured independently, allowing for statistical analysis of variability between biological replicates.

Forty-one experimental glaucoma rats were analyzed. IOP was significantly elevated in all animals and was detected as
Expression Changes Common to or Different between Glaucoma and Axotomy Models

We compared genes that were differentially expressed in both RGC injury models and those that were altered in only one of the two models but not in the other. Our hypothesis was that genes specifically altered only in the glaucoma model might be more likely to reflect a primary or a secondary response to elevated IOP. We also analyzed those genes that were altered at early time points compared with those altered at later time points (this analysis was related to but distinct from the peak analysis referred to because all elevated time points were considered instead of just the time of the peak response). This was based on the hypothesis that early gene expression changes were more likely to be involved in signal transduction and early injury response, whereas later changes were more likely to be related to processes such as RGC death mechanisms, actual loss of RGCs, retinal neuroprotective responses, and results of retinal remodeling.

At the early time points (operationally defined as 1D and 3D), there were 275 differentially expressed genes (186 specific to the glaucoma model, 42 specific to the axotomy model, and 47 common to both models); at the intermediate time points (operationally defined as 1W and 2W) there were 241 differentially expressed genes (111 specific to the glaucoma model, 54 specific to the axotomy model, and 76 common to both models); and at the late time points (operationally defined as 4W and 8W) there were 687 differentially expressed genes (183 specific to the glaucoma model, 377 specific to the axotomy model, and 127 common to both models). Given that RGCs, compared with the glaucoma model, die more quickly and completely after axotomy, the glaucoma late time points may be more equivalent to the axotomy intermediate time points (see also Fig. 1).

To get an overall view of the classes and functions of the differentially expressed genes, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) gene ontogeny analysis program. This analysis revealed, in the early phase of the glaucoma model, an overrepresentation of genes related to immune and stress responses, possibly reflecting a neuroinflammatory response. Of particular interest was the upregulation of many complement components (C3, C4, C1r, C1s, C1q, and C2). In the late phase, there was overrepresentation of genes related to protein synthesis, particularly amino acid tRNA transferases. With the laser model, in the early response group, there was differential representation of genes related to cell death and "negative regulation of cellular physiological process," defined as "any process that stops, prevents or reduces the frequency, rate or extent of a cellular physiological process, the processes pertinent to the integrated function of a cell."
greater than twofold change in expression in the axotomy model (glaucoma upregulated), at least two time points with greater than twofold decreased expression in the glaucoma model and no time points with greater than twofold change in expression in the axotomy model (glaucoma downregulated), at least two time points with greater than twofold increased expression in the axotomy model and no time points with greater than twofold change in expression in the glaucoma model (axotomy upregulated), and at least two time points with greater than twofold decreased expression in the axotomy model and no time points with greater than twofold change in expression in the glaucoma model (axotomy downregulated) (see Appendix 5, Model-specific differential gene expression). Using Ingenuity to perform pathway analysis on these subsets of genes yielded statistically significant overrepresentation of pathways, with the requirement that more than one gene had to be included in the group, only for the glaucoma upregulated group. The most significant pathways identified were complement and coagulation cascade, NRF2-mediated oxidative stress response, and the overlapping signaling pathways for IL-6, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and granulocyte macrophage-colony-stimulating factor (GM-CSF). Ingenuity-based classification of the functional groups to which the differentially expressed sets of genes fall into is shown in Appendix 6. Overall, the glaucoma upregulated group showed the most statistically significant overrepresentation of specific functional classes. Among the functional groups most statistically upregulated by glaucoma were cell cycle, cell death, cellular development, cellular growth and proliferation, neurologic disease, and immune (immune response, immunologic disease, and inflammatory disease–related) genes. Connective tissue disorder–related genes were both upregulated and downregulated in glaucoma. In axotomy, among the most significant changes were downregulation of neurologic disease and immune-related genes.

To confirm select microarray results and to examine biological variability among equivalently treated animals, we examined expression levels in individual animals by QPCR. Genes tested by QPCR were chosen based on a combination of the array results and the potential biological importance of the gene in question. Forty-two and 26 genes, respectively, were tested by QPCR for all time points in the laser and transection models. The correlation between microarray and QPCR was good, and it was better for the transection data than for the glaucoma data. Figure 2 shows a comparison in which each time point is analyzed separately. As shown in the detailed QPCR results below, some genes showed extensive variability among the individual animals at a single time point in a model. This variability presumably reflected variation in laser or surgical injury, variation in the responses of the biological processes studied, and technical variations in the measurement of gene expression changes.

Select QPCR time-course results are shown in Figure 3. Among those genes that were increased by array in the glaucoma model, but not significantly after transection, we confirmed this specificity for glaucoma in five genes. First, endothelin receptor B (Ednrb) was elevated in glaucoma at 4 of 6 time points in QPCR data (P < 0.05 at 1D, 3D, 7D, and 4W [Fig. 3A]; for this gene and others noted, if results are not presented,
the significance level did not reach $P = 0.05$ (Appendix 7, $P$ values for QPCR). Similarly, Edn2 was increased only in glaucoma ($P < 0.05$ at 1D, 1W, and 4W, and $P < 0.01$ at 2W; Fig. 3C). Ednra (endothelin receptor A) was not significantly changed in either model (Fig. 3B). Another significantly up-regulated gene in the glaucoma model alone was tumor necrosis factor receptor superfamily, member 1A (Tnfrsf1a). Its expression peaked at 1D ($P < 0.001$) and decreased thereafter (Fig. 3B). None of the Tnfrsf1a QPCR values for transection eyes were significant, and the glaucoma group was significantly greater than the transection group data for this gene at 3D and 4W ($P < 0.05$ and 0.02). Annexin A3 (Anxa3) was also elevated only in glaucoma, at 4 of 6 time points: 3D ($P = 0.01$), 1W, 2W, and 4W (all $P < 0.05$; Fig. 3P). The related gene, Anxa1, followed a similar trend, but none of the QPCR values were significantly different from control in either model (Fig. 3O). The final gene in this group, Lcn2, was significantly increased in glaucoma at 1D, 1W, 2W, and 4W (all $P < 0.01$) but only at 3D ($P = 0.01$; Fig. 3I) after transection.

Some genes with QPCR confirmation of array findings were significantly altered in transection but not after glaucoma. Only one gene—Sox 11—which was increased at 1D, 3D, and 1W (all $P < 0.01$)—was elevated in transection but not in glaucoma. Mean elevation in transection was significantly higher than in glaucoma at 1D and 3D ($P = 0.01$ and 0.03; Fig. 3G). Two genes, Sn cg and Neft, were decreased more significantly by QPCR in transection (Figs. 3S, 3T). The decreases in Sn cg were significant at all time points after transection from 1D to 8W (all $P < 0.001$), and the decline in transection statistically exceeded that after glaucoma at all time points ($P = 0.01$ or more in four time periods and 0.02 in two others). For Neft, significant decreases occurred after transection from 3D onward ($P = 0.02$ at 3D; $P < 0.001$ thereafter), whereas after glaucoma, the decrease was significant only at 1W ($P = 0.03$). The transection mean was lower than that of glaucoma at 5 of the 6 time points for this gene.

Another group of genes had QPCR values that were increased in glaucoma and transection models, though the times...
at which maximum increases occurred differed in the models. Genes that were significantly increased at earlier times in glaucoma than after transection included Cebpd and cFos. Cebpd was highly elevated in glaucoma at 1D (P = 0.007) and 2W (P = 0.03) and after transection at 2W (P = 0.002; Fig. 3F), with the glaucoma group data higher than the transection data at 1W (P = 0.03). cFos was increased in glaucoma at 1D (P = 0.02) and later after transection at 3D and 2W (P = 0.015; Fig. 3L). Two other genes, Gpnb and Hsp27, were increased in both models but had earlier significant QPCR mean values after transection than after glaucoma. Gpnb was elevated at 1D, 3D, and 1W after transection (all P < 0.01) and at 1W, 2W, and 4W after glaucoma (all P < 0.01; Fig. 3R). This finding of increased retinal expression of Gpnb is intriguing because it is a melanosome-associated protein that is differentially expressed in the pigment epithelium and iris, and, when mutated, it contributes to the iris/glaucoma phenotype of DBA/2J mice. Hsp27 was elevated after transection at 1D, 3D, and 1W (P = 0.01, 0.01, 0.03) and after glaucoma at 1D and 2W (P = 0.01, 0.05; Fig. 3H).

A final group of genes was elevated in both models, with no trend toward different time courses between models. These genes included Atf3, Timp1, cJun, Junb, and Stat3. Atf3 was increased modestly at 1D in glaucoma (P = 0.05) and 1W after transection (P = 0.03; Fig. 3E). Timp1 was highly elevated at four time points in each model in the first 4 weeks, especially at 1D (each model, P = 0.009; Fig. 3Q). cJun and Junb both had had significantly elevated QPCR values at one time point with 1W for each gene in glaucoma, P = 0.02 for both; and 1D for transection, both P = 0.01; Figs. 3J, 3K). Stat3 was modestly elevated in glaucoma at 2W (P = 0.01) but was more variable after transection (significant at 3D, P = 0.003; Fig. 3N). The related transcription factor gene, Stat1, was only significantly elevated at 1D in glaucoma (P = 0.05) and was not increased after transection by QPCR; Fig. 3M).

To determine whether some variability in expression in the glaucoma model derived from the level and duration of IOP elevation, we correlated the degree of exposure to elevated IOP and degree of expression change. As a measure of IOP exposure, we compared the IOP integral2 in rats killed at 1W (each model, P = 0.009; Fig. 3Q). cJun and Junb both had had significantly elevated QPCR values at one time point with 1W for each gene in glaucoma, P = 0.02 for both; and 1D for transection, both P = 0.01; Figs. 3J, 3K). Stat3 was modestly elevated in glaucoma at 2W (P = 0.01) but was more variable after transection (significant at 3D, P = 0.003; Fig. 3N). The related transcription factor gene, Stat1, was only significantly elevated at 1D in glaucoma (P = 0.05) and was not increased after transection by QPCR; Fig. 3M).

Several RGC-Enriched Genes Exhibit Decreased Expression before RGC Loss

Because few, if any, RGCs remain in the retina 8 weeks after optic nerve transection,10 the set of genes substantially downregulated at the 8W time point should include genes that are RGC enriched or RGC specific. Among the genes that showed this behavior in the arrays were Neurofilament-light polypeptide (Neft), Neurofilament 3 (Neft3), γ-synuclein (Sncg), Neuritin 1 (Nrn1), Peripherin1 (Prph1), and Solute carrier family 17 member 6 protein (Slc17a6) (Fig. 5). We also analyzed the expression pattern of Sncg and Neft by QPCR (Fig. 4O-P). All the RGC-enriched genes showed a trend toward decreased expression before detectable RGC loss, suggesting that their decreased expression was attributed, at least in part, to a direct effect of the stress on RGCs, not simply to the loss of the cells themselves.

We also performed in situ hybridization for Sncg and Neft; as expected, they were found predominantly in the ganglion cell layer (GCL) in normal retina. In addition, their hybridization signal decreased after axotomy well before detectable cell loss (Figs. 6B, 6H). In addition to expression by cells in the GCL, an in situ signal for Sncg was observed in some inner nuclear layer cells (Figs. 6A, 6G).

**FIGURE 4.** Correlation between IOP and glaucoma model gene expression changes. Linear regression is shown of the QPCR determined gene expression change (y-axis) compared with the IOP integral at 1 week. Each dot represents QPCR results from an individual animal (11 animals total). Genes shown are (A) Tnfrs1a, (B) Lcn2, (C) Stat1, and (D) Timp1.

**DISCUSSION**

Careful interpretation of microarray data can provide mechanistic insight into the response of RGCs and associated neurons and glia to injury, especially when interpreted in the context of expression changes that occur during retinal development or after experimental retinal or optic nerve injury. Our data, together with previously published work, suggest that the induction of RGC injury leads to complex changes in gene expression. These changes involve the modulation of positive and negative regulatory pathways. The resultant biological effects on the retina, and specific effects on RGCs, are likely to be complex and sometimes opposing. As one example, RGC injury induces expression changes of apoptosis-inducing and survival-promoting genes. Presumably, it is the intricate balance between these various expression changes and translational and posttranslational modifications that determines the fate of RGCs after injury.

We found similarities and differences in the gene expression patterns associated with increased IOP and optic nerve transection, which is logical given the biological similarities and differences between the models. RGC axonal damage occurred in both models, and both likely generated retrograde signals to the cell body that initiated responses to the injury. However, the signaling molecules might have differed quantitatively or qualitatively in the two situations. The glaucoma model induced a modest degree of RGC injury, whereas transection caused nearly complete RGC loss. In addition, the death of RGCs after transection was more rapid and synchronous than that induced by elevated IOP. Furthermore, increased IOP probably altered conditions within the retina and at the optic nerve head, whereas transection had initial effects only outside the eye.

We found rapid increases in expression of immediate early response genes (IEGs) and in members of the mitogen-activated protein kinase pathways. C-Jun, Junb, and Activating transcription factor 3 (Atf3) were elevated in both models, whereas c-Fos, Stat1, and Stat3 were elevated in only the glaucoma model (array data, confirmed by QT-QPCR). Experimental retinal ischemia–reperfusion, like transection, causes increased expression of c-Jun and Junb but not of Fos, Stat1, and Stat3. Stat3 mRNA and protein were increased in rats with glaucoma and might have participated in RGC pro-
that is compatible with published information and our data is pJNK. Its retrograde transport is increased by neuronal injury,51 and it is known to signal neurotrophin withdrawal in neurons.32 Neurotrophin depletion is a likely inducer of apoptotic cell death in glaucoma.31,52,53 Consistent with this hypothesis, pJNK is increased by immunolabeling in RGCs in human glaucoma35 and experimental rat glaucoma.36,37 In addition, pJNK activates expression of the Cebp group of transcription factors,38 which mediate neuronal survival.51 Among this group, Cebpd was significantly increased in rats with glaucoma. We did not find an increase in JNK expression, but this would be expected if signaling took place at a posttranscriptional level, such as increased phosphorylation and transport.

Evidence indicates that endothelin acts on neural cells through the pJNK pathway.49 We found significant increases in endothelin receptor B (Ednrb) gene expression in glaucoma but not after transection, though increased expression of this gene has been reported in rodents with retinal damage and retinal degeneration.41 Ednrb protein increased in glia (astrocytes and Müller cells) after retinal and optic nerve injury42 and in patients with glaucoma.53 Endothelin-1 and the Ednrb receptor proteins were increased in experimental rat glaucoma.44 Several reports have linked tumor necrosis factor alpha (TNFα) to glaucoma injury. There is increased mRNA and immunofluorescence for TNFα in glia, and its inhibition reduces RGC death in vitro.54 In a murine glaucoma model, Nakazawa et al.45 reported increased levels of TNFα and detected significant protection against RGC death by inhibition of TNFα signaling through TNF receptor 2, but not TNF receptor 1. Tezel et al.46 reported protection from RGC death after nerve crush in knockout mice missing the TNF superfamily receptor member 1A gene (Tnfrsf1a). We found a nearly eightfold upregulation of Tnfrsf1a at 1D in the rat glaucoma model, consistent with its upregulation in monkey glaucoma, and an increase in its protein in RGC in human glaucoma retina.55 We speculate that the upregulation of the receptor for TNFα might have resulted from the presentation of increased levels of the agonist to RGC by glia. Furthermore, our data showed in the
FIGURE 6. Downregulation of Sncg and Nefl in rat retina after RGC injury. Cell type-specific localization and expression alterations of Sncg (A–F, A’–F’) and Nefl (G–L, G’–L’) are shown by in situ hybridization with specific riboprobes on rat retinal sections collected from fellow eyes (A–L) and treated eyes (A’–L’) in rat models of glaucoma and optic nerve axotomy. Both Sncg and Nefl are strongly expressed in cells localized in GCL (A, G, arrows) and weakly in upper boundary of INL (A, G, arrowheads). Expression of Sncg (B, D, F) and Nfl (H, J, L) in GCL is strongly downregulated in rat retinas at 1 day, 3 days, and 1 week after optic nerve axotomy, whereas Nefl is also transiently downregulated in GCL on the third day after laser-induced IOP elevation (J’). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelial layer. Scale bar, 100 μm.
Glaucoma model, but not in the transaction model, that there was upregulation of LPS-induced TNF Factor (Litaf), a transcription factor that positively regulates TNFα expression.\textsuperscript{49} However, it should be noted that the upregulation of Litaf is not uniquely specific to glaucoma and that it occurs with other types of retinal injury.\textsuperscript{50} Finally, TNFα is related to upregulation of the Cepb family of transcription factors,\textsuperscript{51} one of which (Cepbda) was increased in our data. Taken together, these data support the participation of the TNFs signaling pathway in mediating glaucoma-induced injury to RGCs.

We identified the upregulation of Anxa1 and Anxa3 in glaucoma but not in the transaction model. Of potential interest, Anxa1 increased rapidly and substantially after IOP elevation (a fourfold upregulation) but was near baseline at 8W, whereas Anxa1 increased to a similar magnitude, but only from 2W to 8W after experimental glaucoma induction. The annexins are intracellular calcium binding proteins that may have additional signaling functions.\textsuperscript{52} Anxa1 is upregulated by heat shock proteins in models of hypoxia and oxidative stress, and it is upregulated in neurons and glia in a spinal cord trauma model.\textsuperscript{53} Anxa1 is a putative regulator of the membrane-associated cytoskeleton. The effect of external force on axons in glaucoma may be transmitted through interactions of the cell membrane and cytoplasmic microtubules that are central to retrograde axonal transport. Hypothetically, increased annexin activity could be a mechanism for membrane repair or it could be related to aggregations of vesicles known to occur in axonal transport blockade.

Some of the expression changes cited provide evidence for glial cell activation, suggesting a neuroinflammatory component in the response to RGC injury. Among the inflammation-related genes whose expression was altered in both models are Lcn2, ceruloplasmin, activity and neurotransmitter-induced early gene protein 4, complement components, and Timp-1. Genes associated with glial activation that were upregulated in both our models, and in previous retinal injury studies and in a mouse glaucoma model,\textsuperscript{3} include Glial fibrillary acidic protein, Vimentin, and Chitinase 3-like 1. Increased ceruloplasmin in the retina has been reported in glaucoma models\textsuperscript{55} and in patients with glaucoma.\textsuperscript{56} Lcn2 is upregulated in Müller glia, as are other acute-phase genes in a rat model of diabetic retinopathy and in other retinal and optic nerve injury models.\textsuperscript{57} Lcn2 is a siderophore-binding protein that sequesters iron. It is likely that the responses we termed inflammatory are a mixture of protective and detrimental effects on the tissue.

In both RGC injury models, several members of the Crystallin family were upregulated. Retinal expression of crystallins is now well accepted,\textsuperscript{55} and increased retinal crystallin expression in glaucoma models has been reported.\textsuperscript{9} Inconsistencies in the magnitude and timing of Crystallin changes in our data, however, led us to suspect that the findings were artifactual. Despite great care in retinal dissection, contamination by lens material could be a factor, as recently documented.\textsuperscript{56}

As expected, some genes preferentially expressed in RGCs\textsuperscript{57} were decreased in both animal models. The greater decreases after transection presumably resulted from the greater RGC loss compared with the glaucoma model. The rapid occurrence of decreased expression 1D and 3D after injury was unexpected because it preceded histologic disappearance of RGCs. Other research groups have also noted alterations in genes characteristic of the normal RGC phenotype with injury at a stage before neuronal death.\textsuperscript{58,59}

The therapeutic implications of our findings include the probability that future neuroprotection of RGCs may be accomplished either by bolstering survival mechanisms or by inhibiting destructive processes, including alterations in neuroinflammatory responses. The increasing availability of drugs that modulate the immune system may make this kind of approach clinically possible.\textsuperscript{60} In addition, the growing body of data on the gene and protein changes associated with early damage may provide avenues to prevent the initiation of RGC injury, when intervention is most likely to be effective.

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References


