Cell Proliferation and Interleukin-6–Type Cytokine Signaling Are Implicated by Gene Expression Responses in Early Optic Nerve Head Injury in Rat Glaucoma

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PURPOSE. In glaucoma, the optic nerve head (ONH) is the principal site of initial axonal injury, and elevated intraocular pressure (IOP) is the predominant risk factor. However, the initial responses of the ONH to elevated IOP are unknown. Here the authors use a rat glaucoma model to characterize ONH gene expression changes associated with early optic nerve injury.

METHODS. Unilateral IOP elevation was produced in rats by episcleral vein injection of hypertonic saline. ONH mRNA was extracted, and retrolbulbar optic nerve cross-sections were graded for axonal degeneration. Gene expression was determined by microarray and quantitative PCR (QPCR) analysis. Significantly altered gene expression was determined by multiclass analysis and ANOVA. DAVID gene ontology determined the functional categories of significantly affected genes.

RESULTS. The Early Injury group consisted of ONH from eyes with <15% axon degeneration. By array analysis, 877 genes were significantly regulated in this group. The most significant upregulated gene categories were cell cycle, cytoskeleton, and immune system process, whereas the downregulated categories included glucose and lipid metabolism. QPCR confirmed the upregulation of cell cycle-associated genes and leukemia inhibitory factor (*Lif*) and revealed alterations in expression of other IL-6–type cytokines and Jak-Stat signaling pathway components, including increased expression of IL-6 (1553%). In contrast, astrocytic glial fibrillary acidic protein (*Gfap*) message levels were unaltered, and other astrocytic markers were significantly downregulated. Microglial activation and vascular-associated gene responses were identified.

CONCLUSIONS. Cell proliferation and IL-6–type cytokine gene expression, rather than astrocyte hypertrophy, characterize early pressure-induced ONH injury. (*Invest Ophthalmol Vis Sci.* 2011;52:504–518) DOI:10.1167/iovs.10-5317

Experimentally elevated IOP in animals produces glaucoma-like retinal ganglion cell (RGC) and optic nerve axonal loss and a characteristic remodeling of the optic nerve head (ONH)

that includes the rearrangement of glial cells and the deposition of extracellular matrix (ECM) proteins. Although experimental glaucoma models have been produced in a number of species, primate and rodent models are the most extensively used. In both human and experimental glaucoma, there is a general consensus that the ONH is the primary site of initial glaucomatous injury to RGC axons.^{1,2} Recently, sophisticated 3-D histomorphometric and biomechanical studies have been used to examine some of the earliest morphologic and structural ONH responses in experimental glaucoma attributed to elevated IOP in primates.^{3–11} However, relatively few studies have examined the early cellular responses of the ONH to elevated IOP exposure. Such studies should help us understand how the ONH cells respond to an environment altered by elevated IOP and how these responses might affect axonal integrity.

To examine the response to acute IOP elevation, the anterior chamber can be cannulated to allow IOP to be elevated for up to a few hours without altering retinal perfusion. After these acute elevations, abnormalities in axonal transport, including the accumulation of mitochondria, dynein, neurotrophins and their receptors, and the distribution of axonal and astrocytic proteins have been observed.^{12–17}

For longer IOP elevation, a number of techniques are available to obstruct aqueous outflow and produce glaucoma-like optic nerve degeneration.^{18–23} In initial studies using our chronic, hypertonic, saline-induced rat glaucoma model and immunohistochemistry, we found characteristic glaucoma-like changes in the rat ONH ECM. As early as 11 days after IOP elevation, we observed depositions of collagen IV, collagen VI, and laminin.²⁴ Next, we used immunohistochemistry to study the chronology of morphologic and protein distribution changes in the ONH. At 3 days after IOP elevation, we observed decreased labeling in ONH glial columns for gap junction protein alpha 1 (connexin 43, *Gja1*) and increases in a cell proliferation marker.²⁵ These changes were followed at 1 week by decreased labeling for neurotrophins and *Gfap*, increased vascular labeling of collagen VI, and the appearance of swollen, degenerating axons in the ONH and focal lesions in optic nerve cross-sections. In a separate study using laser-induced IOP elevation, an accumulation of the retrograde transport motor dynein was observed in the ONH at approximately the same duration of IOP elevation.²⁶

More recently, we have used our glaucoma model to determine gene expression changes in extensively injured ONH.²⁷ As part of that study, we also reported expression changes in a few specific genes in ONH from eyes with focal optic nerve injuries (<50% axon degeneration). These genes had all been found to be highly regulated in ONH with extensive nerve injury. Among these, we found significant upregulation of tissue inhibitor of metalloproteinases 1 (*Timp1*), fibulin 2 (*Fbln2*), and tenascin c (*Tnc*) as well as downregulation of

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aquaporin 4 (*Aqp4*) in these focally injured ONH. In the study presented here, we greatly expand these initial findings by using microarray analysis and quantitative polymerase chain reaction (QPCR) to identify the gene expression changes in glaucoma model ONH from eyes with <15% optic nerve axon degeneration attributed to elevated IOP exposure.

METHODS

Glaucoma Model

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Oregon Health and Sciences University (OHSU) Animal Care and Use Committee. Unilateral IOP elevation was produced in 8-month-old, male Brown Norway rats by episcleral vein injection of hypertonic saline.²⁸ Rats were housed in low-level constant light to minimize circadian IOP fluctuation.^{29,30} IOP was measured frequently in anesthetized animals, primarily using a tonometer (Tono-PenXL; Medtronic, Minneapolis, MN).^{29,31} For some glaucoma model eyes included in the PCR studies, IOP history was collected using a rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH), as described.³² Except where noted, tissues were collected at 5 weeks after injection.

Optic Nerve Grading

Retrobulbar optic nerves were collected, postfixed, embedded in Spurr's resin, and cross-sectioned for light microscopic evaluation.²⁷ Each nerve was graded for injury, on a scale from 1 (normal nerve) to 5 (extensive degeneration affecting nearly all axons), by at least five independent observers, and the mean was defined as the injury grade for that nerve. Previous studies using transmission electron microscopy have demonstrated that each unit increase in the injury grade is equivalent to degeneration affecting approximately 12% to 15% of the optic nerve axons.³⁰

ONH Dissection

Enucleated globes were rapidly chilled in cold phosphate-buffered saline, and the anterior segment, lens, and retina were removed. Using a trephine, the ONH, including the initial portion of myelinated optic nerve, was dissected from the posterior pole of the globe. The ONH was then shortened to a uniform 0.4 mm in length using a razor blade and custom-designed matrix. This initial, largely unmyelinated ONH segment included the pia and pigmented peripapillary border tissue. The dissected ONH was then frozen in liquid nitrogen and stored at -80°C before RNA extraction.

RNA Purification and Amplification

RNA was isolated by first sonicating the frozen nerve heads in 30 μL extraction buffer (Pico-Pure RNA Isolation Kit; Molecular Devices, Sunnyvale, CA) using an MS 0.5 probe (UP50H Ultrasonic Processor; Hielscher USA, Inc., Ringwood, NJ) in accordance with the manufacturer's directions, including DNase treatment. The purified RNA was quantified (RiboGreen RNA Quantitation Kit; Invitrogen, Carlsbad, CA), and 25-ng aliquots underwent two rounds of linear amplification (MessageAmp aRNA Amplification Kit; Ambion, Austin, TX) yielding ample aRNA for both microarray analysis and QPCR. The integrity of the amplified RNA was verified (2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA).

cDNA Microarrays

Aliquots of amplified RNA were reverse transcribed and dye-labeled at the OHSU Gene Microarray Shared Resource (<http://www.ohsu.edu/gmsr/>), which also prepared and processed the cDNA arrays and compiled the data. For each sample, two cDNA arrays (SMCmou8400A and SMCmou6600A) were used, containing a combined total of 15,400 probes. These are listed in Supplementary Table S1, <http://www.iovs.org/>

lookup/suppl/doi:10.1167/iovs.10-5317/-DCSupplemental, and represent the entire initial release of the National Institute on Aging mouse library (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>). The length of the cDNA probes on these arrays makes them relatively insensitive to sequence differences between closely related species (Morrison JC, et al. *IOVS* 2005;46:ARVO E-Abstract 1250).^{33,34} The probes correspond to approximately 8000 individual Entrez gene IDs, 1000 additional transcripts with Unigene designations, and 4000 expressed sequence tags (<http://www.ncbi.nlm.nih.gov/sites/entrez>). Each data point is the mean of four technical replicates. Gene expression data from each array were normalized at the facility using a modified Lowess procedure.³⁵ Expression patterns in each ONH were independently determined relative to an ONH RNA reference standard, derived by pooling aliquots from all samples.

In addition to six control (uninjected, fellow eye) ONH, ONH was collected from 21 eyes with cumulative IOP above the control eye ranging from 34 mm Hg to 490 mm Hg. Mean IOP in control eyes, as measured by tonometer (Tono-PenXL; Medtronic), was 28.6 mm Hg. Although most of the glaucoma model ONH was collected at 5 weeks after episcleral vein injection, the analysis included eight ONH from eyes collected at 10 days after the first IOP reading of 35 mm Hg or more, so that gene expression changes that occurred early in the injury process would be enriched in the analysis.

Physiological IOP Elevation Group

In an additional microarray analysis, gene expression changes with physiological IOP elevation were evaluated to enhance the interpretation of the glaucoma model results. These samples were processed simultaneously with the glaucoma model samples from initial RNA amplification through microarray data preprocessing and normalization in a single experiment at the OHSU Microarray Resource. For this group, animals were housed in standard 12-hour light/12-hour dark conditions. Tissues were collected from normal eyes during the dark-phase peak in circadian IOP elevation, when IOP is approximately 10 mm Hg above the light-phase IOP measurement of approximately 21 mm Hg and 2 to 3 mm Hg above the mean IOP of eyes in animals housed in constant, low-level light.^{29,36} Gene expression alterations in these ONH were independently compared with the control ONH data, described here using the Two Class Unpaired Module of Significance Analysis of Microarrays (SAM, version 3.02; <http://www-stat.stanford.edu/~tibs/SAM/> provided in the public domain by Stanford University, Stanford, CA). Potential gene expression changes in these eyes do not result in significant axon loss over the lifespan of the rat³⁷ and, therefore, represent noninjurious, physiological circadian responses. Significant changes in expression attributed to physiological IOP elevation (1.3-fold change; 2% false discovery rate [FDR]) were noted and used to assist in interpreting the gene expression changes seen in our glaucoma model.

Definition of the Early ONH Injury Group

We reasoned that the ONH from glaucoma model eyes with minimal optic nerve axon degeneration would most consistently display changes in gene expression associated with the earliest phases of axonal injury. Rather than arbitrarily dividing the experimental ONH into injury groups, we used the following visualization strategy to identify a group of ONH with early injury. First, we ordered the microarray data sets by increasing optic nerve injury grade, including all data from the 6 control and 21 glaucoma model ONH, with nerve injury grades ranging from 1.025 to 5. Then we used the Pattern Discovery module in SAM to identify the significant eigengenes (principal components). The SMCmou8400A and SMCmou6600A array data sets were separately analyzed and significant eigengenes ($P < 0.05$) from both were plotted against ONH injury grade. Curve fitting and regression analysis were used to determine the statistically significant best fit line for each eigengene. From this analysis, the Early and Advanced Injury groups of arrays were defined (see Results and Fig. 1)

FIGURE 1. Definition of the Early and Advanced ONH Injury groups. The Pattern Discovery module in SAM was used to identify the significant eigengenes (principal components) in the microarray data sets arrayed by increasing optic nerve injury grade (6 controls, 21 glaucoma model ONH from eyes with optic nerve injury grades from 1.025 to 5.) Each array data set (SMCmou6600A and SMCmou8400A) was separately analyzed, and significant eigengenes ($P < 0.05$) from both were plotted. Curve-fitting and regression analysis were used to determine the statistically significant best-fit line for each eigengene, as plotted here. The two array sets yielded nearly identical eigengene patterns for the first two eigengenes. The most significant pattern (Eigengene 1, red lines) on both arrays was a linear response, with maximal change in the most severely injured ONH. The second most significant pattern (Eigengene 2) for both array sets was a fourth-order polynomial curve, with peak changes below injury grade 2 (approximately 15% axons degenerating). This pattern indicated that a large number of significantly affected ONH genes responded maximally to early pressure-induced damage. Based on nerve injury grade and these two eigengene patterns, data from each sample was assigned to 1 of 3 groups for further analysis: Control ONH (untreated eyes), Early ONH Injury (glaucoma model ONH with optic nerve injury grades <2.0 , $n = 9$), and Advanced ONH Injury (glaucoma model ONH with optic nerve injury grades >2.0 , $n = 12$).

Determination of Genes Significantly Changed in Expression by ONH Injury Group

Next the Multiclass Analysis module of SAM (2% FDR) was used to compare the data from the Early Injury group to both the Control and the Advanced Injury group. This analysis identified all genes that were significantly altered in any group comparison. Then ANOVA and Tukey-Kramer posttest were used to identify genes that differed significantly ($P < 0.05$ corrected for multiple comparisons) and were changed in expression by 1.3-fold between groups so that our early injury group analysis identified all genes significantly regulated in early injury compared with the control group. Finally, DAVID Bioinformatics Resources 2008 (<http://david.abcc.ncifcrf.gov/>) was used to determine significantly regulated functional classes of these genes, using the array probes as background. Official Entrez and Rat genome database (<http://rgd.mcw.edu/>) gene symbols are used throughout the manuscript to refer to specific genes and their protein products.

QPCR Analysis

QPCR was used to verify microarray analysis findings and to examine the expression of genes not included on our microarrays. In addition to aRNA from all the samples analyzed by microarray, aRNA was also prepared from additional control and glaucoma model ONH to increase the final group sizes (12 control, 20 Early Injury, and 24 Advanced Injury). Mean injury grades for these groups were comparable to those in the microarray study (1.0 ± 0.0 , 1.4 ± 0.1 , and 3.9 ± 0.3 , respectively). ONH aRNA (25 ng) from each experimental sample, along with a standard curve ranging from 1.5 to 200 ng pooled control and experimental ONH aRNA, was reverse transcribed to cDNA for QPCR using a kit (SuperScript III Reverse Transcriptase; Invitrogen) according to the manufacturer's protocol. Primers for the 60 messages measured by QPCR were designed from rat nucleotide sequences (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/sites/entrez> and Ensembl <http://www.ensembl.org/index.html>) for products within 350 bases of the target mRNA 3' polyadenylation site using appropriate software (Clone Manager, version 9.0; Sci Ed Software, Cary, NC). Primers used are listed in Supplementary Table S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10.5317/-DCSupplemental>. QPCR was performed (LightCycler DNA Master SYBR Green 1 Kit; Roche Applied Sciences, Indianapolis, IN), and products were verified by sequencing. Each target message was normalized to the average of three glyceraldehyde phosphate dehydrogenase (*Gapdh*) measurements on each sample. *Gapdh* was chosen for normalization because the levels of this message did not differ among groups and there was no significant correlation between ONH *Gapdh* level and IOP level or optic nerve injury grade. ONH gene expression was compared among three groups: controls ($n = 12$), Early Injury (optic nerve injury grade <2 , $n = 20$), and Advanced Injury (optic nerve injury grade >2 , $n = 24$). This analysis was designed to specifically examine gene expression changes associated with the earliest injury. Statistical analyses were performed using statistical software packages (Excel [Microsoft, Redmond WA] and Prism [GraphPad, San Diego, CA]).

ONH DNA Quantitation

To determine whether injury from elevated IOP resulted in increased total ONH DNA content, DNA was extracted from comparable whole control and glaucoma model ONH with injury grades in a range similar to those used in the gene expression studies (PicoPure DNA Extraction Kit; Molecular Devices, Sunnyvale, CA) and was quantitated using an assay kit (Quant-iT PicoGreen dsDNA Assay Kit; Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocols.

RESULTS

The Early ONH Injury Group

Optic nerve injury grades were obtained for all samples, and eigengene analysis was used for data visualization to aid in the separation of glaucoma model sample array data, ordered by injury grade, into Early Injury and Advanced Injury groups for further analysis. The results of this eigengene analysis are shown in Figure 1. Note that the two array sets yielded nearly identical eigengene patterns for the first two eigengenes and that four significant patterns were found for each set. The most significant pattern (Eigengene 1) on both arrays was a linear response, with maximal change in the most severely affected ONH. We found that 52% of array probes were significantly correlated to Eigengene 1 (FDR $<2\%$). This eigengene pattern is representative of genes that are regulated, either up or down in proportion to increasing optic nerve injury, likely reflecting changes related to early injury responses as optic nerve involvement becomes more widespread, and of many genes reflecting all the processes secondary to ongoing axon loss. Regression analysis showed that genes upregulated in propor-

TABLE 1. Microarray Study: IOP Histories by Experimental Group

Group	n	Optic Nerve Injury Grade (Mean $\pm$ SD)	Cumulative Elevated IOP mm Hg (Mean $\pm$ SD)
Controls	6	1.0 $\pm$ 0.0	
Early injury (1 < grade < 2)	9	1.3 $\pm$ 0.3	69 $\pm$ 25
Advanced injury (2 < grade $\leq$ 5)	12	4.3 $\pm$ 0.8	251 $\pm$ 164

tion to injury grade were associated with the gene ontology (GO) categories of extracellular matrix, humoral immune response, lysosome, ribosome, cytoskeleton, cell differentiation, and signaling cascades. Genes that were downregulated in proportion to nerve injury grade included those regulating lipid biosynthesis, glucose metabolism, and mitochondria.

The second most significant pattern (Eigengene 2) for both array sets was a fourth-order polynomial curve, with peak changes in expression below injury grade 2. Fifteen percent of array probes were significantly correlated to this Eigengene pattern, suggesting that a large group of genes demonstrated near maximal changes in expression in ONH from eyes with pressure-induced optic nerve damage of less than grade 2.

Therefore, each sample was assigned to 1 of 3 groups for further analysis: Control ONH (untreated eyes, $n = 6$) and, based on injury grade and the two most significant eigengene patterns, Early ONH Injury (glaucoma model ONH, injury grades <2.0, $n = 9$) and Advanced ONH Injury (glaucoma model ONH, injury grade >2.0, $n = 12$). Table 1 summarizes the injury grade and the cumulative IOP elevation data for the two glaucoma model groups. In the Early Injury group, the mean interval between hypertonic saline injection and tissue collection was 21 ± 9 days. The mean interval between documented IOP elevation (5 mm above mean control eye IOP) and tissue collection was 12 ± 8 days. This group included six of the eyes that were collected at 10 days after the first tonometer (Tono-PenXL; Medtronic) IOP reading of 35 or greater. The peak IOP in the Early Injury group averaged 38.3 ± 1.9 mm Hg. Figure 2 illustrates a typical IOP history for a glaucoma model in the Early Injury group. Figure 3 illustrates an Early Injury group optic nerve lesion. These very early lesions are restricted to, at most, one or a few small, focal areas of the nerve cross-section, with a few degenerating axons intermingled with morphologically normal ones.

FIGURE 2. IOP history of a glaucoma model eye in the Early Injury group. On day 0, hypertonic saline was injected into an episcleral vein to induce elevated IOP, which became apparent at day 20.

FIGURE 3. The area of lesion in a glaucoma model optic nerve with an injury grade of 1.4. Dark condensed degenerating axons (white arrows) occur in a few small, focal areas of the superior portion of the nerve cross-section, and a swollen axon (white arrowhead) is present. These degenerating axons are intermingled with many morphologically intact axons. The remaining optic nerve appears normal. Scale bar, 50 μ m.

Genes Significantly Regulated by Early Injury

The SAM Multiclass and ANOVA analysis yielded a combined total of 877 significantly regulated genes with unique Entrez Gene IDs in the Early Injury group, with 596 upregulated and 281 downregulated genes compared with the control group (Supplementary Table S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>). Fifty-four genes were upregulated to greater than 250% of control ONH levels (Table 2), two-thirds of which have functions associated with the cell cycle and cell proliferation. Table 3 lists genes decreased in expression by at least 50% in the Early Injury group. Supplementary Tables S4 and S5, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>, list genes significantly changed in expression in the other between-group comparisons.

Identification of Functional Gene Categories Significantly Regulated in Early ONH Injury

The most significantly upregulated gene categories by DAVID analysis in the Early Injury group are listed in Table 4. The cell cycle was the most significantly upregulated category and included approximately 14% of all upregulated genes. Among the most upregulated genes in this category were protein regulator of cytokinesis 1 (*Prc1*, 720%), pituitary tumor-transforming 1 (securin, *Pttg*, 622%), and budding uninhibited by benzimidazoles 1 (*Bub1*, 559%), three genes with specific functions in chromosome separation during cytokinesis, suggesting completion of the cell cycle. Note that the most upregulated gene in Table 2 was topoisomerase 2a (*Top2a*, 1424%). Although not a component of the cell cycle GO category, this enzyme is essential for DNA replication and a component of the related category of cell proliferation. The second most upregulated GO category in Early Injury was the cytoskeleton. Several of the most upregulated genes in this category were kinesins with specific mitotic and spindle-associated functions (*Kif18a*, *Kif22*, *Kif23*, *Nek2*, *Tpx2*, and *Nusap1*; 970%–292%), also suggesting cell proliferation. The third category was immune process genes. Among the most affected of these genes were the cytokines chemokine (C-X-C motif) ligand 1 (*Cxcl1*, 271%) and *Lif* (223%), both of which reached peak levels in the Early Injury group. The immune process

TABLE 2. Microarray Analysis Genes Most Upregulated in the Early ONH Injury Group Compared with Control Values

Accession No.	Entrez Gene	Gene Name*	Gene Symbol	Percentage of Control Mean†
BG087390	21973	<i>Topoisomerase IIA</i>	<i>Top2a</i>	1424
BG069098	228421	<i>Kinesin family member 18A</i>	<i>Kif18a</i>	970
AW547246	70021	5'-Nucleotidase domain containing 2	<i>Nt5dc2</i>	808
AW550270	21923	Tenascin C	<i>Tnc</i>	762
BG088644	233406	<i>Protein regulator of cytokinesis 1</i>	<i>Prc1</i>	720
BG063624	52033	<i>PDZ binding kinase</i>	<i>Pbk</i>	698
BG074248	110033	<i>Kinesin family member 22</i>	<i>Kif22</i>	651
BG069168	30939	<i>Pituitary tumor-transforming 1</i>	<i>Pttg1</i>	622
BG073145	80986	<i>Cytoskeleton associated protein 2</i>	<i>Ckap2</i>	608
AW553739	22137	<i>Ttk protein kinase</i>	<i>Ttk</i>	598
BG065692	11504	Adamts1	<i>Adamts1</i>	593
AU018403	51945	<i>Nuclear receptor subfamily 5, A, 2</i>	<i>Nr5a2</i>	591
BG086805	12235	<i>Budding uninhibited by benzimidazoles 1</i>	<i>Bub1</i>	559
BG088110	26934	<i>Rac GTPase-activating protein 1</i>	<i>Racgap1</i>	555
BG072398	14284	Fos-like antigen 2 (Fosl2), mRNA	<i>Fosl2</i>	530
AW553287	50706	Periostin	<i>Postn</i>	521
BG070106	16819	Lipocalin 2	<i>Lcn2</i>	509
BG079289	77022	<i>RIKEN cDNA 2700099C18</i>	<i>2700099C18Rik</i>	499
BG070105	105387	Aldo-keto reductase family 1, member C14	<i>Akr1c14</i>	451
BG072904	19361	<i>RAD51 homolog</i>	<i>Rad51</i>	436
BG078299	52276	<i>Cell division cycle associated 8-Borealin</i>	<i>Cdca8</i>	429
BG068666	71819	<i>Kinesin family member 23</i>	<i>Kif23</i>	407
BG065826	18005	<i>NIMA-related expressed kinase 2</i>	<i>Nek2</i>	405
BG068948	66442	<i>Spindle pole body component 25 homolog</i>	<i>Spc25</i>	385
BG086343	56419	<i>Diaphanous homolog 3</i>	<i>Diap3</i>	376
BG076991	12702	Suppressor of cytokine signaling 3	<i>Socs3</i>	371
BG073234	211770	<i>Tribbles homolog 1</i>	<i>Trib1</i>	360
BG073047	72119	<i>TPX2, microtubule-associated protein</i>	<i>Tpx2</i>	356
BG088567	16009	<i>Insulin-like growth factor binding protein 3</i>	<i>Igfbp3</i>	354
BG076937	16906	<i>Lamin B1</i>	<i>Lmnb1</i>	349
BG071031	17393	Matrix metalloproteinase 7, matrylin	<i>Mmp7</i>	336
BG066442	16647	Karyopherin (importin) α 2	<i>Kpna2</i>	334
BG080700	27279	Tumor necrosis factor receptor superfamily, member 12A	<i>Tnfrsf12a</i>	329
BG079311	238076	Potassium voltage-gated channel, delayed-rectifier, S, 3	<i>Kcns3</i>	328
BG085472	12580	<i>Cyclin-dependent kinase inhibitor 2C</i>	<i>Cdkn2c</i>	318
BG073518	12428	<i>Cyclin A2</i>	<i>Ccna2</i>	307
BG068045	14425	UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	<i>Galnt3</i>	304
BG066443	11806	Apolipoprotein A-I	<i>Apoa1</i>	303
BG072979	108907	<i>Nucleolar and spindle-associated protein 1</i>	<i>Nusap1</i>	292
BG085966	14115	Fibulin 2	<i>Fbln2</i>	288
BG075693	69219	Dimethylarginine dimethylaminohydrolase 1	<i>Ddab1</i>	284
BG067382	74107	<i>Centrosomal protein 55</i>	<i>Cep55</i>	282
BG075357	17219	<i>Minichromosome maintenance deficient 6</i>	<i>Mcm6</i>	281
BG080268	14825	Chemokine (C-X-C motif) ligand 1	<i>Cxcl1</i>	271
BG069808	73710	Tubulin, β 2B	<i>Tubb2b</i>	269
BG085649	216161	Strawberry notch homolog 2	<i>Sbno2</i>	261
BG080666	15937	Immediate early response 3	<i>Ier3</i>	260
AW537534	326618	Tropomyosin 4	<i>Tpm4</i>	259
BG086929	56449	<i>Cold shock domain protein A</i>	<i>Csda</i>	255
BG063171	76448	Phostensin RIKEN cDNA 2310014H01	<i>2310014H01Rik</i>	253
BG080690	20419	<i>Sbc SH2-domain binding protein 1</i>	<i>Sbcbp1</i>	252
BG081419	229841	<i>Centromere protein E</i>	<i>Cenpe</i>	251
BG071607	12457	CCR4 carbon catabolite repression 4-like	<i>Ccrn4l</i>	251
BG084556	17919	Myosin Vb	<i>Myo5b</i>	250

* Italic gene names indicate functions associated with cell proliferation and mitosis.

† Values indicated as percentage of control eye mean, the average of six biologically independent sample values from six separate, independent arrays.

category also included components of the complement cascade (*C1qc*, *C1qa*, *Cfi*, *Cfb*, and *C1r*) that were upregulated to between 215% and 150% of control values in Early Injury.

Table 5 summarizes the significant GO categories of the 271 genes downregulated in the Early Injury group. Most affected were GO categories related to glucose and lipid metabolism. However, the largest category was that for integral membrane proteins. Among the most downregulated genes with known functions in this class were the astrocyte glutamine and gluta-

mate transporters *Slc38a1* (38%) and *Slc1a3* (48%) and the potassium channels *Kcnd2* (42%) and *Kcnj12* (50%).

Inclusion of the few arrays available in the range of 2 to 4 did not have a substantial effect on the identification of genes significantly affected in the Early Injury Group. When we repeated our analysis excluding the data from these intermediate arrays, the probes identified as changed were 96% identical with those identified by the original analysis (Supplementary Table S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/>

TABLE 3. Microarray Analysis Genes Most Downregulated in the Early ONH Injury Group Compared with Control Values

Accession No.	Entrez Gene	Gene Name	Gene Symbol	Percentage of Control Mean*
BG086656	11668	Aldehyde dehydrogenase family 1, subfamily A1	<i>Aldh1a1</i>	15
BG073126	15902	Inhibitor of DNA binding 2	<i>Id2</i>	21
BG071519	223227	HMG-box protein (Sox21)	<i>Sox21</i>	22
BG080229	11676	Aldolase 3, C isoform-L	<i>Aldoc</i>	22
BG067929	75104	Monocyte to macrophage differentiation-associated 2-L	<i>Mmd2</i>	25
BG087678	15360	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2	<i>Hmgcs2</i>	29
BG074398	13602	SPARC-like 1 (hevin)	<i>Sparcl1</i>	33
BG080206	20255	Secretogranin III	<i>Scg3</i>	34
BG067564	21808	Transforming growth factor, β 2	<i>Tgfb2</i>	34
BG073115	18027	Nuclear factor I/A	<i>Nfia</i>	34
BG080743	54418	Formin 2	<i>Fmn2</i>	35
C85331	98582	KH domain containing 1B	<i>Kbdc1b</i>	35
C77281	12388	Catenin, delta 1 transcript variant 1	<i>Ctnnd1</i>	36
BG069709	332175	Zinc finger, DHHC domain containing 23	<i>Zdhhc23</i>	36
AW543487	105727	Solute carrier family 38, member 1	<i>Slc38a1</i>	38
BG080988	244867	Rho GTPase activating protein 20	<i>Arbgap20</i>	38
BG087491	83965	Ectonucleotide pyrophosphatase/phosphodiesterase 5	<i>Enpp5</i>	39
BG078755	76267	Fatty acid desaturase 1-L	<i>Fads1</i>	39
BG065883	52225	DNA segment, Chr 4, ERATO Doi 103	<i>D4Erd103e</i>	39
BG082257	106861	Abhydrolase domain containing 3	<i>Abhd3</i>	39
BG086781	84505	SET domain, bifurcated 1	<i>Setdb1</i>	39
BG080351	75712	Transmembrane protein 14A	<i>Tmem14a</i>	40
BG070174	14866	Glutathione S-transferase, mu 5	<i>Gstm5</i>	41
BG084015	107272	Phosphoserine aminotransferase 1	<i>Psat1</i>	41
BG084178	19049	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	<i>Ppp1r1b</i>	41
BG067863	70757	Protein tyrosine phosphatase-like-L	<i>Ptplb</i>	42
BG073601	13167	Diazepam binding inhibitor	<i>Dbi</i>	42
BG074397	14862	Glutathione S-transferase, mu 1	<i>Gstm1</i>	42
BG087831	16508	Potassium voltage-gated channel, Shal-related family, member 2	<i>Kcnd2</i>	42
BG066209	231051	Myeloid/lymphoid or mixed-lineage leukemia 3	<i>MLL3</i>	42
BG082752	12411	Cystathionine beta-synthase L	<i>Cbs</i>	42
BG073940	228839	TGFB-induced factor 2	<i>Tgif2</i>	43
BG073190	14860	Glutathione S-transferase, α 4	<i>Gsta4</i>	43
BG064512	18631	Peroxisomal biogenesis factor 11a	<i>Pex11a</i>	44
AU018835	58187	Claudin 10A	<i>Cldn10a</i>	44
BG072429	226025	Transient receptor potential cation channel, subfamily M, member 3	<i>Trpm3</i>	44
BG069624	234593	N-myc downstream regulated gene 4	<i>NdrG4</i>	44
BG088903	235180	Fasciculation and elongation protein zeta 1	<i>Fez1</i>	45
BG069040	215821	D10Bwg1379e	<i>D10Bwg1379e</i>	45
BG086971	11487	A disintegrin and metallopeptidase domain 10	<i>Adam 10</i>	45
AW557873	15903	Inhibitor of DNA binding 3	<i>Id3</i>	45
BG088107	50781	Dickkopf homolog 3	<i>Dkk3</i>	46
BG062997	67080	RIKEN cDNA 1700019D03 gene	<i>1700019D03Rik</i>	46
BG076141	108699	Chimerin (chimaerin) 1	<i>Cbn1</i>	46
BG087940	223649	Nuclear receptor binding protein 2	<i>Nrbp2</i>	46
BG079962	236539	3-Phosphoglycerate dehydrogenase	<i>Phgdh</i>	47
BG075920	217480	Diacylglycerol kinase, beta	<i>Dgkb</i>	47
BG069750	17769	5,10-Methylenetetrahydrofolate reductase	<i>Mthfr</i>	47
BG073197	195434	UTP14, U3 small nucleolar ribonucleoprotein, homolog B (yeast)	<i>Utp14b</i>	47
BG075567	259302	SLIT-ROBO Rho GTPase activating protein 3	<i>Srgap3</i>	48
BG075594	384061	Fibronectin type III domain containing 5	<i>Fndc5</i>	48
BG070737	20512	Solute carrier family 1 (glial high-affinity glutamate transporter)	<i>Slc1a3</i>	48
BG087565	54151	Cysteine and histidine rich 1	<i>Cybr1</i>	48
BG066372	17762	Microtubule-associated protein tau	<i>Mapt</i>	48
BG088467	71833	WD repeat domain 68	<i>Wdr68</i>	48
BG087239	226970	Rho guanine nucleotide exchange factor (GEF) 4	<i>Arhgef4</i>	49
BG074754	14081	Acyl-CoA synthetase long-chain family member 1	<i>Acs11</i>	49
BG087975	14183	Fibroblast growth factor receptor 2	<i>Fgfr2</i>	49
BG081752	19791	Rn18s ribosomal RNA	<i>Rn18s</i>	49
BG064101	14897	Thyroid hormone receptor interactor 12	<i>Trip12</i>	49
BG072359	14085	Fumarylacetoacetate hydrolase	<i>Fab</i>	49
BG065128	26399	Mitogen activated protein kinase kinase 6	<i>Map2k6</i>	49
BG073314	16515	Potassium inwardly-rectifying channel, subfamily J, member 12	<i>Kcnj12</i>	49
C87546	20869	Serine/threonine kinase 11	<i>Stk11</i>	49

* Values indicated as percentage of control eye mean, the average of six biologically independent sample values from six separate, independent arrays.

TABLE 4. Upregulated Gene Ontology Categories in Early ONH Injury

Significantly Upregulated Gene Categories	Genes (<i>n</i>)	<i>P</i>
Cell component or biological function*		
Cell cycle	82	<0.00001
Cytoskeleton	77	<0.00001
Immune system process	42	<0.00005
Cell motility	25	<0.005
Extracellular matrix	19	<0.005
Lysosome	16	<0.005
Plasma membrane	65	<0.001
Cell adhesion	32	<0.05
Proteolysis	35	<0.05
Cell differentiation	80	<0.05
Blood vessel development	16	<0.05
Generation of neurons	19	<0.05
Phosphorylation	42	<0.05

* To simplify the table, closely related subcategories are not listed. For example, cell cycle includes 23 significantly affected subcategories of the same genes, grouped by their relationship to different aspects of the cycle.

iovs.10-5317/-DCSupplemental). With the exception of the addition of *Bcas1* as among the most downregulated (41%), there was no change in the genes identified as most significantly affected (Tables 2, 3) or significant GO categories (Tables 4, 5).

Changes in Axonal mRNA Levels as Indicators of Early ONH Injury

In our previous array analysis of extensively injured ONH, we found significant downregulation of several retinal ganglion cell-specific messages that are present in optic nerve axons.²⁷ In the present study, we examined two of these axonal messages, growth-associated protein 43 (*Gap43*) and neurofilament L (*Nefl*) by QPCR. Although both were significantly downregulated in the Advanced Injury group (to 26% ± 5% and 22% ± 5% of control values, respectively), only *Gap43* was significantly reduced in Early Injury (65% ± 10%).

The brain-specific isoform of aldolase, *Aldoc*, was among the most downregulated genes in Early Injury by array analysis (Table 3). *Aldoc* is a component of axonal mRNA,^{38,39} but it is also expressed by astrocytes. By QPCR, *Aldoc* was confirmed to be significantly reduced in both Early (50% ± 9%) and Advanced (24% ± 4%) injury groups. In our arrays, the only distinctly axonal mRNA that was significantly downregulated in the Early Injury group was microtubule-associated protein tau (*Mapt*, 49%). Therefore, as expected based on the small amount of optic nerve degeneration in the Early Injury group,

TABLE 5. Downregulated Gene Ontology Categories in Early ONH Injury

Significantly Downregulated Gene Categories	Genes (<i>n</i>)	<i>P</i>
Cell component or biological function		
Glucose metabolic process	9	<0.0005
Peroxisome	7	<0.005
Lipid metabolic process	20	<0.005
Epithelial cell proliferation	4	<0.01
Serine family amino acid biosynthetic process	3	<0.05
Intrinsic membrane protein	64	<0.05
Positive regulation of progression through cell cycle	3	<0.05

FIGURE 4. Verification of upregulation of selected cell proliferation associated genes in Early and Advanced ONH Injury groups by QPCR. *Top2a* plays a role in DNA replication, whereas *Prc1* and *Esp11* function in cytokinesis. **P* < 0.05, †*P* < 0.01, and ‡*P* < 0.001 compared with the control group.

the axonal mRNA changes in Early Injury ONH were not dramatic.

Confirmation of Upregulation of Cell Proliferation Genes in Glaucoma Model ONH

Our microarray studies identified the cell cycle as the most upregulated biological process in Early Injury. To confirm these observations, we used QPCR to examine expression levels for several genes associated with cell proliferation (Fig. 4). During DNA replication and transcription, *Top2a* controls the topological states of DNA by transient breakage and subsequent rejoining of DNA strands. By QPCR, Early Injury group *Top2a* values were 610% ± 153% of control. *Prc1* regulates the mitotic spindle midzone formation during cytokinesis. Extra spindle pole bodies homolog 1 (*Esp11* or separase) acts together with *Pttg1* (622% in Early Injury on arrays) to regulate chromatid separation at anaphase. By QPCR, *Prc1* and *Esp11* levels were found to be 838% ± 213% and 438% ± 120% of control values in the Early Injury group.

Increased DNA Content with Pressure-Induced ONH Injury

To confirm that cell cycle progression occurs early in ONH injury, total DNA content was measured in groups of glaucoma model ONH with optic nerve injury grades similar to those in the microarray and QPCR studies. ONH DNA content in the Early Injury group was increased to 117% (*P* < 0.05, one way *t*-test) compared with controls, whereas the DNA content was nearly doubled in ONH with Advanced Injury (Table 6).

TABLE 6. Total ONH DNA Content

Group	DNA Content (Percentage of Control Mean)	Grade	<i>n</i>
Control	100 ± 8	1.0 ± 0.0	10
Early injury	117 ± 6*	1.2 ± 0.1	11
Advanced injury	184 ± 12†	4.6 ± 0.2	13

* *P* < 0.05, † *P* < 0.00001, one-tailed *t*-test.

Gene Expression Analysis of IL-6-type Cytokines and Janus Kinase-Signal Transducer and Activator of Transcription Signaling in Early ONH Injury

Our array analysis had demonstrated the upregulation of the cytokine *Lif* (223%) in Early Injury. *Lif* is known to promote glial cell proliferation and astrocytic differentiation.^{40–42} *Lif* signaling by way of the Janus kinase-signal transducer and activator of transcription (Jak-Stat) pathway can lead to the upregulation of the feedback inhibitor suppressor of cytokine signaling 3 (*Socs3*), which was increased to 373% in the Early Injury group (Table 2). Another gene highly upregulated in this group is *Racgap1* (556%), a gene associated with cytokinesis. Interestingly, *Racgap1* has recently been shown to be essential for the Jak phosphorylation of transcription factor, *Stat3* protein, and its entry into the nucleus.⁴³ *Lif* is a member of the IL-6-type cytokine family, all which share a common receptor component (IL-6 signal transducer, *IL-6st*, aka Gp130) to initiate signaling, primarily through Jak-Stat signaling pathways.⁴⁴

Observation of *Lif*, *Socs3*, and *Racgap1* among the most upregulated genes suggested Jak-Stat pathway activation in early ONH injury. Because members of this family play important roles in glial responses to injury, including glial progenitor cell proliferation and differentiation to astrocytes,^{42,45,46} we used QPCR to determine the responses of all IL-6-type cytokines in glaucoma model ONH, many of which were not on our arrays. These studies not only confirmed the upregulation of *Lif*, they also revealed a significant and dramatic upregulation of *IL-6* (1553%), coupled with significant responses by cardiotrophin-like cytokine factor 1 (*Clcf1*, 325%) and downregulation of ciliary neurotrophic factor (*Cntf*, 40%) in the Early Injury group (Fig. 5).

Cellular receptor expression will determine the functional responses to IL-6 family cytokines. We used both our microarray data and QPCR to determine expression levels of the components of all the IL-6-type cytokine receptor components in glaucoma model ONH. By QPCR, we found that *Lif* receptor (*Lifr*) was downregulated to 35% $\pm$ 11% in Early Injury (Fig. 5), whereas the other IL-6 family receptor components (*Il6st* and *Cntfr*, array data, *Il6ra* by QPCR; Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>) were not significantly altered in expression in any experimental group. *Lifr* is the common receptor for *Lif*, *Clcf1*, *Cntf*, and two other cytokines that were unchanged in ONH mRNA expression, oncostatin M (*Osm*) and cardiotrophin 1 (*Ctf1*; Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>).

Among the Jak-Stat pathway components on our arrays, we found moderate, but significant, upregulation of *Jak2* (146%) and *Stat3* (147%) in Early Injury, whereas *Jak1*, *Stat1*, and *Stat2* levels were not significantly affected. We also used QPCR to confirm the upregulation of *Socs3*, finding an increase to 402% $\pm$ 96% in the Early Injury group (Fig. 5). *Socs3* can inhibit signaling through other receptors, such as epidermal growth factor receptor (*Egfr*).⁴⁷ *Socs4*, another inhibitor of *Egfr* signaling, was significantly upregulated (175%) as well by array analysis. By QPCR, *Egfr* levels were unchanged by Early Injury (95% $\pm$ 17%), whereas epidermal growth factor levels fell significantly to 38% $\pm$ 7% (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>).

Cytokine Cxcl1 in Early Injury

Cytokine *Cxcl1*, also highly expressed in the Early Injury group (273%), is expressed by injured astrocytes,⁴⁸ microglia, and oligodendroglial precursors,⁴⁹ is a mitogen for both endothelial cells and oligodendroglia,^{49,50} and may recruit progenitor cells to areas of injury.⁵¹

Other Growth Factors in Early Injury

A number of growth factors have been suggested to play important roles in ONH responses to IOP-induced injury. Transforming growth factor β 2 (*Tgfb2*) is upregulated in the aqueous humor in glaucoma, exerts significant effects in the anterior segment, and may play a role in ONH ECM remodeling in glaucoma.^{52–54} However, our studies show that *Tgfb2* is downregulated in Early Injury ONH (34%, Table 3; QPCR, Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>), confirming our earlier observation.²⁷ In contrast, our QPCR analyses demonstrated that Early Injury had no significant effect on mRNA levels of brain-derived neurotrophic factor, neurotrophin 4/5, nerve growth factor, neurotrophic tyrosine kinase receptor *Trkb*, or p75 neurotrophin receptor (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>).

ECM Gene Expression Changes in Glaucoma Model ONH Injury

Human glaucomatous optic neuropathy involves extensive remodeling of the ECM of the lamina cribrosa, the network of connective, glial, and vascular tissue that spans the scleral opening and supports optic nerve axons as they exit the globe.^{55,56} Our previous studies demonstrate that changes in

FIGURE 5. IL-6-type cytokine, *Lif*, and *Socs3* expression in glaucomatous ONH injury examined by QPCR. This demonstrates the dramatic upregulation of *IL-6* in early ONH injury. In addition, it confirms the upregulation of *Lif* identified by array analysis and reveals significant regulation of 2 of 4 other members of this family that are expressed in the ONH, *Clcf1* and *Cntf*. *Lifr*, the common receptor component for *Lif*, *Clcf1*, and *Cntf* were significantly downregulated in both injury groups. Upregulation of a target of Jak-Stat signaling, *Socs3*, in both injury groups was significant, confirming array results. Note that the scale of the y-axis is log₂ and that Control group variation is indicated by thicker SEM bars. **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001 compared with Controls.

FIGURE 6. Pressure-induced changes in ONH ECM gene expression verified and determined by QPCR. Although changes began in early injury, upregulated gene expression of four matricellular ECM proteins (*Postn*, *Tnc*, *Fbln2*, and *Mgp*) became significant in the Advanced Injury group. In contrast, the integrin component *Itgav* was downregulated in the Early Injury group. *Timp1*, the ECM metalloproteinase inhibitor, was significantly upregulated in both injury groups. Note that the scale of the y-axis is log₂ and that the variation of the Control group is indicated by thicker SEM bars. **P* < 0.05, ‡*P* < 0.01, and †*P* < 0.001 compared with Control group. For *Postn*, *Fbln2*, and *Mgp*, differences between Early and Advanced injury group values are also significant (*P* < 0.01).

ECM gene expression and protein deposition also occur early during IOP-induced injury in rat glaucoma model ONH,^{24,25,27} an observation further supported by the DAVID analysis used in this study (Table 4). By QPCR, we confirmed increased expression levels of four of these ECM genes: *Postn*, *Tnc*, *Fbln2*, and matrix gla protein (*Mgp*; Fig. 6). All four are matricellular proteins, proteins that modulate cell-to-cell and cell-to-matrix interactions and are implicated in processes such as cell proliferation and migration.⁵⁷ Other matricellular proteins significantly affected in early injury were secreted phosphoprotein 1 (osteopontin, *Spp1* 204%) and SPARC-like 1 (hevin, *Sparcl1* 33%). *Spp1* upregulation is associated with microglial activation and stimulates astrocyte migration.⁵⁸ *Sparcl1* has antiadhesion, antiproliferation properties and is present in differentiated astrocytes and endothelial venules.^{59–61}

Increased collagen expression, a characteristic of human and experimental glaucomatous ONH remodeling,^{24,62–64} was indicated in our array study by modest upregulation of collagens *Col4a1* (231%), *Col6a2* (169%), and *Col5a2* (168%) in the Early Injury group.

Integrins are important mediators of cell ECM adhesion.⁵⁶ Among integrin probes on our arrays ($\alpha 1$, $\alpha 3$, $\alpha 5$, αV , and $\alpha 9$; $\beta 1$, $\beta 3$, $\beta 4$, $\beta 5$, and $\beta 7$), only integrins $\beta 1$ (*Itgb1*) and αV (*Itgav*) were significantly altered by Early Injury to 197% and 64% of controls, respectively. *Itgb1* is a common receptor

component for many ECM proteins, whereas *Itgav* receptors are somewhat more selective, linking the cellular cytoskeleton to fibronectin, laminins, tenascins, *Spp1*, vitronectin, L1 cell adhesion molecule, *Postn*, and various RGD-containing proteins.⁶⁵ In neural tissues, *Itgav* is expressed by glia and is often involved in the mediation of vascular interactions.⁶⁶ QPCR confirmed *Itgav* downregulation in the Early Injury group (Fig. 6).

In an earlier study of retinal gene expression changes in our glaucoma model,⁶⁷ *Timp1* was the most upregulated gene. Because probes for this protease inhibitor were not present on the arrays used in the present study, we determined its expression by QPCR, finding it significantly upregulated in the Early (437%) and Advanced ONH Injury group (Fig. 6).

Astrocyte-Specific Marker Expression in Early IOP-Induced ONH Injury

As in our previous report,²⁷ QPCR demonstrated that the expression of the common marker for activated astrocytes, *Gfap*, was not increased in glaucoma model ONH (Fig. 7). Additionally, levels of five other astrocyte-associated messages associated with mature ONH astrocytes were evaluated and found to be either decreased or not significantly changed by QPCR: *Aqp4*, *Gja1*, multiple EGF-like-domains 10 (*Megf10*), and paired box 2 (*Pax2*). *Aqp4* is a water

FIGURE 7. Pressure-induced changes in astrocyte-associated ONH messages determined by QPCR. Levels of four messages associated with mature astrocytes (*Gfap*, *Aqp4*, *Gja1*, and *Megf10*) either were unchanged or were significantly downregulated. Also significantly downregulated was transcription factor *Pax2*, associated with astrocytic proliferative capacity. *Nes* and *Vim* are intermediate proteins expressed in immature astrocytes. Moderate *Vim* upregulation was found in the Advanced Injury group. **P* < 0.05, ‡*P* < 0.01, †*P* < 0.001.

FIGURE 8. Microglial marker message levels determined by QPCR. Upregulation of calcium channel *Aif1* and growth factor receptor *Csf1r* and downregulation of purine receptor *P2ry12* are all associated with microglial activation. Of these messages, only the downregulation of *P2ry12* was significant in the Early Injury group, whereas *Aif1* levels reached significance only in the Advanced Injury group. * $P < 0.05$; † $P < 0.01$.

channel associated with astrocytic endfeet and the blood brain/cerebral spinal fluid barrier.^{68,69} *Gja1* is a key component of astrocytic gap junctions.⁷⁰ *Megf10* is associated with astrocytic phagocytosis.⁴⁰ The transcription factor *Pax2* is specifically expressed by optic nerve astrocytes^{71–73} and associated with proliferative capacity.^{71,74,75} Nestin (*Nes*) and vimentin (*Vim*) are expressed in immature or proliferating astrocytes.⁷⁶ *Vim* levels were increased only in the Advanced Injury group ($145\% \pm 8\%$). Additionally, microarray analysis showed that expression of the glial glutamate transporter *Slc1a3* was significantly reduced in both injury groups (Table 3). Finally, S100b, a macroglial marker, was significantly downregulated in both injury groups (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>).

Microglial-Specific Marker Expression in Early IOP-Induced ONH Injury

Expression levels of some microglial activation markers not present on our arrays were determined by QPCR (Fig. 8). Allograft inflammatory factor (*Aif1*, or IBA1), a calcium channel associated with microglial hypertrophy and proliferation, was significantly upregulated, but only in the Advanced Injury group ($298\% \pm 45\%$). Elevation of Colony stimulating factor 1 receptor (*Csf1r*), which plays a key role in microglial activation, in the Advanced Injury group did not reach statistical significance. Purinergic receptor P2Y12 (*P2ry12*) is an adenine nucleotide receptor abundant in microglia in the brain,⁷⁷ although it is also present in endothelial cells.⁷⁸ *P2ry12* downregulation is associated with microglial activation and process retraction⁷⁹ and was significant in the Early ONH injury group (Fig. 8).

Other messages present on our arrays that are enriched in microglia include cd34 antigen,⁸⁰ ferritin light chain (*Ftl1*), translocator protein (peripheral benzodiazepine receptor, *Tspo*), TGFβ1 receptor 1 (*Tgfb1r1*), and milk fat globule EGF factor 8 protein (*Mfge8*). Of these, only Cd34 was significantly upregulated in the Early Injury group (Supplementary Table S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>).

Oligodendroglia-Specific Marker Expression in Early IOP-Induced ONH Injury

In this study, though we limited our analysis to the initial 0.4-mm portion of the optic nerve, some axons in the distal

portion of this segment were partially myelinated; therefore, our analysis includes a population of oligodendroglia. By QPCR, we found that chondroitin sulfate proteoglycan 4 (*Cspg4*, or NG2), an oligodendroglial precursor marker,⁸¹ was not significantly altered in expression in glaucoma model ONH (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>). By array analysis, platelet-derived growth factor α (*Pdgfa*) was unchanged, and myelin basic protein (*Mbp*) was significantly increased only in the Advanced Injury group (150%, Supplementary Table S4, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>).

Vascular-Specific Marker Expression in Early IOP-Induced ONH Injury

In addition to neural tissues, vascular components of the ONH are likely to play critical roles in the tissue response to elevated IOP, as confirmed by our DAVID analysis of upregulated gene categories (Table 4). The upregulated genes specifically associated by array analysis with the vasculature in the early ONH injury group most frequently were *Adamts1* (593%), tweak receptor (*Tnfrsf12a*, 329%), non-muscle myosin *Myb9* (244%), and the *Vegfb* binding protein neuropilin1 (*Nrp1*, 171%). In addition, among significantly downregulated genes were the endothelin b receptor (*Ednrb*, 55%) and vascular-associated Itgav (Supplementary Table S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>).

To better evaluate the potential contribution of vascular cells to the dramatic gene expression in early pressure-induced ONH injury, we examined some vascular-associated messages by QPCR (Fig. 9). Endothelin 1 (*Edn1*) and adrenomedullin (*Adm*) are potent vasoconstrictor and vasodilator proteins, respectively. Heavy chain myosin 11 (*Myb11*) is a marker for vascular smooth muscle. *Vegfa* and *Vegfb* regulate angiogenesis and blood-brain barrier permeability. The transcription factor hypoxia inducible factor 1α (*Hif1a*) as well as erythropoietin (*Epo*) and heme oxygenase 1 (*Hmox1*) are frequently upregulated under hypoxic conditions (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5317/-/DCSupplemental>). Of these, the only significant change in the Early Injury group was a downregulation of *Vegfb* to $61\% \pm 5\%$.

ONH Gene Expression Change with Physiological IOP Elevation

The physiological IOP elevation associated with the dark phase of the light cycle resulted in moderate, but significant, expression changes in many ONH genes. DAVID analysis indicated that upregulated genes primarily affected signal transduction and the cytoskeleton, whereas downregulated genes were primarily associated with ribosomal protein synthesis. Of the genes significantly regulated by physiological IOP elevation, 170 genes were significantly regulated in the same direction as in the Early Injury group. The magnitude of change was generally greater in Early Injury. For example, of the genes listed in Table 2, only five genes (*Fosl2*, *Trib1*, *Ddab1*, *Tubb2b*, and *Tpm4*) were also upregulated by physiological IOP elevation. For these five, the magnitude was <50% of the level reached in the Early Injury group. Similarly, for the genes downregulated by Early Injury listed in Table 3, Physiological IOP elevation downregulated only *D4Ert103e* and *Ctnnd1* by >50%.

DISCUSSION

Microarray analysis of gene expression is a powerful tool for evaluating tissue responses to injury, such as elevated IOP in the eye. However, the contribution of array analysis to the understanding of disease processes may be limited by a number of factors, including the quality and relevance of the sam-

FIGURE 9. Vascular-associated message levels determined by QPCR. Message levels for the vasoactive compounds, *End1* and *Adm*, were not significantly altered, *Vegf* isoforms were not upregulated, and message levels of *Hif1a* remained unchanged. The smooth muscle myosin *Myh11* was significantly downregulated in the Advanced Injury group. * $P < 0.05$.

ples analyzed, the adequacy of the study design, the array platform and the probe spectrum it contains, the quality of the technical procedure, and the statistical analysis. Additionally, key messages or pathway components may not be present at a level of abundance that allows their detection, or, perhaps more important, pathways may be regulated at the protein level rather than the gene expression level. With these caveats, the microarray analysis and supportive studies reported here lead to the following observations about the earliest ONH responses to elevated IOP exposure.

The ONH Genomic Response to Minimal Pressure-Induced Injury Is Dominated by the Upregulation of Cell Proliferation Genes

Minimal exposure to elevated IOP (cumulative exposure of <100 mm Hg above controls over a 2-week period) results in the significant regulation of a large number of ONH genes. These Early Injury genes are dominated by the increased expression of genes associated with all phases of the cell cycle, indicating that cell cycle progression and mitosis are early and significant events in ONH injury. This study also shows that for most of these cell cycle genes, peak ONH levels are reached when optic nerve axon degeneration is <15%. Our findings of increased DNA content in these minimally injured ONH support these observations. These studies also confirm similar observations made in our earlier study of longer (1-mm) segments of glaucoma model ONH from eyes with extensive optic nerve axon degeneration.²⁷

Altered IL-6-type Cytokine Signaling Is Implicated in the ONH Response to Early Elevated IOP-Induced Injury

This study demonstrates significant upregulation of three IL-6-type cytokines (*IL-6*, *Lif*, and *Ccl1*) and implicates altered Jak-Stat pathway signaling, as evidenced by *Socs3* upregulation, as early ONH responses to elevated IOP exposure. *IL-6* and *Lif* can be produced by all the major cellular components of the ONH (Lambert WS, et al. *IOVS* 2009;50:E-Abstract 2792),^{41,42,82–87} and both can exert axonal neuroprotective, regenerative,^{88–92} and proliferative responses.^{45,93} All three cytokines can induce astrocytic differentiation from precursor cells.^{42,94–96} *Lif* may also promote microglial proliferation.⁹⁷ In addition, these cytokines may act on ONH vascular components because *IL-6* can stimulate smooth muscle cell prolifer-

ation,⁹⁸ although *Lif* has been shown to inhibit retinal endothelial cell proliferation.⁹⁹ Although less is known about *Ccl1*, this cytokine does have both growth factor¹⁰⁰ and neuroprotective properties,¹⁰¹ and its expression is associated with response to stress.¹⁰² In contrast to the other three, *Cntf* was significantly downregulated in Early Injury, as has been observed after nerve crush.¹⁰³ ONH astrocytes express *Cntf* and its tripartite receptor components,¹⁰⁴ and, in retina, *Cntf* represses glial proliferation.¹⁰⁵ Therefore, the downregulation of *Cntf* may complement the proliferation-promoting actions of the upregulated cytokines in this family. Together, these observations suggest that altered expression of these cytokines may contribute to proliferative responses in the ONH to elevated IOP exposure.

In Early Injury ONH, expression levels for the receptor components of the IL-6-family cytokines were unchanged, except for the downregulation of *Lifr*, the common receptor component for *Lif*, *Ccl1*, and *Cntf*. *Lifr* is expressed by most ONH cellular components^{106–112,113} and is important for astrocyte precursor differentiation, including Jak-Stat pathway-regulated *Gfap* expression.^{114–117} Therefore, the downregulation of this receptor is consistent with the lack of increased *Gfap* expression. In addition, in the retina, *Lifr* plays an essential role in endogenous neuroprotective mechanisms triggered by preconditioning-induced stress,¹¹⁸ suggesting that *Lifr* downregulation in the ONH may decrease the effectiveness of potential protective signaling by this receptor.

These observations suggest the involvement of IL-6-type cytokines in early ONH injury; however, we fully realize that the cell-specific locations and impact on ONH integrity of potential activated receptor pathway signaling remains to be determined.

Upregulation of GFAP or Other Astrocyte-Specific Genes Is Not a Characteristic of Early ONH Responses to Elevated IOP Exposure

Upregulation of *Gfap* is such a common marker for astrocytes responding to, or “activated” by, neural injury that the increased expression of this intermediate filament protein is often considered necessary and sufficient evidence of an astrocytic reaction. In glaucoma model retinas and the RGC layer, *Gfap* upregulation is readily observed.^{67,119–121} However, in the ONH, early injury from elevated IOP exposure is not associated with the significant upregulation of *Gfap* or any

other astrocyte-specific gene we examined. Rather, a number of unique astrocytic genes were significantly downregulated. This finding is consistent with the expected downregulation of differentiated cellular functions that would be anticipated if astrocytic mitosis were a major component of the proliferative responses observed in these minimally injured ONH. This finding also suggests an initial passive, rather than active, contribution of astrocytes to further nerve injury because a reduction in differentiated astrocytic functions is likely to include compromised astrocyte-mediated metabolic and functional support for axons. In the ONH, where the metabolic demands of unmyelinated axons are much greater than those of the myelinated nerve¹²² and individual astrocytes are in functional contact with many axons,^{123–125} such a compromise is likely to contribute significantly to further axonal vulnerability to elevated IOP.

Activation and Potential Proliferation of Other Glia Cell Types

In early injury, a purine receptor abundant in brain microglia,⁷⁷ *P2ry12*, was significantly downregulated, a characteristic of microglial activation.^{79,126,127} Additionally, our array data showed that *Nrp1* was significantly upregulated by early injury, and *Nrp1* upregulation has been localized to activated microglia.¹²⁸ Further evidence of microglial activation became apparent in eyes with more advanced injury as *Aif1* expression was significantly increased. However, additional studies are needed to determine specifically whether both microglial proliferation and activation occur early in the time course of pressure-induced ONH injury.

Early Injury and Vasculature-Associated Genes

Our array GO analysis and our QPCR studies indicate that early pressure-induced ONH injury is associated with altered expression of some genes associated with the vascular component of the ONH. Similar *Myb9* and *Myb11* responses are observed in carotid artery injury,¹²⁹ and *Tnfrsf12a* upregulation is associated with endothelial cell proliferation and migration. *Nrp1* is localized to the perivascular region of injured neural tissues,¹³⁰ in proximity to its binding partner, *Vegfb*, which was downregulated in the ONH in early injury. Although the specific functions of *Vegfb* protein are not well understood, it is abundant in brain and retina and is thought to play a critical role in the survival, rather than growth, of vascular cells.^{131–133}

ECM Matricellular Protein Gene Expression

Expression levels of a number of ECM matricellular proteins were significantly altered in early ONH injury. The upregulation of one of these messages, *Postn*, begins in early injury and is one of the most dramatically upregulated genes in advanced injury. In our study of extensively injured ONH, *Postn* was the most upregulated gene.²⁷ *Postn* is expressed in cells subjected to mechanical stress, particularly vascular smooth muscle cells.¹³⁴ *Postn* and the other affected matricellular genes are expressed by astrocytes^{39,59,135,136} and are altered in expression during injury-induced vascular remodeling and repair.^{137–142} These observations suggest that modification of astrocyte-ECM, and possibly vascular cell-ECM, interactions begins early in pressure-induced ONH injury, implying that the interface between astrocyte processes and the vasculature may be particularly vulnerable.

Further Directions

Currently, we are using immunohistochemistry to identify proliferating ONH cells and those that express the IL-6-type cytokines as well as the protein products of other affected genes

identified in this study. In addition, we are in the process of developing a model of glaucomatous ONH injury in which the level and duration of elevated IOP exposure can be precisely controlled. Such a model will allow us to determine the exact onset and time course of these ONH gene expression responses, with the goal of understanding their relationship to the development of glaucomatous axon loss.

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