Microarray Analysis of Retinal Gene Expression in the DBA/2J Model of Glaucoma

Michael R. Steele,¹ *Denise M. Inman*,² *David J. Calkins*,³ *Philip J. Horner*,² *and Monica L. Vetter*¹

PURPOSE. The DBA/2J mouse is a model for secondary angleclosure glaucoma, due to iris atrophy and pigment dispersion, which ultimately lead to increased intraocular pressure (IOP). The study was undertaken to correlate changes in retinal gene expression with IOP elevation by performing microarray analysis of retinal RNA from DBA/2J mice at 3 months before disease onset and at 8 months after IOP elevation.

METHODS. IOP was monitored monthly in DBA/2J animals, and animals with normal (3 months) or elevated IOP (8 months) were identified. RNA was prepared from three individual retinas at each age, and the RNA was amplified and used to generate biotin-labeled probe for high-density mouse gene microarrays (U430.2; Affymetrix, Santa Clara, CA). A subset of genes was selected for confirmation by quantitative RT-PCR, by using independent retina samples from DBA/2J animals at 3, 5, and 8 months of age and compared to retinas from C57BL/6J control animals at 3 and 8 months.

RESULTS. There were changes in expression of 68 genes, with 32 genes increasing and 36 genes decreasing at 8 months versus 3 months. Upregulated genes were associated with immune response, glial activation, signaling, and gene expression, whereas downregulated genes included multiple crystallin genes. Significant changes in nine upregulated genes and two downregulated genes were confirmed by quantitative RT-PCR, with some showing changes in expression by 5 months.

CONCLUSIONS. DBA/2J retina shows evidence of glial activation and an immune-related response after IOP elevation, similar to what has been reported after acute elevation of IOP in other models. (*Invest Ophthalmol Vis Sci.* 2006;47:977–985) DOI: 10.1167/iovs.05-0865

G laucoma is a progressive eye disease that leads to blindness due to loss of retinal ganglion cell (RGC) viability and degeneration of the optic nerve.¹⁻⁵ Elevation in intraocular pressure (IOP) is a significant risk factor for glaucoma and can lead to optic nerve damage.^{6,7} However, although sensitivity to IOP can be a significant initiating event, other factors must contribute to neurodegeneration in this disease. For example,

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Corresponding author: Monica L. Vetter, Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132; monica@neuro.utah.edu.

not all patients with elevated IOP have glaucoma. Conversely, there are also patients with normal IOP who exhibit optic nerve disease and vision loss characteristic of glaucoma.⁸ Furthermore, in the DBA/2J mouse model of glaucoma in which IOP increases with age due to pigment dispersion from the iris and obstruction of the trabecular meshwork, high-dose radiation followed by syngeneic bone marrow transfer almost completely rescues optic nerve damage and RGC loss, without altering the course of IOP elevation.⁹ This raises the question of whether there are factors intrinsic to the retina that contribute to RGC disease as glaucoma progresses.²

Animal models are critical for obtaining a detailed molecular analysis of retinal changes in glaucoma. For example, IOP can be experimentally elevated in the rat or monkey by injecting hypertonic saline into the episcleral vein or by laser photocoagulation of the trabecular meshwork, and this elevation is associated with optic nerve damage, loss of RGC viability, and blindness.^{6,10} These acute models of glaucoma mimic many aspects of the disease and have been used to analyze both cellular and molecular changes associated with increased IOP. Significant changes in the optic nerve correlate with induced elevations in IOP. These include disruption in retrograde axonal transport along the optic nerve¹¹; blockage of both BDNF and TrkB transport, which may result in neurotrophin deprivation at the soma^{3,4}; and remodeling of the optic nerve head, which mimics the cupping of the optic nerve head in humans.^{6,10} Within the retina, RGCs undergo apoptotic death,¹²⁻¹⁵ and there are changes in other retinal cell populations as well. For example, there is evidence that Müller glia and retinal astrocytes become activated after IOP elevation,16-19 and microglial activation has been reported in association with degenerating RGCs.²⁰ Molecular analysis of the retina using microarrays or quantitative reverse transcription polymerase chain reaction (RT-PCR) has shown that IOP elevation elicits changes in the expression of multiple genes, including those involved in iron regulation, glial activation and an immune response.16,21,22

Together, the studies just described reveal a complex retinal response to the insult of elevated IOP. This response raises the question of how gene expression changes in retinal tissue during the progressive development of glaucoma, as occurs in humans, and whether these changes provide insight into the molecular events underlying the loss of RGCs. The inbred DBA/2J mouse strain has emerged as a useful model of secondary, angle-closure glaucoma. This mouse strain carries mutations in two genes, Tyrp1 and Gpnmb, that trigger an immune response in the iris. The immune response leads to iris atrophy and pigment dispersion, $^{23-25}$ which blocks aqueous humor drainage and ultimately causes increased IOP that worsens over time in an age-dependent manner, mimicking the progressive time course associated with human glaucoma.²³⁻²⁵ The DBA/2J is becoming increasingly relevant in light of recent advances in mouse genetics that have made it possible to manipulate gene expression and directly test the role candidate genes play in glaucoma progression. However, to date there has not been an analysis of retinal gene expression in the DBA/2J and an assessment of whether particular molecular

From the ¹Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, Utah; the ²Department of Neurosurgery, University of Washington, Seattle, Washington; and the ³Department of Ophthalmology and Visual Sciences, Vanderbilt University School of Medicine, Nashville, Tennessee.

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changes are associated with IOP elevation in this mouse. Thus, we have undertaken a microarray analysis of whole retina RNA from DBA/2J animals to obtain an initial survey of gene expression changes associated with IOP elevation. We also have used quantitative RT-PCR to compare expression of certain genes identified with the array at early and later time points after IOP elevation. The pattern of gene expression we describe is consistent with a glial response and upregulation of immune-related genes, including complement components, suggesting that these genes represent a response to elevated IOP in the DBA/2J retina. These changes in gene expression bear similarity to gene array results from other glaucoma models^{16,22} and also highlight parallels with other neurodegenerative diseases of the central nervous system.

METHODS

Animals and IOP Measurement

DBA/2J and C57BL/6J animals were bred and handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve DBA/2J animals and seven C57BL/6J animals were used for the study. All animals were female. IOP in each eye was measured in DBA/2J animals every month, generally beginning at 3 months of age, with a handheld tonometer (Tono-Pen XL; Medtronic Solan, Jacksonville, FL) (Supplementary Table S1, available online at http://www.iovs.org/cgi/content/full/47/3/977/DC1), although there were a few animals for which complete IOP history was not available. A minimum of 20 Tono-Pen measurements were recorded for each eye at each time point as described in Inman et al. (manuscript submitted). Because the working range of the Tono-Pen is 5- to 80-mmHg, pressures obtained outside this range were discarded. Animals were selected for each group based on IOP elevation, with a focus on identifying animals with IOP elevation of comparable duration, generally beginning at 5 to 6 months of age (Supplementary Table S1). We confirmed that the Tono-Pen could reliably detect changes in IOP by raising (with adenosine) or lowering (with MRS-1191) IOP in DBA/2J mice, including in older mice with substantial corneal occlusion (Inman et al., manuscript submitted; and data not shown).

RNA Isolation, Amplification, and Labeling

Whole retinas were removed from 3-, 5-, and 8-month-old animals and rinsed in ice-cold 0.1 M phosphate-buffered saline to remove blood. Individual retinas were homogenized in buffer (RLT; Qiagen,Valencia, CA) by using a micropestle and were pulled five times through a 22-gauge needle and flash frozen in liquid nitrogen. Total RNA was then isolated (RNeasy Kit; Qiagen). The quantity and quality of the RNA was assessed with a spectrophotometer (ND1000; NanoDrop Technologies, Rockland, DE) and a bioanalyzer (model 2100; Agilent, Palo Alto, CA). For the 3- and 8-month samples, 20 ng of each retinal RNA sample was amplified, fractionated, and labeled with a biotin kit (Ovation; NuGen Technologies, San Carlos, CA) to generate probes for Affymetrix (Santa Clara, CA) microarray analysis.

Microarray Data Analysis

Biotinylated probes were hybridized to high-density mouse Affymetrix arrays (U430.2), according to the manufacturer's protocol. All probes had highly similar 5'-3' probe degradation plots. All preprocessing of Affymetrix data was performed using the Bioconductor Package²⁶ in the R statistical environment (R: a language and environment for statistical computing. http://www.r-project.org/). Quality control evaluation was performed for each microarray by confirming similarity of density distributions of the raw and normalized data, allowing valid normalizations to the final data set. Data set analysis was performed with R² version 1.9.0 on i686-pc-linux-gnu: Bioconductor package "affy" version 1.4.32, "gcrma" version 1.1.0, "siggenes" (R significance analysis of microarray [SAM]) version 1.0.6. Generally, *.cel files were read in and analyzed in appropriate groups. They were background corrected using gcrma, normalized with quantile normalization, and summary measures for probe sets were obtained by median polish. The Affymetrix data files are available through the National Center for Biotechnology Information's (NCBI's) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo/ provided in the public domain by the National Institutes of Health, Bethesda, MD) and have been assigned a series record number of GSE3554. For the SAM in R, the package siggenes was used on preprocessed data with a delta of 0.1112. The results of the SAM analysis were then filtered for a change of 1.8-fold or greater. Similar results were obtained using a rank product analysis (data not shown and Ref. 27).

Quantitative RT-PCR

Reverse transcriptase reactions were performed starting with 500 ng of total RNA for each sample and using the reverse transcription portion of a qRT-PCR lot (SuperScript III Platinum Two-Step qRT-PCR; Invitrogen, Carlsbad, CA) to produce cDNA. The real-time PCR reactions were completed (SYBR Green PCR Master Mix; Applied Biosystems, Inc. [ABI], Foster City, CA) and the following primer sets: β-actin, forward (F) 5'-TATTGGCAACGAGCGGTTCC-3' and reverse (R) 5'-GGCATA-GAGGTCTTTACGGATGTC; ceruloplasmin, F 5'-CTGATGTCTTTGAC-CTTTTCCCTG-3' and R 5'-TTCTCGTTTTCCACTTATCGCC-3'; chemokine ligand 12, F 5'-CAGGAGAATCACAAGCAGCCAG-3' and R 5'-GGGAACTTCAGGGGGGAAATACG-3'; chitinase 3-like 1, F 5'-TGGTG TGGGCACTGGATTTG-3' and R 3'-TCCTTGATGGCGTTGGTGAG-3'; complement compement1qβ, F 5'-ATGGATGCGTAATCACGGGG-3' and R 5'-GTCTGGGTTTCAGGCAGTCAAG-3'; interferon-induced transmembrane protein1, F 5'-CCTGTTCTTCACCATCCTCACG-3' and R 5'-TTTGGGCAGCGATAGACAAGG-3'; lipocalin 2, F 5'-ACTGAATGGGT-GGTGAGTGTGG-3' and R 5'-TCTGGCAACAGGAAAGATGGAG-3' or F 5'-AGCCACCATACCAAGGAGCATC-3' and R 5'-TTTATTCAGCA-GAAAGGGGACG-3'; P-lysozyme structural, F 5'-GCTTTCCCCTCCAAG-TAACAGGAC-3' and R 5'-TGGCTTTGCTGACTGACAAGG-3'; b2-Microglobulin: F 5'-CCTGTATGCTATCCAGAAAACCCC-3' and R 5'-GCAGTTCAGTATGTTCGGCTTCC-3'; major intrinsic protein of eye lens fiber (MIP), F 5'-TTTCTCTCGGCTATCCTTTCTGC-3' and R 5'-TTCCTTGTTCGTTCAGGGTCAC-3'; and crystallin beta A4, F 5'-GCTGAACGATGACTATCCCTCTCTG-3' and R 5'-ACTGAAAACCTCG-GTAGCCAGG-3'. All qRT-PCR was performed in triplicate using three to seven independent retinal samples (Prism 7700 Sequence Detection System; ABI). Cycling conditions were the same for all primer sets: 95°C for 10 minutes and cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 40 seconds. A computer program (SDS2.1 software; ABI) was used to visualize the data. For each primer set, a dissociation curve was performed to confirm that there was a single peak corresponding to a single product and no primer dimer. The Comparative Critical Threshold Method²⁸ was used to determine relative changes in gene expression levels, using β -actin as a reference, which did not change significantly in our samples. Standard deviation was calculated as described by the manufacturer,²⁸ and a Student's t-test was used to calculate statistical significance between 3-month samples and either 5- or 8-month samples. To assess the relative efficiencies of the primer pairs, we also performed a standard curve for each primer pair and calculated the changes using the Standard Curve Method,²⁸ with results similar to those of the Comparative Critical Threshold Method (not shown).

RESULTS

Identification of Animals for Microarray Analysis

Because there is variability in the age of onset of IOP elevation in the DBA/2J strain,²³ we monitored IOP in the mice chosen for this study generally beginning at 3 months of age, using a Tono-Pen (Medtronic Solan; Fig. 1; Supplementary Table S1, http://www.iovs.org/cgi/content/full/47/3/977/DC1). Elsewhere, we have shown that the Tono-Pen measurements are reliable for repetitive measurements over time, but consistently overpredict IOP measured by cannulation by a factor of



FIGURE 1. Plot of IOPs values for the DBA/2J colony and final IOP values for eyes used in this study as a function of age. (•) The average IOPs for all animals in the colony (mean \pm SEM) were fit with the best-fitting sigmoidal. The following shows the number of eyes measured in each age group: 2 (n = 195), 3 (n = 446), 4 (n = 412), 5 (n = 401), 6 (n = 357), 7 (n = 336), 8 (n = 332), 9 (n = 269) and 10 (n = 69) months. (\triangle) The final IOPs for individual eyes at 3 or 8 months were used to prepare retinal RNA for microarray analysis. (\Box) Final IOPs for individual eyes at 3, 5, or 8 months that were used to prepare retinal RNA for quantitative RT-PCR confirmation.

1.5 (Inman et al., manuscript submitted). This is consistent with other published Tono-Pen measurements in mice.²⁹ Average IOPs for the colony were 15 to 16 mm Hg (n = 195 eves) at 2 months of age and rose approximately linearly to 22 to 23 mm Hg (n = 69 eyes) at 10 months of age (Fig. 1), although elevated IOP could be detected in individual eyes as early as 4 to 5 months of age (Fig. 1). Three eyes at each age were selected for microarray analysis from 3- and 8-month-old DBA/2J animals (Table 1). The 3-month eyes were chosen based on the clustering of their IOPs near or below the colony average, whereas the 8-month eyes were chosen because they demonstrated a 40% to 50% increase in IOP compared to 3-month eyes and because their IOPs were 20% to 40% greater than the colony average at 8 months (Fig. 1). The 8-month eyes selected for analysis also generally showed evidence of IOP elevation starting at 5 to 6 months (see Supplementary Table S1, http://www.iovs.org/cgi/content/full/47/3/977/DC1) and remained high, typically exceeding the colony average at each month by 20%. Parallel studies have shown that sustained elevation in IOP in DBA/2J animals is associated with glaucoma-like optic nerve disease.³⁰

Microarray Analysis of Retinal RNA

To allow us to correlate changes in gene expression with the IOP history for individual retinas, we avoided pooling of samples and isolated total RNA from three individual retinas at each age. We then performed a linear amplification of the RNA and incorporated biotin label to prepare probe for hybridization to high-density mouse gene chips (U430.2; Affymetrix), which represents more than 39,000 transcripts. SAM analysis³¹ using a 1.8-fold cutoff and a delta of 0.1112 revealed significant changes in expression of 68 genes, with 32 genes increasing (Table 2) and 36 genes decreasing (Table 3) at 8 months versus 3 months. Similar results were obtained using rank product analysis (data not shown and Ref. 27). Most notable among the upregulated genes were multiple immune-related genes, including complement factors, lipocalin2, chemokine ligand 12, and chitinase 3-like (gp39), as well as genes associated with activated glia such as glial fibrillary protein (GFAP) and ceruloplasmin. The downregulated genes included a large number of crystallin genes, as well as cytoskeletal and extracellular matrix components.

Verification of Upregulated Genes by qRT-PCR

Seven of the upregulated genes were selected for confirmation by qRT-PCR, with a focus on genes identified in other microarray studies after acute IOP elevation in an attempt to identify common retinal responses to IOP elevation.^{16,22} Gene-specific primers were designed to yield PCR products between 80 and 200 bp in length, and β -actin was used a reference gene. We compared gene expression at 3 (n = 6 eyes), 5 (n = 4 eyes), and 8 months (n = 7 eyes) from retinas of DBA/2J animals (see Table 1), using individual retinal RNA samples that were independent of those used for the microarray analysis. For these experiments, we chose 3-month DBA/2J animals whose IOPs were at or below the colony average, 5-month animals with a broader spectrum of IOPs, and 8-month animals with IOPs at or above the colony average (Fig. 1). We chose 5 months as an intermediate time point to address which gene expression changes are evident shortly after elevation in IOP. To control for age-dependent changes in gene expression, we also compared levels of gene expression in the retinas of 3-month (n =4 eyes) versus 8-month (n = 5 eyes) C57BL/6J animals.

We found that the seven genes tested were significantly upregulated at 8 months (Fig. 2). Several genes, including ceruloplasmin, complement component C1q, chemokine ligand 12, and chitinase 3-like 1, were modestly upregulated by 5 months, suggesting that they may represent an early response of the retina to elevated IOP. In general, the level of gene upregulation was significantly higher using the qRT-PCR method than was observed from the microarray results. For example, lipocalin2 was 6.1-fold upregulated on the array (Table 2), but was 30-fold upregulated by qRT-PCR in 8-month DBA/2J retinas (Fig. 2), suggesting that qRT-PCR is more sensitive for detecting changes in gene expression. No statistically significant changes in the expression of any of the genes tested

TABLE 1. Final IOP for DBA/2J Retinas

Animal # (L or R eye)	Age (mo)	Final IOP (mm Hg)		
Retinas for microarray a	analysis			
1024L	3	19.1		
1025L	3	15.3		
1026L	3	18.2		
543L	8	25.3		
544R	8	26.5		
552R	8	30.4		
Retinas for qRT-PCR co	nfirmation analysis			
1024R	3	16.0		
1026R	3	17.1		
1175L	3	15.6		
1175R	3	15.1		
1188L	3	15.0		
1188R	3	14.4		
1078L	5	20.0		
1078R	5	24.2		
1087L	5	25.8		
1087R	5	21.8		
543R	8	23.2		
544L	8	23.2		
552L	8	28.6		
724L	8	29.4		
724R	8	28.3		
740L	8	25.2		
740R	8	21.4		

TABLE	2	List	of	Genes	$\mathbf{L}\mathbf{b}$	pregulated	in	8-Month	versus	3-Month	DBA	21	Retina
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Accession Number	Gene Name			
Immune response	se			
NM_008491	Lipocalin 2 (Lcn2/NGAL)	6.1		
NM_007695	Chitinase 3-like 1 (Chi $311 = gp39$)	3.5		
NM_021365	X-linked lymphocyte-regulated 4 (Xlr4)	2.8		
NM_026820	Interferon-induced transmembrane protein 1 (Ifitm1)	2.7		
NM_025378	Interferon-induced transmembrane protein 1 (Ifitm3/Fgls)	2.5		
NM_178083	Interferon regulatory factor 6 (lrf6)	2.1		
NM_009777	Complement component 1, q subcomponent, beta polypeptide (C1qb)	2.1		
NM_010745	Lymphocyte antigen 86 (LY86/MD-1)	1.9		
NM_009780	Complement component 4 (within H-2S) (C4)	1.9		
NM_009252	Serine (or cysteine) proteinase inhibitor, clade A, member 3N (Serpina3n/ Spin2c)	1.9		
NM_011331	Chemokine (C-C motif) ligand 12 (Cel12/MCP-5)	1.8		
Glial activation				
NM_007752	Ceruloplasmin (Cp)	1.9		
NM_010277	Glial fibrillary acidic protein (Gfap)	2.2		
Regulators of tra	inscription/translation/splicing			
NM_026375	Embryonic large molecule derived from yolk sac (Elys)	2.0		
NM_016809	RNA binding motif protein 3 (Rbm3)	2.0		
A54691	Splicing factor proline/glutamine rich (SFPQ)	1.8		
NM_013507 AK011687	Eukaryotic translation initiation factor 4, gamma 2 (Eif4g2) RIKEN cDNA 2610036A22 gene (predicted DNA binding Zn-finger protein C2H2 type, KRAB box containing)	1.8 2.3		
Regulators of sig	naling			
NM_019444	Receptor (calcitonin) activity modifying protein 2 (Ramp2)	1.8		
AK031675	Angiopoietin-like factor (CDT6 protein)	1.8		
Enzymes/kinases	3			
NM_013590	P lysozyme structural (Lzp-s)	2.7		
NM_009773	Budding uninhibited by benzimidazoles 1 homolog, beta (Bub1b)	2.0		
XM_134707	RIKEN cDNA 4921509F24 gene	4.1		
AF358867	Tribbles homolog 2 (Trib2)	2.1		
Cytoskeleton/ex	tracellular matrix			
NM_013808	Cysteine and glycine-rich protein 3 (Csrp3/MLP)	2.2		
NM_133859	Olfactomedin-like 3 (Olfmi3)	1.9		
Miscellaneous/u	aknown function			
BC004722	RIKEN cDNA 2210401K01	1.8		
NM_144906	RIKEN CDNA 3110007P09	2.3		
AK039433	RIKEN CDNA A330042121	2.1		
XM_354975	ribronectin type III domain-containing 1 (Fndc1)	2.0		
AK0/8020	REUNIOR ACIU-INUUCEU 10 (KAI10) DIKEN CDNA A220076H08 gene	1.9		
AK040049	AIKLIV CDIVA AJJUU/UHUO gene	1.7		

Genes in bold were selected for confirmation by qRT-PCR.

were observed in C57BL/6J animals at 8 months compared with 3 months, suggesting little contribution by age-dependent changes in gene expression. Because the microarray may have underrepresented the number of genes that were significantly increased at 8 months versus 3 months, we also tested for upregulation of one other candidate gene from the microarray results that fell below the 1.8-fold cutoff. β 2-Microglobulin is a light chain component necessary for expression of most major histocompatibility complex (MHC) class I proteins and was found to be upregulated twofold at 5 months and threefold by 8 months in DBA/2J retinas.

Verification of Downregulated Genes by qRT-PCR

In addition to the upregulated genes we also observed downregulation of multiple genes, including crystallin genes and cytoskeletal and matrix components. Two of the most significantly downregulated genes, major intrinsic protein of the lens and crystallin beta A4, were selected for confirmation by qRT-PCR on retinal RNA from 3-, 5-, and 8-month-old DBA/2J animals, as described earlier. Both genes were significantly downregulated by 8 months, with only a slight decrease apparent at 5 months (Fig. 3).

DISCUSSION

In this study, we were able to monitor IOP in DBA/2J animals and profile changes in gene expression on individual retinas with a known IOP history. We were further able to use qRT-PCR to assess whether gene expression changes were evident at early or later time points after IOP elevation. As discussed in detail below, a subset of the gene expression changes identiTABLE 3. List of Genes Downregulated in 8-Month versus 3-Month DBA/2J Retina

Accession Number	Gene Name	Change (x-fold)
Lens proteins		
NM_144761	Crystallin, gamma B (Crygb)	-3.4
NM_007776	Crystallin, gamma D (Crygd)	-1.8
NM_153076	Crystallin, gamma N (Crygn)	-2.3
NM_021352	Crystallin beta B3 (Crybb3)	-2.6
NM_008600	Major intrinsic protein of eye lens fiber (Mip)	-4.8
NM_021351	Crystallin, beta A4 (Cryba4)	-4.4
NM_177693	Lens intrinsic membrane protein 2 (Lim2)	-2.1
NM_023695	Crystallin, beta B1 (Crybb1)	-3.0
NM_021541	Crystallin, beta A2 (Cryba2)	-2.4
NM_013501	Crystallin, alpha A (Cryaa)	-1.9
XM_132470	Galectin-related interfiber protein (Grifin)	-2.0
NM_009965	Crystallin, beta A1 (Cryba1)	-2.1
Cytoskeletal/mat	rix proteins	
NM_007733	Procollagen, type XIX, alpha 1 (Col19a1)	-1.9
NM_198104	T-complex-associated testis expressed 3 (Tcte3)	-2.1
AF064749	Procollagen, type VI, alpha 3 (Col6a3)	-2.2
NM_175836	Spectrin beta 2 (Spnb2)	-2.0
Enzymes/metabo	lic	
NM_172466	Adamts18	-1.8
NM_010256	Phosphoribosylglycinamide formyltransferase (Gart/Prgs)	-2.1
NM_013743	Pyruvate dehydrogenase kinase, isoenzyme 4 (Pdk4)	-2.2
NM_009388	Transketolase (Tkt)	-1.8
NM_007513	Solute carrier family 7 (cationic amino acid transporter, y+1 system), member 1 (Slc7a1)	-1.8
NM_024406	Fatty acid binding protein 4, adipocyte (Fabp4)	-1.9
Nucleic acid bind	ing proteins	
NM_027497	Enhancer of polycomb homolog 1 (Epc1)	-1.8
NM_183029	RIKEN cDNA C330012H03 gene (predicted nucleic acid binding)	-2.9
NM_011757	Zinc finger proliferation 1 (Zipro1)	-1.9
Regulators of sign	naling	
NM_010303	Guanine nucleotide binding protein, alpha 13 (Gna13)	-1.8
NM_028472	BMP-binding endothelial regulator (Bmper/Cv2)	-1.9
NM_009846	CD24a antigen	-2.2
NM_029571	RIKEN cDNA 1110001AI2 gene (predicted nucleotide kinase)	-1.9
NM_013834	Secreted frizzled-related sequence protein 1 (Sfrp1)	-1.9
NM_010050	Deiodinase, iodothyroinine, type II (Dio2)	-2.1
Miscellaneous/un	known function	
NM_009150	Selenium binding protein 1(Selenbp1)	-2.5
NM_008220	Hemoglobin beta, adult chain 1 (Hbb-b1)	-2.1
NM_172205	Suprabasin (Sbsn)	-1.8
AK014666	RIKEN cDNA 4833408G04	-2.3
XM 283848	RIKEN cDNA 2810407C02	-1.8

Genes in bold were selected for confirmation by quantitative RT-PCR.

fied were comparable to those identified in previous microarray studies in rat and monkey after acute elevation of IOP.^{16,22} This suggests that these gene expression changes may represent a shared retinal response to IOP elevation that may enhance or limit the development of disease.

Glial Activation

Glia play important roles in the homeostasis of the retina, and include Müller glia, retinal astrocytes, and microglia. We found upregulation of GFAP, consistent with reports of glial activation in both human tissue and animal models of glaucoma.^{17,22,32,33} After IOP elevation in monkey GFAP expres-

sion increased in both Müller glia and astrocytes,^{16,32} and ultrastructural analysis has also shown evidence of Müller glial activation in DBA/2J animals.³⁴ Other glial populations such as retinal astrocytes or microglia show similar evidence for activation in glaucoma,^{16,17,20,32,33} and there is also activation of astrocytes in the optic nerve head.³⁵ Thus, multiple glial populations may respond to IOP elevation in glaucoma, with potentially beneficial or detrimental consequences.³⁶

Complement Pathway in Retinal Disease

Several complement components were elevated in DBA/2J retinas, including C4 and C1q β , which were significantly up-



FIGURE 2. Quantitative RT-PCR confirmation of select upregulated genes identified by microarray analysis. Gene-specific primers were used to perform quantitative RT-PCR on individual retinal RNA samples from DBA/2J animals at 3 (n = 6 eyes), 5 (n = 4 eyes), and 8 (n = 7 eyes) months or C57BL/6J animals at 3 (n = 4 eyes) and 8 (n = 5 eyes) months. Graphs represents average change relative to gene expression levels at 3 months for either DBA/2J or C57BL/6J mice. *Statistically significant differences (P < 0.01) by Student's *t*-test.

regulated by 5 months. Microarray analysis in both rat and monkey also showed elevated expression of multiple complement components after acute IOP elevation.^{16,22} In addition,

increased complement gene expression has been reported in retinas from a mouse photoreceptor degeneration model and in Müller cells from a rat model of diabetic retinopathy.^{37,38}



FIGURE 3. Quantitative RT-PCR confirmation of select downregulated genes identified by microarray analysis. Gene-specific primers were used to perform quantitative RT-PCR on individual retinal RNA samples from DBA/2J animals at 3 (n = 6), 5 (n = 4) and 8 (n = 7) months. Graphs represent average change relative to gene expression levels at 3 months. (A) Major intrinsic protein of eye lens fiber and (B) crystallin beta A4.

This is intriguing, given the recent finding that polymorphisms in complement factor H are associated with age-related macular degeneration in humans.³⁹⁻⁴³ Thus, changes in the expression of complement genes may be associated with retinal damage or disease.

The complement system is a complex cascade consisting of approximately 30 proteins that can act through several possible pathways that ultimately converge on complement component C5, leading to formation of the terminal membrane attack complex. Although we detected increased expression of early pathway components in the DBA/2J retina with IOP elevation, the terminal complement pathway is compromised in this inbred mouse strain due to a deficiency in the C5 complement components still contribute to disease in the DBA/2J retina, even in the absence of C5, due to their opsonization or chemoattractant activity,⁴⁵ although this remains to be investigated.

Immune-Related Responses

Other immune-related genes were also increased with disease progression in the DBA/2J retina, specifically genes associated with acute inflammatory responses, including lipocalin2, interferon-induced transmembrane proteins, chemokine ligand 12, Serpina3n, and chitinase 3-like 1 (also known as gp39 or CD40 ligand). There is loss of immune privilege in the anterior chamber of the DBA/2J eye, and inflammatory leukocytes infiltrate and accumulate within the iris,²⁵ raising the possibility that the inflammatory genes identified in our retinal microarray analysis are due to loss of ocular immune privilege. However, despite substantial inflammation of the iris, there is no evidence of macrophage infiltration into the retina.³⁴ Furthermore, other microarray studies have shown similar upregulation of inflammatory genes in the retina after acute IOP elevation in the absence of iris inflammation.^{16,22} There is evidence that many immune-related genes can be upregulated in Müller glia, astrocytes, and RGCs in response to retinal stress or injury.^{16,22,37,38} In addition, microglia can function as antigen-presenting cells and have immune response properties.⁴⁶ Thus, the gene expression changes could reflect a local response within the retina to IOP elevation.⁴⁷

Other Gene Expression Changes

Other genes were also increased by 8 months in the DBA/2J retina, including some that were not identified by microarray analysis after acute IOP elevation.^{16,22} This includes candidate regulators of gene expression and cell signaling, as well as multiple genes of unknown function. Further work is needed to validate these gene expression changes and determine their significance in glaucoma. Multiple downregulated genes were also identified, including many crystallin genes and other lens-related genes. Although the function of these genes is best understood in the lens, some are known to function outside of the lens as small heat shock proteins that act as chaperones and prevent protein aggregation in response to heat or oxidative stress.⁴⁸ It will be interesting to determine which retinal cell populations express these genes and analyze how their expression changes after IOP elevation.

Timing of Gene Expression Changes after IOP Elevation

We found that some gene expression changes were apparent by 5 months, which is shortly after the onset of detectable disease in the optic nerve in animals with elevated IOP.³⁰ In this parallel study, Inman et al.³⁰ found that 4-month DBA/2J animals with IOP higher than the colony average already had iridocorneal angle closure, increased corneal thickness, and reduced axon density in the optic nerve compared with animals with normal IOP. Thus, the gene expression changes that we identified by microarray analysis parallel the disease in the DBA/2J. Changes in immune response and complement genes may represent an early response to IOP elevation in multiple glaucoma models, because similar immune-related genes were upregulated in rat as soon as 8 days after acute IOP elevation.²² It is essential to assess whether these early responses play a role in glaucoma progression and whether these pathways are reasonable targets for therapeutic intervention.

CONCLUSIONS

In summary, our work has revealed changes in retinal gene expression in the DBA/2J mouse model of glaucoma, with some changes apparent relatively soon after detectable elevation of IOP. Changes in genes associated with immune responses and glial activation are shared with other glaucoma models after acute elevation of IOP.^{16,22} Future work is needed to determine the relationship between altered gene expression and the progression of neuronal disease in glaucoma.

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