Anti-inflammatory effect of pigment epithelium-derived factor in DBA/2J mice

Xiaohong Zhou, Feng Li, Li Kong, James Chodosh, Wei Cao

Dean McGee Eye Institute, Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

Purpose: Glaucoma is the second leading cause of blindness. The ultimate cause of vision loss in glaucoma is thought to be retinal ganglion cell (RGC) death. Neuroprotection of RGC is therefore an important goal of glaucoma therapy. Several lines of evidence suggest that pigment epithelium derived factor (PEDF) is a potent anti-angiogenic, neuroprotective, and anti-inflammatory factor for neurons. In this study, we examined the potential role of PEDF in protection of RGC in the DBA/2J mouse, an animal model of inherited glaucoma.
Methods: DBA/2J mice at two months of age were transfected intravitreally with adeno-associated virus (AAV)-PEDF or AAV-green fluorescent protein (AAV-GFP). RGC and nerve fiber layer protection were evaluated in retinal cross sections. Biochemical alterations in the retinas of DBA/2J mice in response to intravitreal transfection of PEDF were also examined by reverse transcriptase PCR (RT–PCR) and western blot. Cellular localization of PEDF and glial fibrillary acidic protein (GFAP) was determined by immunohistochemistry. Visual acuity was determined by optomotor testing.
Results: PEDF protein levels in the retina and optic nerves of DBA/2J mice declined with age. The expression of tumor necrosis factor (TNF), GFAP, and interleukin-18 (IL-18) increased with age in the retina and optic nerve of DBA/2J mice. Intravitreal PEDF transfection in DBS/2J mice reduced loss of RGC and nerve fiber layer, delayed vision loss, and reduced TNF, IL-18, and GFAP expression in the retina and optic nerve.
Conclusions: Transduced PEDF potently and efficaciously reduces RGC loss and vision decline in DBA/2J mice, possibly via the reduction of TNF and IL-18, and downregulation of GFAP. The anti-inflammatory effect of PEDF represents a novel approach to the prevention of glaucomatous RGC death.

Pigment epithelium-derived factor (PEDF), a 50 kDa glycoprotein, belongs to the serine protease inhibitor (serpin) superfamily. PEDF is thought to be both neurotrophic and anti-angiogenic. The neuroprotective capacity of PEDF protein has been documented by extensive experimental data both in vitro and in vivo. PEDF can significantly prevent glutamate-induced apoptotic cell death in cerebellar granular cells [1,2], hippocampal neurons [3], and spinal motor neurons [4]. PEDF protects retinal photoreceptor cells from light damage in the rat [5], delays progression of photoreceptor degeneration, and reduces apoptosis of photoreceptor cells in retinal degeneration slow (rds) mutant mice [6]. PEDF has also been shown to protect adult rat retinal ganglion cells (RGC) from glutamate- and trophic factor withdrawal-mediated cytotoxicity [7], and reduce retinal ischemia-induced RGC loss [8,9]. In addition, PEDF has been recognized as an important endogenous anti-inflammatory factor [10]. Vitreous levels of PEDF were significantly decreased in proliferative diabetic retinopathy, suggesting that reduced PEDF in the retina may contribute to

the pathogenesis of diabetic retinopathy, perhaps by augmentation of retinal inflammation [10,11].

Glaucoma, the second leading causes of blindness, is characterized by loss of RGC and excavation of the optic nerve head [12]. At the present time, medical treatments of glaucoma include the non-specific reduction of intraocular pressure (IOP) with medications that suppress aqueous production or enhance aqueous outflow, laser trabeculoplasty, and filtering surgery. Although little is known about the molecular/biochemical mechanism(s) underlying the glaucoma family of disorders, RGC degeneration represents a final step in glaucomatous vision loss. Therefore, neuroprotection of RGC is a critical element in management of the disease [13].

Recent and quickly accumulating evidence demonstrates that the degeneration of RGC in glaucoma is associated with low-grade, subclinical inflammation. Increased levels of pro-inflammatory mediators such as tumor necrosis factor (TNF) have been demonstrated in glaucoma patients [14]. In glaucomatous eyes, the expression of TNF and TNF receptor-1 was upregulated in the retina and optic nerve head [14,15]. It has been reported that the expression of TNF and TNF receptor-1 parallel the progression of optic nerve degeneration [16]. Moreover, a functional blockade of TNF with a TNF blocking antibody or deletion of the gene encoding TNF

Correspondence to: Dr. James Chodosh, Massachusetts Eye and Ear Infirmary, Howe Laboratory, 243 Charles Street, Boston, MA, 02114; Phone: (617) 573-3311; FAX: (617) 573-4290; email: james_chodosh@meei.harvard.edu
Dr. Wei Cao passed away during the preparation of this manuscript.

in mice completely prevented ocular hypertension-induced oligodendrocyte degeneration and the secondary loss of RGC. These results support the notion that inflammation plays an important role in the development and progression of glaucoma.

The DBA/2J mouse has been described as an animal model for human inherited glaucoma [17,18]. This mouse strain develops several hallmarks of human pigmentary glaucoma including iris atrophy, pigment dispersion, peripheral anterior synechiae, elevated IOP, retinal ganglion cell loss, and optic nerve head excavation [17,19,20]. In our previous work with DBA/2J mice, we demonstrated a correlation between elevated expression of interleukin-18 (IL-18) and the death of RGC [21]. We also showed age dependent decreases in PEDF gene expression and protein in the iris/ciliary body and aqueous humor, respectively. In human patients with advanced glaucoma, intraocular PEDF is significantly lower than in those with cataract alone [8]. It has been suggested that lower levels of PEDF in aqueous humor of eyes with neuroretinal dystrophy – including those with advanced glaucoma – may be related to the loss of the RGC or pigment epithelium cells that synthesize PEDF [8,22]. Furthermore, exogenous PEDF protects RGC from pressure-induced ischemia [9,23]. However, the mechanisms for the protective effect of PEDF in the glaucoma are undefined.

In the present study, we make several novel observations. First, PEDF protein levels in the retina and optic nerve of DBA/2J mice decrease significantly with age in comparison to C57BL/6J (control) mice. Second, the expression of TNF, IL-18, and glial fibrillary acidic protein (GFAP) in the retina and optic nerve increases with age. Third, PEDF transduction reduces the loss of RGC and nerve fiber layer, delays vision loss, and leads to reduced TNF, IL-18, and GFAP in the retina and optic nerve of DBA/2J mice.

METHODS

Animals: The DBA/2J and C57BL/6J mice used in this study were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were born and raised in a 12 h on versus 12 h off cyclic light environment at an illumination of 50–60 lx. Animals were cared for and handled according to the Association for Research in Vision and Ophthalmology statement for the use of animals in vision and ophthalmic research and with approval of the University of Oklahoma Health Sciences Center (Oklahoma City, OK) IACUC.

Identification of retinal ganglion cells by retrograde labeling: The neuronal tracer 4-di-10-ASP, a long-chain dialkylcarbocyanine, is widely used to label living tissues, and with injection of 4-di-10-ASP into the superior colliculus, will label RGC by retrograde axoplasmic transport [24]. It has been shown that 7 days after injection of 4-di-10-ASP, more than 80% of the RGC are labeled [25]. We used 4-di-10-ASP (Molecular Probes, Eugene, OR) to label RGC as previously described [21]. Briefly, 4 weeks-old mice were anesthetized by intraperitoneal administration of a mixture of xylazine and ketamine. The skin over the cranium was incised, and the skull was exposed. Holes ~1 mm in diameter were drilled in the skull 4 mm posterior to the bregma and 1 mm lateral to the midline on both sides of the midline raphe. These positions correspond to the superior colliculi as determined from a stereotactic mouse brain atlas. Retinal ganglion cells project to the superior colliculus and dorsal lateral geniculate nucleus. 4-di-10-ASP (3 μl of a 25 mg/ml concentration) was injected through both holes in the superior colliculi region by inserting a syringe 2 mm deep. The skull openings were then sealed with a petroleum-based antibiotic ointment. The overlying skin was sutured and antibiotic ointment was applied externally. The mice were euthanized by CO_2 inhalation at 1, 6, and 15 months following 4-di-10-ASP administration. Whole retina flat mounts were prepared by detachment of the retina at the ora serrata followed by eight radial relaxing incisions. Imaging of the labeled retinas was performed using a digital camera and a fluorescence microscope (Nikon Instruments, Inc., Melville, NY) at 20X magnification.

Measurement of visual acuity: The Virtual Optomotor System (OptoMotry; CerebralMechanics, Lethbride, Alberta, Canada) was used to measure visual acuity as an index of visual function, as previously shown [26], at 1 to 11 months of age. The grating spatial acuity was measured starting with a low spatial frequency (0.1 cyc/deg) sine wave grating at the same mean luminance and 100% contrast. The spatial frequency of the grating was incrementally increased until the animal failed to respond. Each test was performed at least 5 times. The threshold was identified as the highest spatial frequency that mice could track.

Preparation and intravitreous delivery of adeno-associated virus expressing human PEDF: Adeno-associated virus expressing human PEDF (AAV-PEDF) has been used to protect neurons in animal models [27-29]. AAV-PEDF was constructed by cloning a full-length human *PEDF* cDNA under the control of the CMV promoter. The construct sequence was confirmed by DNA sequencing (Oklahoma Medical Research Foundation, Oklahoma City, OK). A control virus containing a green fluorescent protein cDNA under the same promoter (AAV-GFP) was obtained from a commercial source (InVivoGen, San Diego, CA). AAV-PEDF and AAV-GFP vector titers were 2.0×10^{13} particles/ml, and 2.5×10^{12} particles/ml, respectively. Intravitreal injection was

performed under general anesthesia after intramuscular injection of ketamine (85 mg/kg) and xylazine (14 mg/kg). Anesthetic drops (0.5% proparacaine hydrochloride; Alcon, Fort Worth, TX) were also applied topically to each eye before injections. A 30 gauge hypodermic needle was used to perforate the sclera 1 mm behind the limbus. AAV-PEDF (1 μl) was then injected into the left vitreous and AAV-GFP (1 μl) was injected into the right vitreous using a 10 μl Hamilton Syringe (Hamilton Co, Reno, NV) under an ophthalmic operating microscope (Carl Zeiss Meditec, Inc., Thornwood, NY). Care was taken not to damage the lens. Following intraocular injections, the needle was held in place for one min and withdrawn slowly. Animals with retinal bleeding or lens injury following the injection procedure were excluded from the study. Animals were euthanized nine months after the injection, and eyes rapidly enucleated. Retinas were dissected free from the choroid and sclera, and whole mounted on a glass slide. They were fixed briefly in 4% (w/v) paraformaldehyde in PBS. Whole retina was mounted with Vectorshield mounting medium (Vector Laboratories, Inc., Burlingame, CA).

Semiquantitative reverse transcription (RT)-PCR: Reverse transcription RT–PCR was performed as described previously [30,31]. For RT–PCR, 4 μg RNA was mixed in water with random hexamers (50 ng/μl) and 10 mM dNTPs heated to 65 °C for 5 min. The 20 μl RT reaction, containing RNA, primers, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, 40 units of RNase inhibitor (RNase OUT), and 50 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), was assembled, heated at 42 °C for 1 h and then at 70 °C for 15 min. After chilling on ice, 2 units of RNase H were added to each reaction and the mixture was incubated at 37 °C for 20 min. PCR was performed in 50 μl reaction volumes, containing 2 μl of cDNA, 2 units of TaqDNA polymerase (Promega, Madison, WI), 0.2 mM dNTPs, 10 μM each of forward and reverse primers, and 1.5 mM $MgCl_2$. Amplification cycles were: one cycle at 94 °C for 3 min, followed by 35 cycles of 94 °C for 20 s, 58 °C for 45 s, and 72 °C for 1 min, terminating with 72 °C for 10 min. The amplification products were run on 1% agarose gel containing 10ng/ml ethidium bromides and visualized under UV light. PEDF, TNF, and IL-18 gene expression were examined by semi-quantative RT–PCR using the following primers: PEDF (AF017057) Forward: TGA TCA CCA ACC CTG ACA TCC ACA, Reverse: GCT GGG CAA TCT TGC AGT TGA GAT; TNF (NM_013693) Forward: ACA CCG TCA GCC GAT TTG CTA TCT, Reverse: TGG ACA TTC GAG GCT CCA GTG AAT; IL-18 (NM_008360) Forward: GAC AAC TTT

GGC CGA CTT CAC TGT, Reverse: CAC AAG GCG CAT GTG TGC TAA TCA.

Western blot analysis: Western blot analysis was performed as described previously [32,33]. Retinal tissues were sonicated in 0.0625 M Tris-HCl, pH 6.8, then centrifuged for 15 min and the supernatants assayed for protein using a Bradford assay. Aliquots (10 μg) of the sonicated supernatant were loaded onto SDS–PAGE mini-gels, electrophoresed, and transferred to nitrocellulose paper. After transferring, blots were washed for 2X 10 min in TTBS (0.1% Tween-20 in 20 mM Tris-HCl, pH 7.4, and 410 mM NaCl) and blocked with 10% BSA in TTBS with 5% milk for 2 h at room temperature or overnight at 4 °C. Blots were incubated with rabbit anti-IL-18 polyclonal antibody (Millipore, Billerica, MA), rabbit anti-TNF polyclonal antibody (Millipore, Billerica, MA), rabbit anti-PEDF polyclonal antibody (Santa cruz, Delaware, CA), or rabbit anti-GFAP polyclonal antibody (Abcam, Cambridge, MA) for 2 h at room temperature. Following primary antibody incubations, blots were washed three times for 5 min each with TTBS, then incubated for 1 h with HRP-linked secondary antibodies, washed four times for 10 min each with TTBS, and developed by enhanced chemiluminescence. In some instances, membranes were stripped by incubation in stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol) for 30 min at 50 °C and reused.

Histopathology: Mouse eyes were enucleated, rinsed in PBS, and fixed with 10% neutral buffered formalin for 24 h at room temperature. After paraffin embedding, whole eyes were cut into 5 μm thick sections, mounted on positively charged slides and air dried overnight. After deparaffinization and rehydration, slides were stained with hematoxylin and eosin.

Immunohistochemistry: Immunohistochemistry staining was performed as described previously [34]. Briefly, Eyes were enucleated and then fixed with 4% paraformaldehyde in phosphate-buffered saline for 4 h. The whole eye was cut along the vertical meridian, and tissue sections incubated in primary antibodies. The secondary antibody (Vector Laboratories, Burlingame, CA) was labeled with fluorescein isothiocyanate. In control experiments, the primary antibody was incubated for 2 h at 4 °C with its blocking peptides before being applied to the section. Sections were photographed and analyzed with a confocal laser scanning microscope (IX81-FV500; Olympus, Melville, NY).

Statistical analysis: Each experiment was performed at least three times. Mean values were calculated and expressed as means±SD. Statistical analyses were performed using Student's *t*-test, or ANOVA with the Scheffé multiple comparison test. A statistical difference was considered significant at $p < 0.05$.
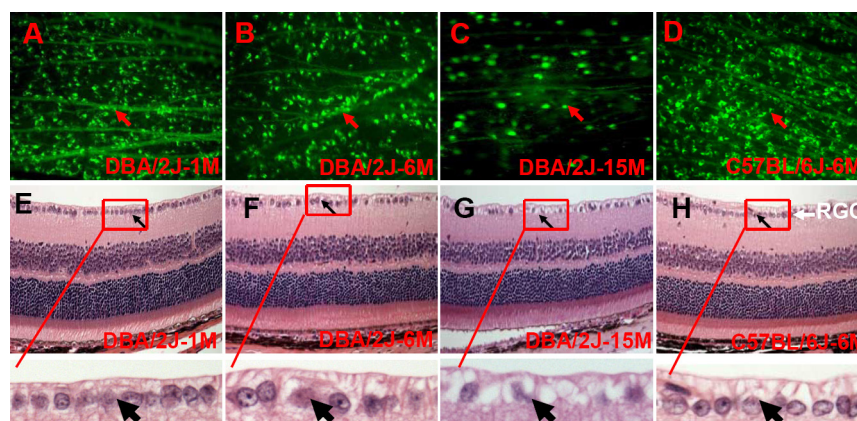
Figure 1. Retinal ganglion cell (RGC) degeneration in DBA/2J mice. **A**: RGC labeled by neurotracer in flat-mounted retina of DBA/2J mice at 1 month of age. **B**,**C**: Substantial loss of RGC in flat-mounted retina from DBA/2J mice at 6 and 15 months of age, respectively. **D**: Normal RGC density in whole mount retina from C57BL/6J mice. Red arrows indicate 4-di-10-ASP labeled RGC. **E**: RGC density in paraffin cross-section from DBA/2J mice at 1 month of age. **F**,**G**: Substantial loss of RGC in retina from DBA/2J mice at 6 and 15 months old, respectively. **H**: Normal RGC density in paraffin cross-section from C57BL/6J mice. Black arrows indicate RGC. Insets show higher magnification.

RESULTS

Loss of retinal ganglion cells in eyes of DBA/2J mice: RGC density was determined by counting 4-di-10-ASP-labeled RGC in flat whole-mount retinas. Significant loss of RGC was observed in flat whole-mount retina from DBA/2J mice at 6 months of age (Figure 1B) in comparison to age-matched control C57BL/6J mice (Figure 1D). This loss of RGC appeared to worsen at 15 months of age (Figure 1C). The morphological difference between ganglion cell layers in the retina of DBA/2J mice and C57BL/6J control animals at matched age was also compared by histology. Loss of RGC was apparent in DBA/2J mice at 6 months, and appeared to have dramatically worsened at 15 months of age (Figures 1F,G, respectively), as compared with retina from C57BL/6J mice (Figure 1H).

Reduction in PEDF gene and protein expression with age in eyes of DBA/2J mice: It was previously shown that PEDF levels in eyes with advanced glaucoma are significantly lower than in eyes with cataract alone. It has also been suggested that the lower levels of PEDF in eyes with neuro-retinal dystrophy may be related to the loss of the retinal ganglion or pigment epithelial cells that synthesize PEDF [22]. In DBA/2J mice, semi-quantitative RT–PCR revealed significantly decreased retinal *PEDF* gene expression with age (Figure 2A,B). A decrease of PEDF protein expression with age in retina from DBA/2J mice was also detected by western blot; the expression of PEDF was significantly reduced at the age of 4 months, and this decrease continued to the age of 6 months (Figure 2C,D). Western blot analysis also showed a significant reduction of PEDF expression in the optic nerve of DBA/2J mice at 6 months of age (Figure

2E,F). Immunohistochemistry demonstrated substantial expression of PEDF in the RGC and retinal pigment epithelial (RPE) layers of DBA/2J mice by 1 month of age (Figure 3A). At 6 months of age, decreased PEDF expression in the RGC layer with residual staining in the RPE layer of DBA/2J mice (Figure 3B) was detected when compared with the age-matched C57BL/6J controls (Figure 3C).

Inflammatory markers in retina and the optic nerve of DBA/2J mice: TNF is an important inflammatory mediator and is expressed in the retina and optic nerve head of patients with glaucoma [15,16,35]. To investigate whether changes in TNF expression occur in DBA/2J mice, we isolated total RNA and protein from retina and optic nerve from 1 to 6 months old mice, and performed RT–PCR and ELISA, respectively. *TNF* gene and protein expression increased significantly by 3 months of age in both the retina and optic nerve of DBA/2J mice compared with control tissue from one month old mice (Figure 4A-D), and remained elevated through age 6 months. Significant increases in TNF protein expression was also seen in optic nerve of DBA/2J mice from 3 to 6 months of age (Figure 4E,F).

We have previously reported that *IL-18* gene expression in the iris/ciliary body and IL-18 protein in the aqueous humor of DBA/2J mice are significantly increased, and further demonstrated that these increases occur before the development of elevated IOP and loss of RGC [21]. To determine whether *IL-18* is upregulated in the retina and optic nerve of DBA/2J mice, we performed semi-quantitative RT–PCR and western blot. mRNA for *IL-18* was elevated by 3 months of age, and increased almost twofold by 6 months of age as compared to *IL-18* expression at 1 month of age (Figure
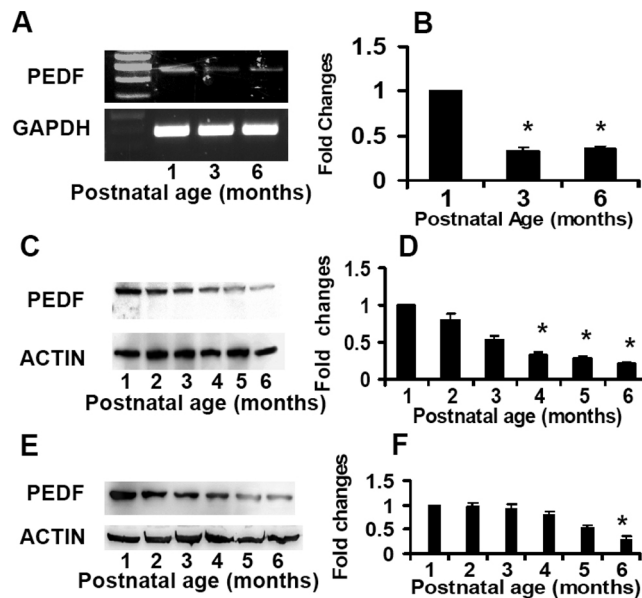
Figure 2. Reduced PEDF gene and protein expression in retina and optic nerve from DBA/2J mice. **A**: RT–PCR shows reduced PEDF gene expression in th retina of DBA/2J mice at 3 and 6 months of age. **B**: Graphical representation of the RT–PCR data in **A** after normalization with GAPDH loading control (mean±SD, n=3). **C**: Reduced PEDF protein expression in retina of DBA/2J mice with age, as detected by western blot. **D**: Densitometric analysis of PEDF protein expression in 3 independent experiments from **C** after normalization with actin loading control. (n=3). **E**: Western blot analysis shows decreased PEDF protein expression in optic nerve from DBA/2J mice at 6 months old. **F**: Densitometric analysis of PEDF protein expression in 3 independent experiments, after normalization to actin loading control. Statistical analyses for this experiment was performed using ANOVA with the Scheffé multiple comparison test (the asterisk indicates a $p<0.05$).

5A,B). By western blot analysis, IL-18 protein increased approximately twofold at 4–6 months of age (Figure 5C,D). Upregulation of *IL-18* in the optic nerve of DBA/2J mice at 5 and 6 months of age was also found (Figure 5E,F).

Increased GFAP expression in retina and the optic nerve of DBA/2J mice: Glial cell activation has been proposed as an important factor contributing to RGC death in glaucoma [36,37]. Chronic IOP elevation results in significantly increased GFAP expression in the rat retina and optic nerve [38]. By immunohistochemistry in DBA/2J mice at 1 month of age, GFAP expression was mainly localized to the vicinity of the inner limiting membrane and nerve fiber layer (Figure

6A,B). However at 6 months of age, GFAP expression was significantly increased in both retina (Figure 6C) and optic nerve (Figure 6D). Western blot analysis demonstrated significantly increased GFAP protein in retina and optic nerve of DBA/2J mice by 3 months of age (Figures 6E-H).

Ocular PEDF gene transfection in DBA/2J mice leads to increased intraocular PEDF expression, improved retinal ganglion cell survival, and better vision: In situ expression of GFP can be observed from cross-section or whole-mount techniques, whereas the expression of PEDF is detected using western blot. Injections of AAV vectors encoding GFP or PEDF were performed in mice at 2 months of age.
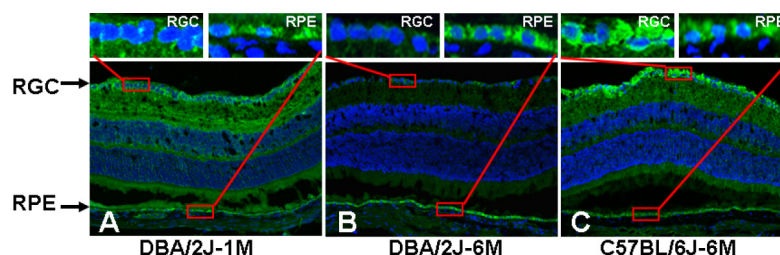

Figure 3. Immunofluorescent localization of PEDF in retina from DBA/2J and C57BL/6J mice. **A**: PEDF immunoreactivity localizes to the RGC and RPE layers in DBA/2J mice at 1 month old. **B**: Decreased PEDF expression in the RGC layer of DBA/2J mice at 6 months, with residual staining in the RPE. **C**: In C56BL/6J mice, PEDF is localized mainly in the RGC and RPE layers. Insets show higher magnification.
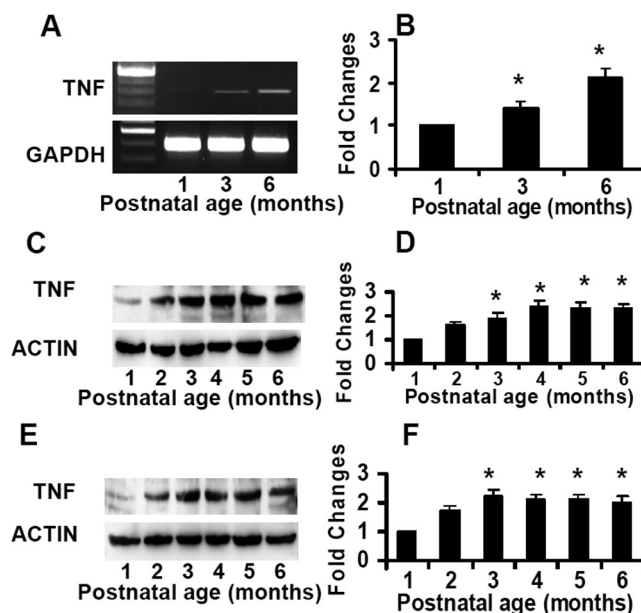
Figure 4. Increased *TNF* gene and protein expression in retina and optic nerve from DBA/2J mice. **A**: RT–PCR shows increased *TNF* gene expression in retina from DBA/2J mice at 3 and 6 months of age. **B**: Graphical representation of 3 independent experiments in **A** normalized with *GAPDH* loading control (mean±SD). **C**: Western blot shows increased TNF protein expression in retina from DBA/2J mice at 3 to 6 months of age. **D**: Densitometric analysis of 3 independent experiments from **C**, using actin as a loading control. **E**: Western blot shows increased TNF protein expression in optic nerve from DBA/2J mice from 3 to 6 months of age. **F**: Densitometric analysis of three independent experiments in **E**. Statistical analyses for this experiment was performed using ANOVA with the Scheffé multiple comparison test (the asterisk indicates a $p < 0.05$).
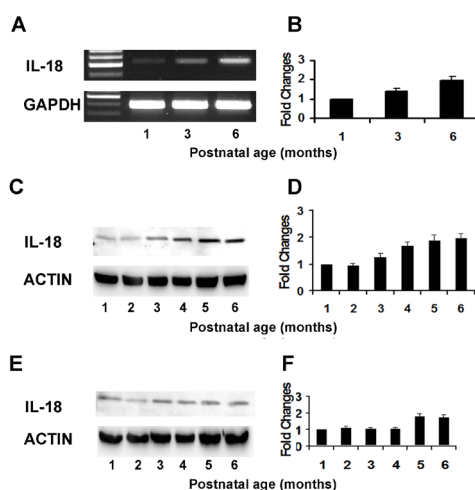

Figure 5. Increased *IL-18* gene and protein expression in retina and optic nerve from DBA/2J mice. **A**: RT–PCR analysis shows increased *IL-18* gene expression in retina from DBA/2J mice at 3 and 6 months old. **B**: Graphical representation of 4 independent experiments in **A** normalized with *GAPDH* loading control (mean±SD). **C**: Western blot of IL-18 protein expression in retina from DBA/2J mice at 1 to 6 months of age. **D**: Densitometric analysis of 3 independent experiments from **C**, using actin as a loading control, revealed significantly higher expression of IL-18 in retina from DBA/2J mice from 4 to 6 months when compared with 1 month of age. **E**: Western blot of IL-18 protein expression in optic nerve from DBA/2J mice at 1 to 6 months of age. **F**: Densitometric analysis of 3 independent experiments from **E**, shows increased IL-18 protein expression compared to actin loading control in optic nerve from DBA/2J mice by 5 months of age. Statistical analyses for this experiment was performed using ANOVA with the Scheffé multiple comparison test (the asterisk indicates a $p < 0.05$).
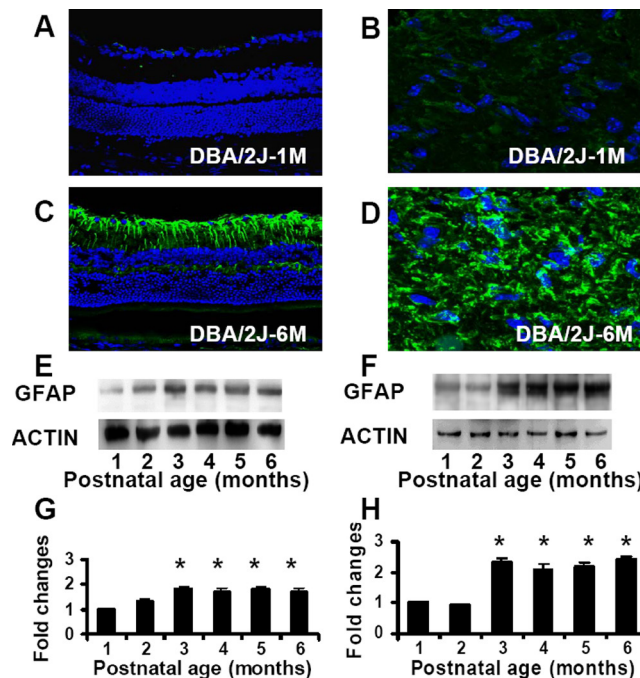
Figure 6. Localization of glial fibrillary acidic protein (GFAP) in retina and optic nerve from DBA/2J mice. **A**,**B**: Respective retina and optic nerve sections from DBA/2J mice at 1 month of age show minimal staining for GFAP. **C**,**D**: Respective retina and optic nerve sections from DBA/2J mice at 6 months of age show prominent immunostaining for GFAP in retinal Müller cells and optic nerve. **E**,**F**: western blot analysis for GFAP protein in the retina and optic nerve, respectively. **G**,**H**: Densitometric analysis of 3 independent experiments from **E** and **F**, using actin as a loading control. Statistical analyses for this experiment was performed using ANOVA with the Scheffé multiple comparison test (the asterisk indicates a $p<0.05$).

As demonstrated by assessment of GFP expression in retinal whole mount and cross section (Figures 7A,B, respectively) at 11 months of age (9 months after transfection), the retina can be successfully transduced by intravitreal injection. Transfection of AAV-PEDF resulted in a significant increase in PEDF protein level in retina from DBA/2J mice at 11 months of age (Figure 7C). The long-term effect of *PEDF* gene transfer on RGC survival was also examined. AAV-PEDF transfected eyes appeared to retain more RGC at 11 months of age (Figure 7D) than age-matched AAV-GFP transfected eyes (Figure 7E). As an index of visual function, we compared optomotor acuity in AAV-GFP, AAV-PEDF, and control transfected mice from 1 to 11 months of age (until 9 months post transfection; Figure 7F). Through 3 months of age, average acuity was approximately 0.33 cyc/deg in all 3 groups. In AAV-GFP transfected and control DBA/2J mice, acuity thresholds decreased rapidly thereafter, dropping to near 0.02 cyc/deg at 11 months of age. In contrast, AAV-PEDF transfected mice maintained near normal acuity throughout the study (Figure 7F).

Ocular PEDF transfection reduces TNF and IL-18 expression in retina and optic nerve of DBA/2J mice: To explore

a possible link to inflammation in understanding the salutary effect of PEDF on morphology and visual function in DBA/2J mice, we next determined levels of TNF and IL-18 in retina and optic nerve after AAV-PEDF transfection. For these experiments, eyes were analyzed at 6 months of age (4 months after transfection). By RT–PCR, mRNA and protein expression of both *TNF* and *IL-18* were significantly reduced in the retina and optic nerve of AAV-PEDF transfected mice as compared to AAV-GFP mice (Figure 8A-F), suggesting an anti-inflammatory effect of PEDF in the DBA/2J mice.

PEDF inhibits GFAP overexpression in retina and optic nerve of DBA/2J mice: GFAP expression has been implicated in the pathogenesis of glaucoma, and shown to increase with progression of glaucoma [39]. We examined the effects of PEDF transfection on GFAP expression in retina (Figures 9A,C) and optic nerve (Figures 9B,C) of DBA/2J mice at 6 months of age. As demonstrated by western blot and subsequent densitometry, GFAP protein expression in retina and optic nerve of PEDF transfected DBA/2J mice was significantly reduced compared to that of GFP transfected mice.
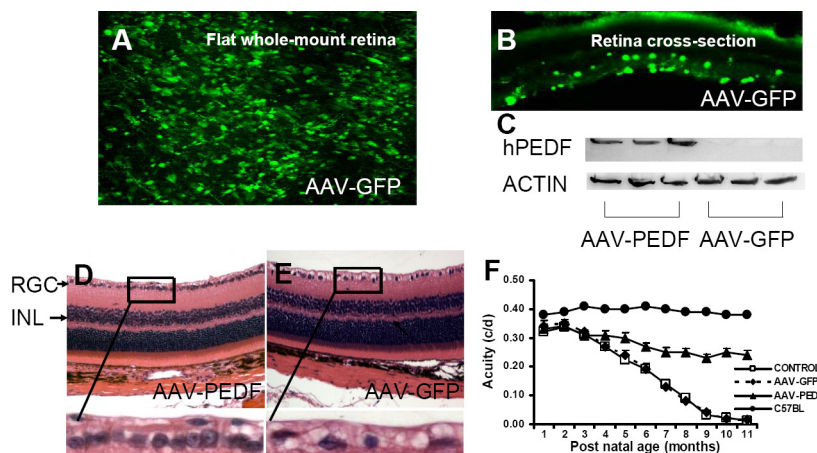
Figure 7. *PEDF* gene transfection protects against the loss of RGC in DBA/2J mouse retina and delays vision loss. **A**: Retina flat whole-mount shows extensive GFP labeling 9 months after transfection with AAV-GFP (at 11 months of age). **B**: Retina cross section shows GFP labeling in ganglion cell layer and in inner nuclear layer. **C**: Western blot for PEDF in retina from DBA/2J mice 9 months after AAV-PEDF transfection. **D**: Protection of RGC in DBA/2J mice retina 9 months after transfection with AAV-PEDF. **E**: RGC loss in retina 9 months after transfection with AAV-GFP. Insets show higher magnification. **F**: Visual acuity in mock transfected, AAV-GFP, and AAV-PEDF transfected DBA/2J mice, in comparison to C57BL/6J mice. Acuity decreased progressively beginning at 4 months of age (2 months post transfection) in control transfected and AAV-GFP treated mice, but was maintained to age 11 months in AAV-PEDF transfected mice. Abbreviations: retinal ganglion cell (RGC) layer; inner nuclear layer (INL).
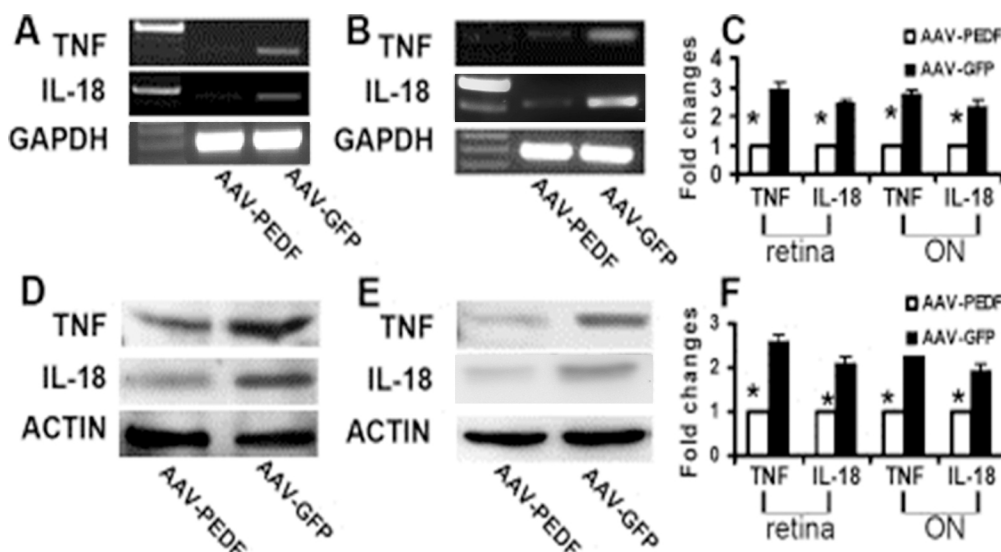

Figure 8. Changes in *TNF* and *IL-18* expression after PEDF transfection in DBA/2J mice. **A**,**B**: Reduced *TNF* and *IL-18* gene expression in retina and optic nerve, respectively, 4 months after AVV-PEDF transfection in DBA/2J mice. **C**: Graphical representation of 3 independent experiments in **A** and **B** normalized with *GAPDH* loading control. As compared with AAV-GFP, intravitreal injection of AAV-PEDF significantly reduced *TNF* and *IL-18* expression in retina and optic nerve. **D**,**E**: Reduced expression of TNF and IL-18 protein in DBA/2J mice retina and optic nerve, respectively, at 4 months after AAV-PEDF transfection as compared with AAV-GFP. **F**: Densitometric analysis using actin as a loading control from 3 independent experiments in **D** and **F** shows a significant reduction of TNF and IL-18 protein expression. ON: optic nerve. Statistical analysis for these experiments was performed using unpaired Student's *t*-test (the asterisk indicates a p<0.05).

DISCUSSION

We demonstrate in this study of DBA/2J mice that: 1) PEDF protein levels in retina and optic nerve decrease significantly with age; 2) expression of TNF, IL-18, and GFAP in retina and optic nerve increase with age; 3) PEDF transfection reduces age-related RGC loss and vision decline; and 4) PEDF transduction decreases TNF, IL-18, and GFAP expression. These findings indicate that PEDF potently and efficaciously reduces RGC loss in DBA/2J mice, reduces expression of TNF and IL-18, and downregulates GFAP.

TNF is upregulated in several neurodegenerative disorders including multiple sclerosis, Parkinson disease, and Alzheimer disease [40] and is increased in optic nerve microglia and astrocytes of glaucoma patients [15,16,35]. Furthermore, *TNF* gene polymorphisms increase the risk of glaucoma [41], suggesting that *TNF* may contribute to the pathogenesis of the disease. Meanwhile, TNF is toxic to immunopurified RGC and to RGC in mixed cultures under conditions of glial stress [42,43]. In vivo, exogenous TNF leads to loss of oligodendrocytes and a delayed loss of RGC. Furthermore, a functional blockade of TNF with TNF blocking antibody or deletion of the gene encoding TNF completely prevents ocular hypertension-induced oligodendrocyte degeneration and the secondary loss of RGC [44]. In the present study, using the DBA/2J mouse as a model of inherited glaucoma, we demonstrate that TNF expression increases with age in the retina and optic nerve, suggesting that TNF may contribute to the pathophysiology of the glaucoma.

IL-18 is a proinflammatory cytokine in the IL-1 family [45,46], and is an important regulator of innate and acquired immune responses. IL-18 appears to play a role in many autoimmune and inflammatory diseases, including rheumatoid arthritis, ischemic renal and heart disease, atherosclerosis, and multiple sclerosis [47-50]. We previously demonstrated that *IL-18* gene and protein expression is significantly increased with age in iris/ciliary body and aqueous humor of DBA/2J mice. IL-18 in the aqueous humor of DBA/2J mice increases with age [21]. Our present study demonstrates that *IL-18* gene and protein expression increase significantly with age in retina and optic nerve of DBA/2J mice. These novel findings suggest a role for *IL-18* in the pathogenesis of glaucoma in this animal model.

GFAP is well known to be a sensitive marker of glial activation in response to neural injury [51]. GFAP is expressed in the normal retina mainly in astrocytes, and is localized to the vicinity of the inner limiting membrane and nerve fiber layer. Müller cells do not normally express GFAP, but they can be induced to increase their expression in response to injury [52,53]. Glial cell activation has been proposed as an important factor contributing to RGC death in glaucoma [36,37]. GFAP immunoreactivity was increased in Müller cells from the inner to the outer limiting membranes in retina from rats with elevated intraocular pressure [37]. Optic nerve head astrocytes demonstrate increased GFAP expression in glaucoma and under conditions of elevated hydrostatic pressure [54]. In our present study, GFAP expression increased dramatically with age in retina and optic nerve of DBA/2J mice. This finding is in agreement with the view that activation of retinal glial cells is a prominent feature of the glaucomatous retina. Furthermore, it has been demonstrated that activated glial cells may have noxious effects on neuronal tissue by alteration of the neuron microenvironment [14,55,56]. For example, activated glial cells in glaucomatous eyes produce neurotoxic substances, such as nitric oxide synthase and TNF [15,16]. These data suggest that astrocytes and Müller cells may be important contributors to the pathogenesis of glaucoma.

PEDF has neuroprotective and antiangiogenic functions in the mammalian eye, and was recently shown to have antiinflammatory properties as well. PEDF levels in vitreous humor are decreased in patients with diabetes and proliferative retinopathy [57]. PEDF treatment decreased retinal levels of proinflammatory cytokines in experimental diabetes,
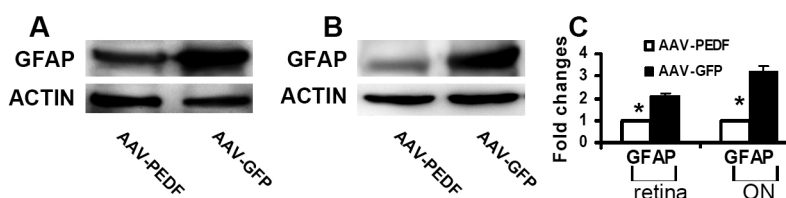

Figure 9. Changes in GFAP expression after PEDF transfection. **A**,**B**: Reduced GFAP expression in DBA/2J mice retina and optic nerve, respectively, 4 months after AAV-PEDF transfection. **C**: Graphical representation of 3 independent experiments in **A** and **B**, normalized with actin loading control. As compared with AAV-GFP control, AAV-PEDF transduced mice show significantly reduced expression of GFAP in retina and optic nerve. ON: optic nerve. Statistical analysis for these experiments was performed using unpaired Student's *t*-test (the asterisk indicates a p<0.05).

suggesting a possible role as an endogenous anti-inflammatory factor [10]. In cultured retinal capillary endothelial cells, PEDF significantly decreased TNF expression due to hypoxia. Moreover, downregulation of *PEDF* expression by siRNA treatment of retinal Müller cells resulted in significantly increased *TNF* expression [10,58]. PEDF levels in aqueous humor were significantly reduced in advanced glaucoma as compared to normal controls [8]. Pressure-induced ischemia and subsequent reperfusion led to extensive RGC death in the absence of PEDF, whereas addition of PEDF protected RGC [9,59]. In summary, the published literature strongly supports a role for PEDF as an anti-inflammatory molecule in a variety of ocular conditions.

The studies reported herein strongly suggest a role for PEDF in the maintenance of RGC in the DBA/2J mouse model of glaucoma, and add indirectly to the evidence for reduced PEDF as a possible pathogenic mechanism in human glaucoma. We demonstrate that endogenous PEDF expression in retina and optic nerve of DBA/2J mice declines with age, while PEDF transfection maintains RGC viability, inhibits TNF and IL-18 upregulation, and reduces GFAP expression. Our data suggest that PEDF may be involved in the pathogenesis of glaucoma in this mouse model, that decreased PEDF levels in aging eyes may contribute to subclinical inflammation in glaucoma, and that the protective effect of PEDF on glaucoma in DBA/2J mice may be mediated, at least in part, by its anti-inflammatory activity.

Development of glaucoma in DBA/2J mice takes months; while the activity of PEDF applied by a periocular route is approximately 6 h [60]. Repeated intravitreal injection of PEDF would be impractical, but gene transfer in our model was effective in the prevention of visual loss, and could be considered in the prevention of human glaucoma. Our results support a role for PEDF as a potent and efficacious agent to reduce RGC death and vision loss in DBA/2J mice. An anti-inflammatory approach to glaucoma, as illustrated by the use of PEDF, represents a novel advance in the potential prevention of glaucomatous RGC death.

ACKNOWLEDGMENTS

REFERENCES

1. Taniwaki T, Becerra SP, Chader GJ, Schwartz JP. Pigment epithelium-derived factor is a survival factor for cerebellar granule cells in culture. J Neurochem 1995; 64:2509-17. [PMID: 7760030].

2. Araki T, Taniwaki T, Becerra SP, Chader GJ, Schwartz JP. Pigment epithelium-derived factor (PEDF) differentially protects immature but not mature cerebellar granule cells against apoptotic cell death. J Neurosci Res 1998; 53:7-15. [PMID: 9670988].

3. Lynch S, Yanagi G, DelBono E, Wiggs JL. DNA sequence variants in the tyrosinase-related protein 1 (TYRP1) gene are not associated with human pigmentary glaucoma. Mol Vis 2002; 8:127-9. [PMID: 12011806].

4. Houenou LJ, D'Costa AP, Li L, Turgeon VL, Enyadike C, Alberdi E, Becerra SP. Pigment epithelium-derived factor promotes the survival and differentiation of developing spinal motor neurons. J Comp Neurol 1999; 412:506-14. [PMID: 10441236].

5. Cao W, Tombran-Tink J, Elias R, Sezate S, Mrazek D, McGinnis JF. In vivo protection of photoreceptors from light damage by pigment epithelium-derived factor. Invest Ophthalmol Vis Sci 2001; 42:1646-52. [PMID: 11381073].

6. Cayouette M, Smith SB, Becerra SP, Gravel C. Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degenerations. Neurobiol Dis 1999; 6:523-32. [PMID: 10600408].

7. Pang IH, Zeng H, Fleenor DL, Clark AF. Pigment epithelium-derived factor protects retinal ganglion cells. BMC Neurosci 2007; 8:11-[PMID: 17261189].

8. Ogata N, Matsuoka M, Imaizumi M, Arichi M, Matsumura M. Decrease of pigment epithelium-derived factor in aqueous humor with increasing age. Am J Ophthalmol 2004; 137:935-6. [PMID: 15126162].

9. Takita H, Yoneya S, Gehlbach PL, Duh EJ, Wei LL, Mori K. Retinal neuroprotection against ischemic injury mediated by intraocular gene transfer of pigment epithelium-derived factor. Invest Ophthalmol Vis Sci 2003; 44:4497-504. [PMID: 14507898].

10. Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma JX. Pigment epithelium-derived factor (PEDF) is an endogenous anti-inflammatory factor. FASEB J 2006; 20:323-5. [PMID: 16368716].

11. Murugeswari P, Shukla D, Rajendran A, Kim R, Namperumalsamy P, Muthukkaruppan V. Proinflammatory cytokines and angiogenic and anti-angiogenic factors in vitreous of patients with proliferative diabetic retinopathy and eales' disease. Retina 2008; 28:817-24. [PMID: 18536597].

12. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. Br J Ophthalmol 2006; 90:262-7. [PMID: 16488940].

13. Levin LA. Retinal ganglion cells and neuroprotection for glaucoma. Surv Ophthalmol 2003; 48:Suppl 1S21-4. [PMID: 12852431].

14. Tezel G, Li LY, Patil RV, Wax MB. TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. Invest Ophthalmol Vis Sci 2001; 42:1787-94. [PMID: 11431443].

15. Yan X, Tezel G, Wax MB, Edward DP. Matrix metalloproteinases and tumor necrosis factor alpha in glaucomatous optic nerve head. Arch Ophthalmol 2000; 118:666-73. [PMID: 10815159].

16. Yuan L, Neufeld AH. Tumor necrosis factor-alpha: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. Glia 2000; 32:42-50. [PMID: 10975909].

17. John SW, Smith RS, Savinova OV, Hawes NL, Chang B, Turnbull D, Davisson M, Roderick TH, Heckenlively JR. Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2J mice. Invest Ophthalmol Vis Sci 1998; 39:951-62. [PMID: 9579474].

18. Schuettauf F, Quinto K, Naskar R, Zurakowski D. Effects of anti-glaucoma medications on ganglion cell survival: the DBA/2J mouse model. Vision Res 2002; 42:2333-7. [PMID: 12350421].

19. Anderson MG, Smith RS, Hawes NL, Zabaleta A, Chang B, Wiggs JL, John SW. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. Nat Genet 2002; 30:81-5. [PMID: 11743578].

20. Chang B, Smith RS, Hawes NL, Anderson MG, Zabaleta A, Savinova O, Roderick TH, Heckenlively JR, Davisson MT, John SW. Interacting loci cause severe iris atrophy and glaucoma in DBA/2J mice. Nat Genet 1999; 21:405-9. [PMID: 10192392].

21. Zhou X, Li F, Kong L, Tomita H, Li C, Cao W. Involvement of inflammation, degradation, and apoptosis in a mouse model of glaucoma. J Biol Chem 2005; 280:31240-8. [PMID: 15985430].

22. Ogata N, Matsuoka M, Imaizumi M, Arichi M, Matsumura M. Decreased levels of pigment epithelium-derived factor in eyes with neuroretinal dystrophic diseases. Am J Ophthalmol 2004; 137:1129-30. [PMID: 15183804].

23. Ogata N, Wang L, Jo N, Tombran-Tink J, Takahashi K, Mrazek D, Matsumura M. Pigment epithelium derived factor as a neuroprotective agent against ischemic retinal injury. Curr Eye Res 2001; 22:245-52. [PMID: 11462162].

24. Perry VH, Oehler R, Cowey A. Retinal ganglion cells that project to the dorsal lateral geniculate nucleus in the macaque monkey. Neuroscience 1984; 12:1101-23. [PMID: 6483193].

25. Vidal-Sanz M, Villegas-Perez MP, Bray GM, Aguayo AJ. Persistent retrograde labeling of adult rat retinal ganglion cells with the carbocyanine dye diI. Exp Neurol 1988; 102:92-101. [PMID: 3181354].

26. Prusky GT, Alam NM, Beekman S, Douglas RM. Rapid quantification of adult and developing mouse spatial vision using a virtual optomotor system. Invest Ophthalmol Vis Sci 2004; 45:4611-6. [PMID: 15557474].

27. Bilak MM, Becerra SP, Vincent AM, Moss BH, Aymerich MS, Kuncl RW. Identification of the neuroprotective molecular region of pigment epithelium-derived factor and its binding sites on motor neurons. J Neurosci 2002; 22:9378-86. [PMID: 12417663].

28. Streck CJ, Zhang Y, Zhou J, Ng C, Nathwani AC, Davidoff AM. Adeno-associated virus vector-mediated delivery of pigment epithelium-derived factor restricts neuroblastoma angiogenesis and growth. J Pediatr Surg 2005; 40:236-43. [PMID: 15868591].

29. Li Q, Miller R, Han PY, Pang J, Dinculescu A, Chiodo V, Hauswirth WW. Intraocular route of AAV2 vector administration defines humoral immune response and therapeutic potential. Mol Vis 2008; 14:1760-9. [PMID: 18836574].

30. Zhou X, Li F, Ge J, Sarkisian SR Jr, Tomita H, Zaharia A, Chodosh J, Cao W. Retinal ganglion cell protection by 17-beta-estradiol in a mouse model of inherited glaucoma. Dev Neurobiol 2007; 67:603-16. [PMID: 17443811].

31. Kong L, Li F, Soleman CE, Li S, Elias RV, Zhou X, Lewis DA, McGinnis JF, Cao W. Bright cyclic light accelerates photoreceptor cell degeneration in tubby mice. Neurobiol Dis 2006; 21:468-77. [PMID: 16216520].

32. Yu X, Rajala RV, McGinnis JF, Li F, Anderson RE, Yan X, Li S, Elias RV, Knapp RR, Zhou X, Cao W. Involvement of insulin/phosphoinositide 3-kinase/Akt signal pathway in 17 beta-estradiol-mediated neuroprotection. J Biol Chem 2004; 279:13086-94. [PMID: 14711819].

33. Kong L, Tanito M, Huang Z, Li F, Zhou X, Zaharia A, Yodoi J, McGinnis JF, Cao W. Delay of photoreceptor degeneration in tubby mouse by sulforaphane. J Neurochem 2007; 101:1041-52. [PMID: 17394579].

34. Cao W, Chen W, Elias R, McGinnis JF. Recoverin negative photoreceptor cells. J Neurosci Res 2000; 60:195-201. [PMID: 10740224].

35. Yuan L, Neufeld AH. Activated microglia in the human glaucomatous optic nerve head. J Neurosci Res 2001; 64:523-32. [PMID: 11391707].

36. Wang X, Tay SS, Ng YK. An immunohistochemical study of neuronal and glial cell reactions in retinae of rats with experimental glaucoma. Exp Brain Res 2000; 132:476-84. [PMID: 10912828].

37. Woldemussie E, Wijono M, Ruiz G. Muller cell response to laser-induced increase in intraocular pressure in rats. Glia 2004; 47:109-19. [PMID: 15185390].

38. Jiang C, Moore MJ, Zhang X, Klassen H, Langer R, Young M. Intravitreal injections of GDNF-loaded biodegradable microspheres are neuroprotective in a rat model of glaucoma. Mol Vis 2007; 13:1783-92. [PMID: 17960131].

39. Savagian CA, Dubielzig RR, Nork TM. Comparison of the distribution of glial fibrillary acidic protein, heat shock protein 60, and hypoxia-inducible factor-1alpha in retinas from glaucomatous and normal canine eyes. Am J Vet Res 2008; 69:265-72. [PMID: 18241025].

40. Shohami E, Ginis I, Hallenbeck JM. Dual role of tumor necrosis factor alpha in brain injury. Cytokine Growth Factor Rev 1999; 10:119-30. [PMID: 10743503].

41. Funayama T, Ishikawa K, Ohtake Y, Tanino T, Kurosaka D, Kimura I, Suzuki K, Ideta H, Nakamoto K, Yasuda N, Fujimaki T, Murakami A, Asaoka R, Hotta Y, Tanihara H, Kanamoto T, Mishima H, Fukuchi T, Abe H, Iwata T, Shimada N, Kudoh J, Shimizu N, Mashima Y. Variants in optineurin gene and their association with tumor necrosis factor-alpha polymorphisms in Japanese patients with glaucoma. Invest Ophthalmol Vis Sci 2004; 45:4359-67. [PMID: 15557444].

42. Tezel G, Wax MB. Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induces apoptosis in cocultured retinal ganglion cells. J Neurosci 2000; 20:8693-700. [PMID: 11102475].

43. Fuchs C, Forster V, Balse E, Sahel JA, Picaud S, Tessier LH. Retinal-cell-conditioned medium prevents TNF-alpha-induced apoptosis of purified ganglion cells. Invest Ophthalmol Vis Sci 2005; 46:2983-91. [PMID: 16043875].

44. Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, Michaud N, Hafezi-Moghadam A, Miller JW, Benowitz LI. Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. J Neurosci 2006; 26:12633-41. [PMID: 17151265].

45. Gracie JA. Interleukin-18 as a potential target in inflammatory arthritis. Clin Exp Immunol 2004; 136:402-4. [PMID: 15147340].

46. Gracie JA, Robertson SE, McInnes IB. Interleukin-18. J Leukoc Biol 2003; 73:213-24. [PMID: 12554798].

47. Huang WX, Huang P, Hillert J. Increased expression of caspase-1 and interleukin-18 in peripheral blood mononuclear cells in patients with multiple sclerosis. Mult Scler 2004; 10:482-7. [PMID: 15471361].

48. Mahmoud RA, el-Ezz SA, Hegazy AS. Increased serum levels of interleukin-18 in patients with diabetic nephropathy. Ital J Biochem 2004; 53:73-81. [PMID: 15646011].

49. Nicoletti F, Conget I, Di Marco R, Speciale AM, Morìnigo R, Bendtzen K, Gomis R. Serum levels of the interferon-gamma-inducing cytokine interleukin-18 are increased in individuals at high risk of developing type I diabetes. Diabetologia 2001; 44:309-11. [PMID: 11317661].

50. Narins CR, Lin DA, Burton PB, Jin ZG, Berk BC. Interleukin-18 and interleukin-18 binding protein levels before and after percutaneous coronary intervention in patients with and without recent myocardial infarction. Am J Cardiol 2004; 94:1285-7. [PMID: 15541247].

51. Wang L, Cioffi GA, Cull G, Dong J, Fortune B. Immunohistologic evidence for retinal glial cell changes in human glaucoma. Invest Ophthalmol Vis Sci 2002; 43:1088-94. [PMID: 11923250].

52. Erickson PA, Fisher SK, Guerin CJ, Anderson DH, Kaska DD. Glial fibrillary acidic protein increases in Muller cells after retinal detachment. Exp Eye Res 1987; 44:37-48. [PMID: 3549345].

53. Sethi CS, Lewis GP, Fisher SK, Leitner WP, Mann DL, Luthert PJ, Charteris DG. Glial remodeling and neural plasticity in human retinal detachment with proliferative vitreoretinopathy. Invest Ophthalmol Vis Sci 2005; 46:329-42. [PMID: 15623793].

54. Salvador-Silva M, Ricard CS, Agapova OA, Yang P, Hernandez MR. Expression of small heat shock proteins and intermediate filaments in the human optic nerve head astrocytes exposed to elevated hydrostatic pressure in vitro. J Neurosci Res 2001; 66:59-73. [PMID: 11599002].

55. Hernandez MR. The optic nerve head in glaucoma: role of astrocytes in tissue remodeling. Prog Retin Eye Res 2000; 19:297-321. [PMID: 10749379].

56. Agapova OA, Ricard CS, Salvador-Silva M, Hernandez MR. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human optic nerve head astrocytes. Glia 2001; 33:205-16. [PMID: 11241738].

57. Spranger J, Osterhoff M, Reimann M, Möhlig M, Ristow M, Francis MK, Cristofalo V, Hammes HP, Smith G, Boulton M, Pfeiffer AF. Loss of the antiangiogenic pigment epithelium-derived factor in patients with angiogenic eye disease. Diabetes 2001; 50:2641-5. [PMID: 11723044].

58. Wang JJ, Zhang SX, Mott R, Chen Y, Knapp RR, Cao W, Mao JX. Anti-inflammatory effects of pigment epithelium-derived factor in diabetic nephropathy. Am J Physiol Renal Physiol 2008; 294:F1166-73. [PMID: 18322021].

59. Li H, Tran VV, Hu Y, Mark Saltzman W, Barnstable CJ, Tombran-Tink JA. PEDF N-terminal peptide protects the retina from ischemic injury when delivered in PLGA nanospheres. Exp Eye Res 2006; 83:824-33. [PMID: 16822505].

60. Demetriades AM, Deering T, Liu H, Lu L, Gehlbach P, Packer JD, Gabhann FM, Popel AS, Wei LL, Campochiaro PA. Trans-scleral delivery of antiangiogenic proteins. J Ocul Pharmacol Ther 2008; 24:70-9. [PMID: 18370877].