

Retinal synthesis and deposition of complement components induced by ocular hypertension

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Abstract

Inappropriate activity of the complement cascade contributes to the pathophysiology of several neurodegenerative conditions. This study sought to determine if components of the complement cascade are synthesized in the retina following the development of ocular hypertension (OHT) and if complement accumulates in association with retinal ganglion cells. Toward this goal the gene expression levels of complement components 1qb (*C1qb*) and 3 (*C3*) were determined in the retina by quantitative polymerase chain reaction in human eyes with elevated intraocular pressure (IOP) and healthy retinal tissue as well as in a rat model of OHT induced by laser cauterization of the trabecular meshwork and episcleral veins. Immunohistochemical methods were employed to determine the sites of complement deposition in the retina and optic nerve head. Our data demonstrate that transcript levels for *C1q* and *C3* are significantly elevated in retinae subjected to OHT, both in the animal model as well as in human eyes. Immunohistochemical analyses indicate that *C1q* and *C3* accumulate specifically in the retinal ganglion cell layer and the nerve fiber layer. In addition, we demonstrate that the terminal complement complex, or membrane attack complex, is formed both in the human and rat model as a consequence of OHT. Complement activation, particularly formation of membrane attack complexes, has the potential to exacerbate ganglion cell death through bystander lysis or glial cell activation. The results show that complement activation occurs in the retina that has been subjected to elevated IOP, and may have implications in pathophysiology of glaucoma.

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1. Introduction

Elevated intraocular pressure (IOP) is an important risk factor for glaucoma and to date pharmacological and surgical reduction of IOP remains the only accepted treatment of this disease (Alward, 1998). Glaucoma is a leading cause of vision loss worldwide and, despite many recent improvements in the diagnosis and treatment of this disease, continues to pose a clinical challenge. This group of diseases is characterized

by the slow, progressive loss of retinal ganglion cells (RGC) and their axons, and is clinically recognized by progressive excavation of the optic nerve head and resultant visual field loss. Many aspects of the molecular pathogenesis of glaucoma remain unclear and the loss of vision is irreversible. In particular, the biochemical, cellular and molecular events that are initiated by elevated IOP in the retina and in the optic nerve head (ONH) remain active areas of research (Kuehn et al., 2005). A number of studies suggest that RGC die through apoptosis, but the molecular events that precede or initiate this process in the glaucomatous retina remain unclear (Farkas and Grosskreutz, 2001).

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Preliminary microarray data generated by our laboratories indicated that retinal expression of a number of components of the complement cascade is elevated following experimental elevation of IOP in a rat model of ocular hypertension (OHT) induced by laser cauterization of the episcleral veins and trabecular meshwork (Kim et al., 2005). Elevated transcript levels were most consistently observed for the members of complement component 1 complex and complement component 3. The complement system consists of approximately 25 plasma- and membrane-bound proteins that function together to protect the host from bacterial or parasitic infection through T-cell independent mechanisms. Complement can be activated through several pathways, all of which converge to generate multimolecular enzyme complexes that cleave the complement components 3 (C3) and 5 (C5). If left uninhibited, complement activation results in the formation of the terminal complement complex or membrane attack complex (MAC). This macromolecular complex is formed by complement components C5b, C6, C7, C8, and multimers of C9 and forms a transmembrane channel that enables ions and small molecules to diffuse freely through cell membranes via this newly formed pore, resulting in severe disturbance of cellular homeostasis and cell lysis. In addition, complement initiates local inflammatory responses through production of the anaphylatoxins C3a and C5a and promotes phagocytosis of the bacterial cells through opsonization. Recent reports suggest that opsonization is also required for the efficient phagocytosis of an organism's own cellular debris (Taylor et al., 2000). For example, inherited deficiencies of the classical complement pathway components, particularly C1q, are strongly associated with the development of systemic lupus erythematosus or glomerulonephritis presumably by causing autoimmunity through delayed or insufficient clearance of apoptotic cells (Botto et al., 1998).

On the other hand, inappropriate activation of complement appears to be involved in a number of neurodegenerative conditions. Complement plays a crucial role in the pathobiology of many neuroinflammatory conditions, including traumatic or ischemic brain injury, Guillain–Barre syndrome, multiple sclerosis, as well as Alzheimer's and Parkinson's diseases (Cowell et al., 2003; Rancan et al., 2003; Yamada et al., 2004). Recently, genomic variations in the factor H gene, an inhibitor of the complement cascade, have been reported to be associated with the development of age-related macular degeneration (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). The precise role of complement in the etiology of these conditions is unresolved but it seems clear that local inflammation can exacerbate neuronal loss and that complement inhibition often results in neuroprotection (van Beek and Elward, 2003; Fonseca et al., 2004).

The majority of complement proteins are synthesized in the liver and circulate in the plasma. However, extrahepatic synthesis of complement components has been documented in many cell types including fibroblasts, synovial cells, adipocytes, kidney glomerular mesangial cells, keratinocytes and macrophages (reviewed in (Morgan and Gasque, 1997)). In recent years, it has been reported that complement gene

transcription and protein synthesis also occurs in glia and neurons, (Nataf et al., 1999) including retinal cells and in the optic nerve following crush (Bora et al., 1993; Sohn et al., 2000; Ohlsson et al., 2003). Endogenous production of complement by neuronal cells may be particularly important in immune privileged sites because it provides a level of protection against invading pathogens, but it introduces the possibility that inappropriate activation or insufficient regulation of complement damages neuronal cells.

The goals of this study were to determine whether complement components are deposited in retinae subjected to OHT, which retinal structures are associated with complement and if the final mediator of complement cell lysis, MAC, is formed. Toward this goal we conducted an immunohistochemical evaluation of the retina and optic nerve head of a rat model of OHT induced by laser cauterization of the episcleral veins and trabecular meshwork (Grozdanic et al., 2004). These data were correlated to those obtained from human donor eyes. Our findings indicate that complement components C1q and C3 are transcribed in the retina and accumulate in the nerve fiber layer (NFL) and ganglion cell layer (GCL) of eyes with OHT. Our data further suggest that MAC is formed in this region, which may further exacerbate RGC damage.

2. Material and methods

2.1. Rat model of ocular hypertension

Elevated intraocular pressure was induced in one eye of adult Brown Norway rats as described in detail previously (Grozdanic et al., 2004). Briefly, animals were anesthetized by isoflurane inhalation and indocyanine green was injected into the anterior chamber of one eye. Twenty minutes post-injection, a diode laser (DioVet, Iridex Corp.) was used to externally deliver 810 nm energy pulses through a 50- μ m fiberoptic probe to the region of the trabecular meshwork and episcleral veins in close proximity to the limbal region. Approximately 50 laser spots were delivered through a 300° range of the limbal radius (300 mW energy, 400 ms pulse time). The resulting IOP elevation was measured weekly in isoflurane anesthetized animals with a hand-held tonometer (Tonopen XL, Mentor, Norwell, MA). Only animals that developed elevated IOP were included in this study. Rat eyes were harvested either 14 or 28 days after the induction of elevated IOP.

All research conducted in this study was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Iowa State University Committee on Animal Care Regulations.

2.2. Evaluation of optic nerve damage

Thin sections of the rat optic nerve were prepared using standard electron microscopy methods as described by others (Smith et al., 2002). Immediately after death optic nerves were carefully dissected and a 3-mm long portion of optic nerve, located approximately 2 mm posterior to the eye cup, was carefully excised and preserved in 2% glutaraldehyde and 2%

paraformaldehyde. Following osmium fixation, tissue was stained with uranyl acetate and embedded in Eponate 12 resin. One-micrometer sections were taken in a plane perpendicular to the length of the optic nerve. Sections were stained with 1% Paraphenylenediamine (PPD) and photomicrographs were taken at a magnification of 400 \times .

The degree of optic nerve damage was evaluated using a grading scheme similar to those used by other investigators (Jia et al., 2000; Libby et al., 2005) and optic nerves were assigned to one of four grades of damage. Grade 1: healthy nerve—only a very small number (<50) of darkly stained (damaged) axons were detected across the entire optic nerve; Grade 2: slight damage—damaged axons are readily discernible although they are sparse (5–10% per nerve); Grade 3: moderate damage—11–40% of axons stain darkly, some axon loss may be evident; Grade 4: severe damage—more than 40% of axons are damaged. Axon loss is obvious, and swollen axons and/or glial cell proliferation can be detected. The degree of damage was evaluated independently and in a masked fashion by two investigators. In cases where the investigators disagreed a third investigator rated the optic nerves. In all cases the evaluation of the third investigator corresponded with one of the first two and served as a tie-breaker.

2.3. Human tissue

Donor eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA) and preserved within 5 h postmortem. Following chart review, donors with elevated IOP ($n = 9$) and healthy age-matched control donors ($n = 9$) were chosen. Four donors from each group were used for Q-PCR and the remainder for immunohistochemistry. Control donor eyes did not have any ocular disease except for cataract or pseudophakia. The study eyes had a clinical diagnosis of primary open angle glaucoma ($n = 5$) or ocular hypertension ($n = 4$); no other ocular disease except for cataract or pseudophakia was present. All OHT eyes had been treated with anti-glaucoma topical medications.

2.4. Quantitative PCR analyses

Total retinal RNA was extracted from retinas of both human and rat eyes using Qiagen RNeasy (Qiagen, Valencia, CA) columns and treated with DNase. Samples of human retina were obtained from the superior-temporal quadrant of each eye and stored at -80°C until RNA extraction. In the rat model retina from the entire eye was harvested for RNA extraction. 200 ng RNA was reverse transcribed in a random primed reaction.

PCR primers to human and rat *Clqb*, *C3* and ubiquitin C (*Ubc*) were designed to span at least one intron to avoid the generation of PCR signal from potentially remaining genomic DNA (see Table 1).

Samples underwent 35 cycles of amplification in an ABI type 7700 machine using SYBR Green as the reporter dye (QuantiTect, Qiagen). Data from each sample was obtained in triplicate; amplification controls included wells containing genomic DNA only and those containing no target (water controls). Transcript levels were determined based upon standard curves for each primer pair. Melt curve analyses were carried out following each amplification reaction to ascertain the absence of unspecific amplification products or primer dimers. Expression values obtained for *Clqb* and *C3* were normalized to transcript levels for *Ubc*.

2.5. Immunohistochemistry

Tissue samples for immunohistochemistry were fixed in 4% paraformaldehyde in phosphate buffered saline for 4 h. Tissue was then infiltrated with increasing concentrations of sucrose, frozen in optimal cutting temperature (OCT) compound and sectioned to a thickness of 7 μm . Sections were blocked with bovine serum albumin and incubated with polyclonal antibodies directed against human C1q (Calbiochem, San Diego, CA), human C3 (Calbiochem) or with monoclonal antibodies directed against human C5b-9 (clone aE11, Dako, Carpinteria, CA). These antibodies were used at 1:100 dilution. In addition polyclonal antibodies, diluted 1:200, directed against human glial fibrillary acidic protein (GFAP, Chemicon, Temecula, CA) were used.

All sections were counterstained with the nuclear dye DAPI (Invitrogen, Carlsbad, CA) to facilitate orientation and viewed on an Olympus (Melville, NY) BX41 microscope. Digital images were collected with a SPOT RT camera (Diagnostic Instruments; Sterling Heights, MI).

3. Results

Consistent with findings from our previous studies (Grozdanic et al., 2003, 2004) laser cauterization of indocyanine green infused episcleral veins and trabecular meshwork in rat eyes resulted in a gradual elevation of IOP in treated eyes. The IOP reached an average maximum value of 27.7 mmHg (control: 18.5 mmHg) 3 days after the procedure (Fig. 1). Thereafter pressure slowly declined but remained elevated throughout the study period (1 day postop $P = 0.0001$;

Table 1
Sequences of primers used for real-time PCR

Primer name	Forward primer (5' to 3')	Reverse primer (5' to 3')
UBC-human	ATTGGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
C1qb-human	AGGGAACCTGTGCGTGAAC	CMACATGCCAGTAGTGAGTT
C3-human	TCAGCATGTCCGACAAGAAAGGGA	TGCAGAAGGCTGGATTGTGGAGTA
<i>Ubc</i> -rat	ATCTTTGCAGGCAAGCAGCTGGAA	TCTTGCTGTGAGGGTCTTCACAA
<i>Clqb</i> -rat	AACCAGGCACMACAGGGATAAA	TTGTAGTCMACAGAGCCACCTT
<i>C3</i> -rat	AGCTGGCTTTCAAACAGCCCAT	TGTMACGGAAGCCACCAATCAT

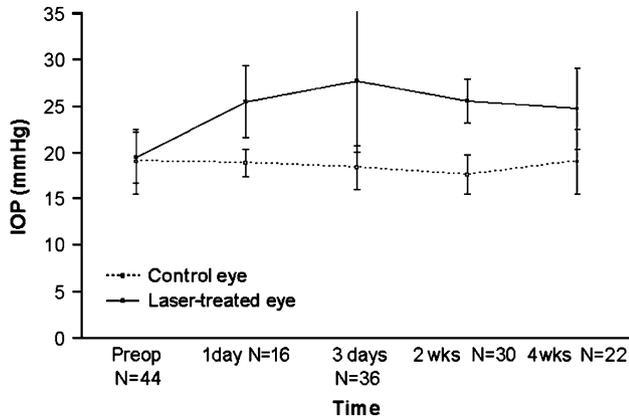


Fig. 1. Development of elevated IOP in the rat model of OHT. Error bars signify standard error.

3 days $P = 7.6 \times 10^{-12}$; 14 days $P = 9.6 \times 10^{-7}$; 28 days $P = 0.0002$, using ANOVA with Bonferroni multiple comparison as post hoc test). The pressure in the control eyes remained unaffected ($P = 0.23$).

Elevated IOP was accompanied by a progressive development of optic nerve damage (Fig. 2). For this study, optic nerve damage in two groups of animals was evaluated using a grading scheme similar to those employed by other investigators (Jia et al., 2000; Libby et al., 2005). Fourteen days after laser surgery, only mild to moderate axon damage could be observed in optic nerves of induced eyes ($n = 8$) (Fig. 3). However, 28 days after IOP elevation optic nerve damage had progressed and some nerves were severely damaged ($n = 8$). These changes were statistically significant; $P = 0.007$ at 14 days and $P = 0.01$ at 28 days (by t -test).

In order to establish the local synthesis of complement components in the OHT retina, real-time PCR quantitation of mRNA levels for *C1qb* and *C3* was carried out. Expression values were normalized to those of ubiquitin C (*Ubc*), a gene with very stable transcript levels in many organs (Vandesompele et al., 2002; Szabo et al., 2004). For each molecule transcript levels from the control and laser-treated eyes of the same animals were compared. Analyses of RNA obtained

from the retinas of rat eyes with elevated IOP confirmed significantly increased levels for both *C1qb* and *C3* ($P = 0.01$ and 0.008 by t -test, respectively) 14 days after surgery when compared to the contralateral control eyes from the same animals (Fig. 4A). A slight reduction of the elevation of normalized *C1q* and *C3* expression values was observed 28 days after surgery, concomitant with increased transcript levels in the contralateral eyes of individual animals (*C1qb* $P = 0.4$; *C3* $P = 0.02$ by t -test).

Since it is conceivable that the surgical procedures used in the induction of IOP elicit an inflammatory response in our animal model or that complement activation is a rodent-specific phenomenon, we sought to determine if elevated synthesis of complement components also occurs in human eyes with elevated IOP. Toward this end RNA was extracted from the retina of human eye donors ($n = 4$) with clinically documented glaucoma or OHT as well as from control donors ($n = 4$). Data obtained demonstrated significant elevation of both *C1qb* and *C3* expression ($P = 0.02$ and 0.04 by t -test, respectively) in donors with elevated IOP when compared to the control donors (Fig. 4B). The observed gene expression changes for both *C1qb* and *C3* in humans are less pronounced when compared to those observed in the animal model.

To verify the synthesis of complement proteins and to establish their distribution in the retina we determined the location of *C1q*, *C3* and the membrane attack complex C5b-9 (MAC) by immunohistochemistry (Fig. 5). Incubation of rat retinal sections with antibodies directed against *C1q*, *C3*, and MAC demonstrated accumulation of all three molecules associated with the GCL and NFL in response to laser induced OHT. Binding of anti-*C1q* antibody appeared to be concentrated along the vitreal surface of the retina, possibly associated with ganglion cell axons, whereas anti-*C3* and anti-MAC immunoreactivity was observed both along the vitreal surface as well as with cell bodies of the GCL. In the optic nerve head labeling was only evident in the NFL. Significant binding of anti-complement antibodies associated with other retinal cell types was not observed, although weak immunoreactivity was occasionally detected in the inner nuclear layer (INL). The labeled structures in the INL might represent glial

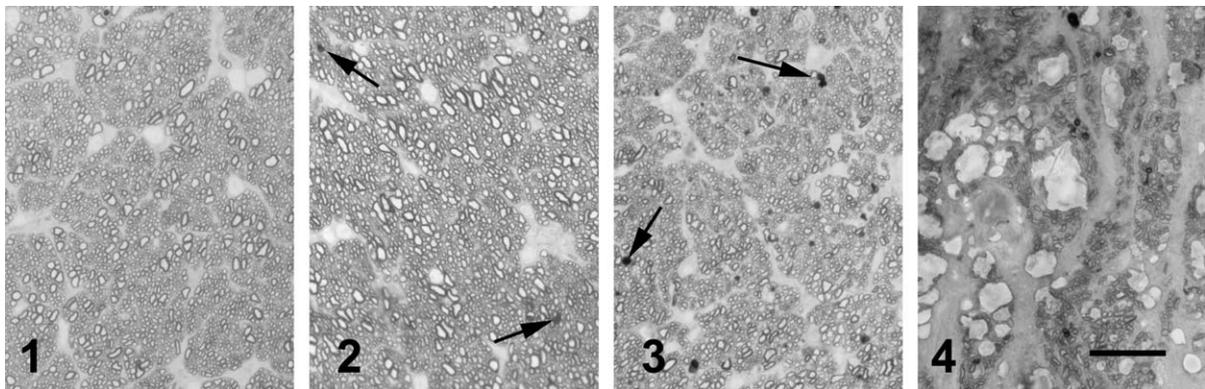


Fig. 2. Morphological evaluation of optic nerve damage in rat eyes with elevated IOP. Grade 1: Healthy nerve; Grade 2: slight damage—a small number of dark stained and damaged axons become detectable (arrows); Grade 3: moderate damage—increasing amounts of axons are damaged; Grade 4: severe damage—noticeable loss of axons and apparent gliosis. Scale bar = 20 μm .

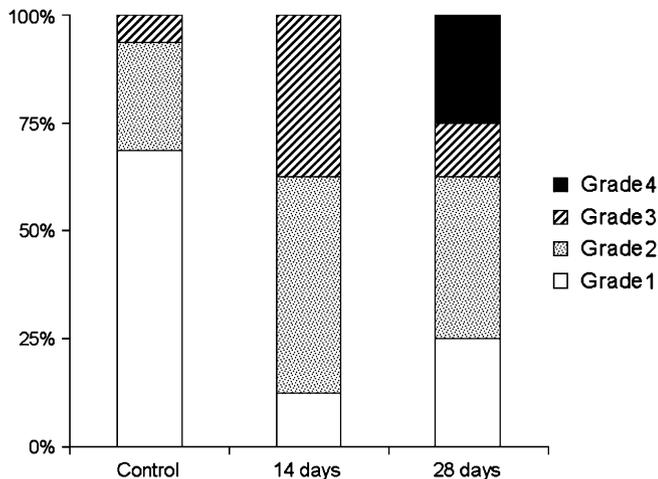


Fig. 3. Development of optic nerve damage in response to elevated IOP. Fourteen days after induction of elevated IOP, most optic nerves displayed slight to moderate damage. Twenty-eight days after laser treatment several animals had developed severe optic nerve damage. Control $n = 16$, 14 days $n = 8$, 28 days $n = 8$.

elements, a possible source of complement. In contrast, increased GFAP immunoreactivity was evident both in the retina as well as in the ONH in patterns similar to those described by others (Wang et al., 2000; Woldemussie et al., 2004). GFAP immunoreactivity did not appear to co-localize with complement deposition (data not shown).

Accumulation of C1q, C3 and MAC could also be detected in all human donor eyes with glaucoma or OHT examined ($n = 5$). Complement could not be discerned in the NFL or RGC layer in human control eyes ($n = 5$), although some complement accumulation in the INL and the choroidal space was occasionally noted. The observed binding patterns in the human retina closely resembled those observed in the rat model, although the amount of complement detected was less in human eyes (Fig. 6). Furthermore, complement components could only be observed focally in human eyes with elevated IOP, in contrast to the near uniform labeling of the RGC in rat retina.

4. Discussion

Our findings presented herein demonstrate that persistent OHT is accompanied by the synthesis and accumulation of components of the complement cascade. In the rat complement components C1qb and C3 appear to be locally synthesized in the retina and optic nerve head and can be detected as early as 14 days following elevation of intraocular pressure in a rat model coincident with the development of optic nerve damage. These findings are in accordance with gene array data made available by other laboratories that suggest that components of the complement cascade are also expressed at elevated levels in the retina of other animal models of glaucoma or OHT (Miyahara et al., 2003; Ahmed et al., 2004; Nagel et al., 2004). Our study further demonstrates that complement deposition in response to OHT occurs largely in the ganglion cell and nerve fiber layers, in close apposition to RGC. Interestingly, we did not detect significant amounts of complement components in the pre- and retrolaminar regions or associated with the lamina cribrosa of the ONH, suggesting a limited role for complement in the pathophysiology of these tissues. Complement activation also occurs in the human retina in eyes with elevated IOP; C1qb, C3 and MAC deposition was evident in all human eyes with either OHT or glaucoma examined, although immunoreactivity and gene expression levels in the human retina are less pronounced than those observed in the rat and are typically restricted to discrete areas of the retina. One explanation for these findings may be that all human donor eyes were derived from persons with pharmacologically controlled IOP.

Perhaps most importantly, we demonstrate the presence of MAC in the GCL of eyes with elevated IOP. While it is possible that the immunohistochemical methods employed detect soluble and not membrane intercalated MAC, our findings suggest that complement activation may not only serve to support clearance of apoptotic cell debris but could damage RGC through lytic or sublytic injury. Data presented by others have demonstrated that neurons express low levels of complement inhibitors and are highly susceptible to complement attack

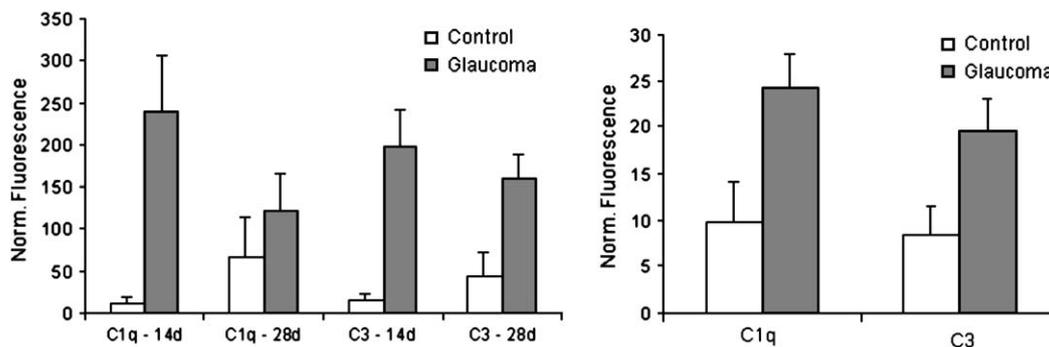


Fig. 4. Expression levels of C1QB and C3 in the rat model of OHT (A) and in human retinas with and without OHT (B). mRNA levels for complement components C1qb and C3 were determined in rat retinas harvested 14 (14d) or 28 (28d) days after laser-induced elevation of IOP. Each group included five animals, the uninduced eye from the same animals served as the control. C1qb and C3 expression is also elevated in human eyes with OHT ($n = 4$) when compared to normotensive control eyes ($n = 4$). In comparison to the animal model the response in the human retina is markedly reduced in magnitude (note Y-axis units). Error bars signify standard error.

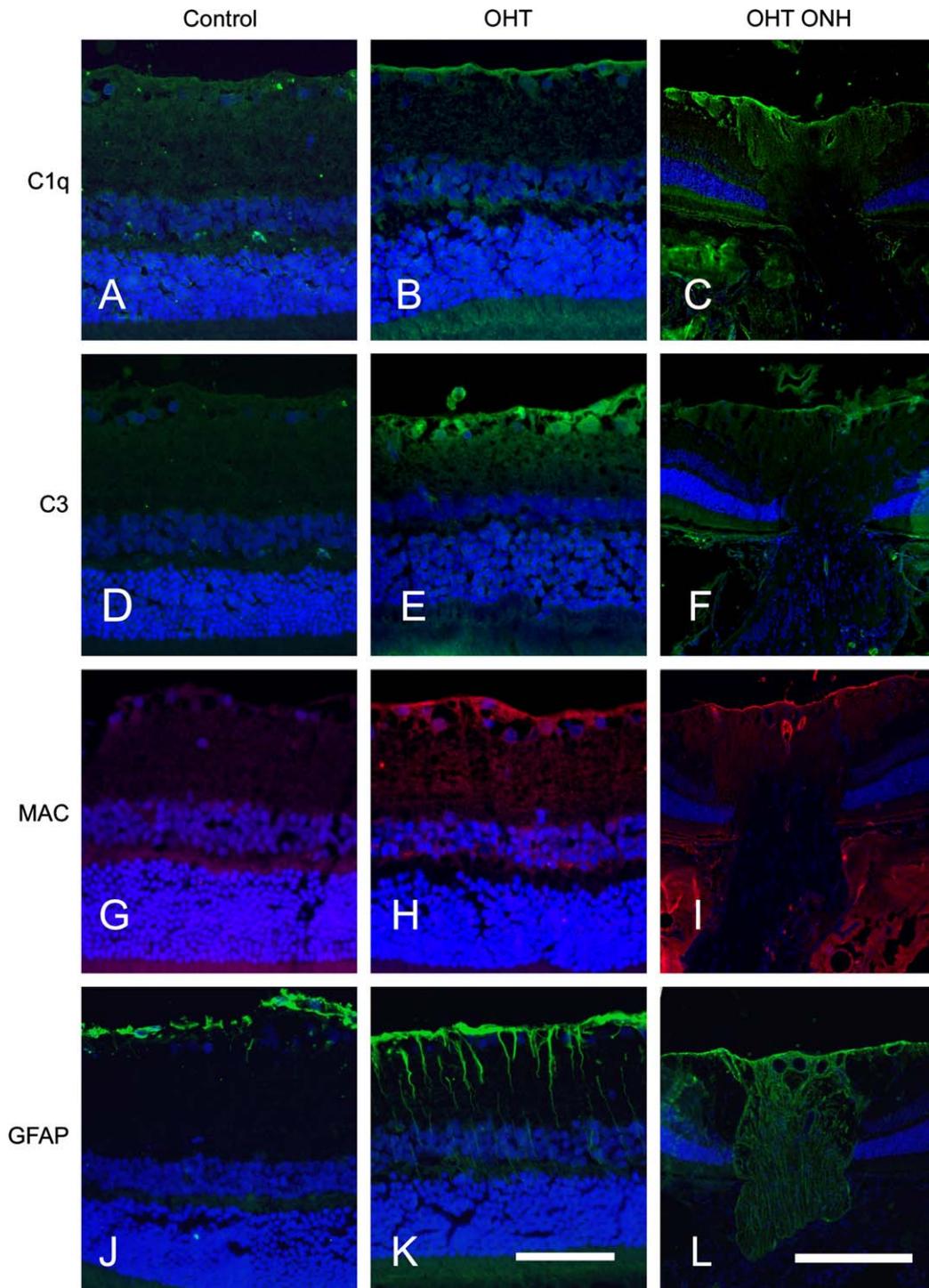


Fig. 5. Immunohistochemical localization of C1q, C3, MAC and GFAP in the rat model 28 days after the induction of OHT. Strong labeling was detected for C1q, C3 and MAC in OHT, but not control eyes, in the nerve fiber and ganglion cell layers. In the OHT retina anti-GFAP antibodies also labeled reactive Mueller glial cells (J). Complement labeling was evident in the NFL of the OHT optic nerve head (ONH) but not in the pre- or postlamellar regions. Scale bars = 50 μ m (retinal images) and 200 μ m (ONH images).

(Singhrao et al., 2000). In contrast, astrocytes and microglia are relatively well protected from complement-mediated damage since they express high levels of complement inhibitors (Singhrao et al., 1999). Thus the presence of MAC in the GCL is likely to affect predominately RGC and may target both compromised as well as additional healthy RGC through

bystander lysis. As such, formation of MAC in response to OHT introduces the possibility that complement contributes to retinal degeneration and may represent a previously undescribed mechanism of secondary damage in glaucoma. The existence of secondary damage in glaucoma has been proposed but the molecular pathways involved have not yet been

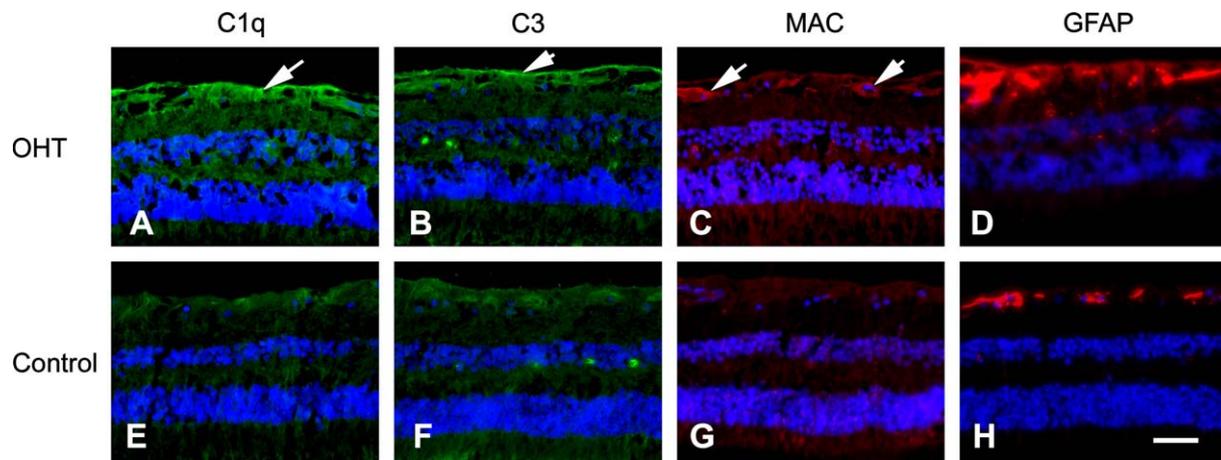


Fig. 6. Immunohistochemical detection of complement deposition in the superior-temporal region of human OHT (top) and normal (bottom) retinas. Immunoreactivity (arrows) for C1q (A and E), C3 (B and F), and MAC (C and G) can be observed associated with distinct focal areas of the nerve fiber layers. GFAP staining is strongly enhanced in eyes with OHT (D and H). Scale bar = 50 μ m.

determined (Yoles and Schwartz, 1998; Levkovitch-Verbin et al., 2003).

A second potential consequence of complement accumulation in the retina may be the activation of retinal or ONH glial cells. Astrocytes and microglia can become reactive when exposed to complement, causing them to synthesize inflammatory mediators such as cytokines, superoxide radicals and additional complement components (Kulkarni et al., 2004). Synthesis of these molecules by adjacent reactive retinal glial elements could further damage RGC. The activation of glial cells has been suggested to cause the characteristic reorganization of the glaucomatous ONH and may damage RGC through the release of nitric oxide, TNF- α , or other neurotoxic molecules (Hernandez, 2000; Tezel and Wax, 2000; Tezel et al., 2004; Neufeld and Liu, 2003).

Currently the events initiating the complement cascade in glaucoma are unclear. A number of recent reports have suggested an autoimmune component to the development of glaucomatous damage (Maruyama et al., 2001; Wax et al., 2001; Yang et al., 2001; Schori et al., 2002). Clearly, the presence of autoantibodies to RGC could serve to initiate the classical complement pathway through their interaction with C1q. On the other hand, C1q and C3 might be synthesized primarily to aid phagocytic removal of apoptotic ganglion cell debris. Expression of classical pathway components appears to represent a relatively unspecific response to retinal damage, e.g. synthesis of these molecules has been described following mechanical injury to the retina (Vazquez-Chona et al., 2004). In this case, formation of the MAC might result from insufficient inhibition of the downstream portions of the complement cascade.

While complement activation has the potential to hasten RGC degeneration other, primary, events inducing RGC death undoubtedly exist. In the DBA/2J mouse model of pigmentary glaucoma RGC are clearly lost despite the fact that these animals are genetically C5 deficient (Wetsel et al., 1990; Anderson et al., 2002). Since C5 is an essential component of MAC, lytic damage to RGC is unlikely to occur in this

model. However, it is conceivable that RGC loss would occur more rapidly in these animals if they were able to carry out all complement mediated functions.

Chronic activation of complement often contributes to the pathobiology of neurodegenerative disorders, but recent data have also shown that certain aspects of the cascade appear to promote neuronal survival and ultimately contribute to the protection and healing of the host (Osaka et al., 1999; van Beek et al., 2001). Given the highly diverse consequences of complement activity, it is difficult to predict which role this system plays in the retina and whether inhibition of the cascade would support ganglion cell survival in glaucomas associated with elevated IOP. We anticipate that future studies using complement deficient animals will resolve the functional significance of the findings reported herein.

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