Oxidative Stress and the Regulation of Complement Activation in Human Glaucoma

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PURPOSE. As part of ongoing studies on proteomic alterations during glaucomatous neurodegeneration, this study focused on the complement system.

METHODS. Human retinal protein samples obtained from donor eyes with ($n = 10$) or without ($n = 10$) glaucoma were analyzed by a quantitative proteomic approach using mass spectrometry. Cellular localization of protein expression for different complement components and regulators were also determined by immunohistochemical analysis of an additional group of human donor eyes with glaucoma ($n = 34$) compared with age-matched control eyes without glaucoma ($n = 20$). In addition, to determine the regulation of complement factor H (CFH) by oxidative stress, in vitro experiments were performed using rat retinal cell cultures incubated in the presence and absence of an oxidant treatment.

RESULTS. Proteomic analysis detected the expression and differential regulation of several complement components in glaucomatous samples, which included proteins involved in the classical and the lectin pathways of complement activation. In addition, several complement regulatory proteins were detected in the human retinal proteome, and glaucomatous samples exhibited a trend toward downregulation of CFH expression. In vitro experiments revealed that oxidative stress, which was also prominently detectable in the glaucomatous human retinas, downregulated CFH expression in retinal cells.

CONCLUSIONS. These findings expand the current knowledge of complement activation by presenting new evidence in human glaucoma and support that despite important roles in tissue cleaning and healing, a potential deficiency in intrinsic regulation of complement activation, as is evident in the presence of oxidative stress, may lead to uncontrolled complement attack with neurodestructive consequences. (*Invest Ophthalmol Vis Sci.* 2010;51:5071–5082) DOI:10.1167/iovs.10-5289

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Clinical and experimental studies over the past decade highlight the involvement of the immune system in glaucomatous neurodegeneration. Different components, including both innate and adaptive immunity, exhibit prominent activity in glaucoma.^{1–3} Despite the fact that immune activity is a necessary intrinsic response to promote the tissue cleaning, healing, and regeneration process, if there is a failure in the immune system regulation because of increasing risk factors, initially beneficial immune activity may turn into an autoimmune injury process. In addition to the potential cytotoxicity of autoreactive T cells⁴ and autoantibodies,⁵ present evidence suggests that uncontrolled complement activation may also contribute to the progression of degenerative injury to retinal ganglion cells (RGCs), their synapses, and axons in glaucoma. Recent histopathologic studies of human tissues and in vivo studies using different animal models have demonstrated that complement components, including C1q and C3, are synthesized and terminal complement complex is formed in the glaucomatous retina.^{6,7} Findings of another study using mice deficient in complement components C1q and C3 have also provided evidence to suggest that the classical complement cascade may be involved in synapse elimination during neurodegenerative injury.⁸ These findings support that injured RGCs in glaucoma may be similarly targeted and destroyed through complement-mediated processes involving reactive glia.

This study aimed to further explore complement activation in glaucoma by focusing particularly on proteomic and immunohistochemical findings in human donor eyes. In addition, based on potential immunostimulatory consequences of oxidative stress in glaucoma,³ including the recently identified regulatory roles of oxidative stress in T-cell-mediated immunity,⁹ this study aimed to determine whether oxidative stress may be involved in the regulation of complement activation in glaucoma. Therefore, we also performed in vitro experiments using primary cultures of retinal cells in the presence and absence of oxidative stress. Findings of these studies collectively support complement activation in the glaucomatous human retina. In addition to the classical pathway, the lectin pathway is likely involved in complement activation during glaucomatous neurodegeneration. By targeting and removing the toxic debris from dying neurons in glaucoma, complement activation may participate in tissue healing and may minimize inflammatory insults. However, a potential deficiency in the intrinsic regulation of complement activation, as is evident in the presence of oxidative stress, may facilitate the progression of neurodegenerative injury by collateral cell lysis, inflammation, and autoimmunity.

MATERIALS AND METHODS

Experimental Design

Proteomic analysis with mass spectrometry used retinal samples obtained from human donor eyes with or without glaucoma. Selected

findings were further validated by quantitative Western blot analysis, and cellular localization of different complement components and regulators was studied using histologic sections of the retina obtained from an additional group of glaucomatous and nonglaucomatous human donors. All human donor eyes were handled according to the tenets of the Declaration of Helsinki. We also performed *in vitro* experiments with primary cultures of rat retinal cells to determine the regulation of complement factor H (CFH) expression by oxidative stress. All animals used in *in vitro* experiments were handled according to the regulations of the Institutional Animal Care and Use Committee, and all procedures adhered to the principles set forth in ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Human Donor Eyes

Retinal protein samples were obtained from 10 human donor eyes with glaucoma (age, 84.7 ± 8) and 10 eyes without glaucoma (age, 83.7 ± 7). Retinal tissue punches were collected as previously described¹⁰ within <6 hours after death (average postmortem time: 4:33 hours for glaucoma, 4:53 hours for controls). All glaucomatous donor eyes had primary open-angle glaucoma with high intraocular pressure that was well documented by intraocular pressure readings, optic disc assessments, and visual field tests. Four glaucomatous and four nonglaucomatous donor eyes (samples 7–10 [see Figs. 2, 7]) had age-related macular degeneration (AMD). Protein lysis used a buffer containing 50 mM HEPES-KOH [pH 8.0], 100 mM KCl, 2 mM EDTA, 0.10% NP-40, 2 mM dithiothreitol, 10% glycerol, and protease and phosphatase inhibitors, as previously described.^{10–12}

An additional group of human donor eyes, including 38 donor eyes with a diagnosis of glaucoma (age, 76.8 ± 11) and 30 eyes from donors without glaucoma (age, 71.0 ± 15 years), was used for immunohistochemical analysis. All these donor eyes were fixed within 12 hours after death and processed for 5 μ m paraffin-embedded sagittal tissue sections. Detailed information on donor demographics and clinical data of glaucomatous donor eyes has recently been published.¹³

Proteomic Analysis

Trypsin-digested protein samples were analyzed through a gel-free mass spectrometric approach using two-dimensional capillary liquid chromatography and linear ion trap mass spectrometry (LC-MS/MS), as previously described.^{10,14} Database searching was performed with tandem mass spectra extracted by ReAdW and converted to mzXML format. The acquired MS/MS spectra were analyzed using a data analysis system (SEQUEST Sorcerer; Sage-N Research, Inc., San Jose, CA), which was set up to search a FASTA formatted human protein database (Human RefSeq) with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. Iodoacetamide derivative of cysteine was specified as a fixed modification, and oxidation of methionine and iodoacetamide derivatives of cysteine were specified as variable modifications. A specific software (Scaffold version 3.0.00; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability, as specified by the PeptideProphet algorithm.¹⁵ Protein probabilities were assigned by the ProteinProphet algorithm,¹⁶ and protein identification was based on the criteria of greater than 99.0% probability and at least two identified peptides. The abundance of each identified protein was determined by normalizing the number of unique spectral count matching to the protein by its predicted molecular weight. This value has been termed a protein abundance factor.^{14,17} Bioinformatics analysis used a pathways analysis system (Ingenuity Pathways Analysis; Ingenuity Systems, Mountain View, CA) to define functional patterns within the human retinal proteome composition.

Cell Cultures

Primary cultures of RGCs and macroglia were derived from adult rats as previously described.^{9,10,12,18–20} Briefly, retinas dissociated in a papain solution were used in a two-step immunomagnetic cell selection pro-

cess using antibody-coated magnetic beads (Dyna, Oslo, Norway). In the first step, an antibody to macrophage/microglia surface antigens was used. In the second step, the macrophage/microglia-depleted cell suspension was incubated with magnetic beads bound to a monoclonal antibody specific to Thy-1.1 (Millipore/Chemicon, Billerica, MA). Selected Thy-1.1-positive RGCs were incubated in a serum-free culture medium, as previously described.¹⁸ RGCs isolated by this procedure were identified based on retrograde fluorescence labeling, cell morphology, and immunolabeling for specific markers.¹⁸ In addition, the purity of selected RGCs was further validated by Western blot analysis²¹ and quantitative RT-PCR analysis of different retinal cell markers.¹⁰

The unselected fraction of retinal cells was cultured in a medium that does not allow residual neurons to survive (Dulbecco's minimum essential medium, 10% fetal bovine serum, 2 mM glutamine, 1 mM Na-pyruvate, and antibiotics) but contains macroglial cells, including astrocytes and Müller cells, as previously documented.¹⁸ During the experimental period, macroglial cell cultures were incubated in a serum-free medium containing DMEM, 1.3% bovine albumin fraction V, 1 μ L/mL culture supplement (ITS+ Premix; BD Biosciences, San Diego, CA), and antibiotics. To better simulate *in vivo* conditions and astrocyte-derived signals involved in complement regulation,⁸ *in vitro* experiments used cocultured cells by seeding RGCs on the monolayer of macroglial cells, as previously described.²² Although we initially studied separate cultures of these cell types, no prominent alteration in complement regulation was detectable. Therefore, cocultures were used to better simulate contact-dependent and -independent cellular interactions under *in vivo* conditions.

Oxidative stress was generated by treating the cocultures with H₂O₂ (50 μ M) for 24 hours. Additional cocultures were treated with staurosporine (100 nM; Sigma-Aldrich, St. Louis, MO), a broad-spectrum protein kinase inhibitor, to induce apoptosis. Control cultures, prepared using an identical passage of cells, were simultaneously incubated in the absence of H₂O₂ and staurosporine.

Cell viability was determined using a live/dead kit containing calcein AM (Molecular Probes, Eugene, OR) as previously described.^{9,10,18,19} The survival rate was expressed as the percentage of the total cell number in control wells. All *in vitro* experiments were repeated at least three times for each experimental condition. Data are presented as mean $\pm$ SD.

Western Blot Analysis

Because of the limited amount of protein samples obtained from human donor eyes with glaucoma, comparative Western blot analysis of glaucomatous and nonglaucomatous human samples determined only the expression of CFH among multiple complement components and regulators identified by LC-MS/MS analysis. In addition, human retinal protein samples were used to determine oxidative stress. Western blot analysis was also used in *in vitro* experiments.

Immunoblot analysis, performed as previously described,^{10–12} used a monoclonal antibody to CFH (1:200; Lifespan Biosciences, Seattle, WA) and a monoclonal antibody to 4-hydroxy-2-nonenal (HNE; 15 μ g/mL; Abcam, Cambridge, MA) to detect HNE-adducts generated by free-radical attack. In addition, a β -actin antibody (Sigma-Aldrich) was used to reprobe the stripped immunoblots for loading and transfer control. Secondary antibody incubation was performed using a specific IgG conjugated with horseradish peroxidase (1:2000; Sigma-Aldrich). The primary antibody was omitted to serve as a control. After normalization to β -actin, the average band intensity value obtained from control samples was used to calculate the fold change in protein expression in glaucomatous samples.

Morphologic Analysis

To determine the cellular localization of different complement components and regulators, immunoperoxidase labeling and double-immunofluorescence labeling were performed using histologic sections from human donor eyes. All procedures were similar to those previ-

ously described.^{10,12,13,23} Monoclonal antibodies to C3b (1:200; Genway Biotech, San Diego, CA), C1q, C5b-9, CD35, CD59 (1:100; Abcam), and CFH (1:200; Lifespan Biosciences) served as primary antibody. In addition, polyclonal antibodies against neuron-specific nuclear protein (NeuN; 1:200; Abcam), a neuronal marker, or Brn-3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), an RGC marker, were used to identify RGCs, and a polyclonal antibody to glial fibrillary acidic protein (GFAP; 1:200; Santa Cruz Biotechnology) was used to identify macroglia during double-immunofluorescence labeling. A biotinylated IgG (1:400; Millipore/Chemicon, Billerica, MA) was used as the secondary antibody for immunoperoxidase labeling and hematoxylin for counterstaining. For double-immunofluorescence labeling, a mixture of Alexa Fluor 488- or 568-conjugated species-specific IgG (1:400; Molecular Probes) was used for secondary antibody incubation. For each procedure, at least four histologic sections were used from each eye, including those obtained from the superior and inferior halves of the retina. All slides subjected to immunohistochemical analysis were masked for the identity and diagnosis of donors before their immunolabeling. Double immunolabeling of cultured cells grown on coverslips in triplicate wells was performed as previously described^{12,18,19} using the same antibodies to CFH or cell markers described. Negative controls were performed by replacing the primary antibody with serum. In addition, slides used for double-immunofluorescence labeling were incubated with each primary antibody, followed by the inappropriate secondary antibody to determine that each secondary antibody was specific to the species it was made against.

RESULTS

Differential Expression of Complement Components and Regulators in the Glaucomatous Human Retina

Proteomic analysis of human retinal samples obtained from donor eyes with or without glaucoma detected the expression

and differential regulation of several complement components. As listed in Table 1, identified proteins included different complement components (C1s, C1r, C1q, C3, C4b, C7-9) and receptors (CR1, CR2, C5aR). On the basis of the label-free quantitative LC-MS/MS analysis, expression levels of some complement components were higher in glaucomatous samples than in controls, or some of them were detectable only in glaucomatous retinas. Besides the proteins involved in the classical pathway of complement activation, differentially expressed proteins in the glaucomatous human retina also included those linked to the lectin pathway, such as mannan-binding lectin serine proteases 1 and 2, C-type lectin, and galectin. LC-MS/MS analysis also identified some complement regulatory molecules in the retinal proteome. In addition to CR1 (CD35), identified regulatory proteins included CFH-related 4, C4 binding protein, and clusterin. Despite an overall prominent difference between glaucomatous and nonglaucomatous samples, glaucomatous donor eyes exhibited individual differences in the increased expression of different complement components. However, the data presented in Table 1 were consistent in at least six glaucomatous samples for each of the listed proteins. Figure 1 shows the integration of identified proteins into canonical pathways of complement activation using bioinformatics analysis tools (Ingenuity Pathways Analysis).

As presented in Table 1, the label-free quantitative analysis detected a downregulation of a CFH-related complement regulatory protein. This observation motivated us to determine the expression of CFH protein by Western blot analysis using a specific antibody. As shown in Figure 2, findings of Western blot analysis supported decreased expression of CFH in glaucomatous human retinas. Quantitative analysis of Western blot analysis detected a more than two-fold decrease in the expression level of CFH in 7 of 10 glaucomatous samples compared

TABLE 1. Proteomic Analysis of Complement Activation in Human Glaucoma

RefSeq Accession	Protein Acronym	Fold Change
NP_958850	Complement component 1s	∞
NP_001724	Complement component 1r	∞
NP_115532	C1q domain containing 1 isotope S	∞
NP_000055	Complement component 3	∞
NP_000583	Complement component 4b	0.9
NP_000578	Complement component 7	1.2
NP_000553	Complement component 8 alpha	∞
NP_000057	Complement component 8 beta	1.1
NP_001728	Complement component 9	1.4
NP_000642	Complement component (3b/4b) receptor-1; CD35	1.1
NP_001868	Complement component receptor 2	1.2
NP_001727	Complement component 5a receptor	∞
NP_001870	Mannan-binding lectin serine protease 1	∞
NP_631947	Mannan-binding lectin serine protease 2	∞
NP_005743	C-type lectin, superfamily member 1	2.9
NP_982297	Lectin, galactoside-binding	2.6
NP_006675	Complement factor H-related 4	-2.4
NP_000706	Complement component 4 binding protein alpha	-0.9
NP_001822	Clusterin isoform 1	-1.4
NP_000053	C1 inhibitor, SERPING 1	1.6

Quantitative LC-MS/MS analysis used retinal protein samples obtained from human donor eyes with (n:10) or without (n:10) glaucoma. Identified proteins included complement components (C1s, C1r, C1q, C3, C4b, C7-9) and receptors (CR1, CR2, C5aR) and some complement regulatory molecules, such as CFH-related 4, C4 binding protein, clusterin, and the C1 inhibitor SERPING 1. In addition to the proteins involved in the classical pathway of complement activation, differentially expressed proteins in the glaucomatous human retina included those linked to the lectin pathway, such as mannan-binding lectin serine proteases 1 and 2, C-type lectin, and galectin. Note that some of the identified proteins (with a fold change shown as ∞) were detectable only in glaucomatous samples. However, glaucomatous donor eyes exhibited individual differences in the increased expression of different complement components; the data reflect at least six glaucomatous samples for each of the listed proteins.

Proteomic findings were also supported by the findings of immunohistochemical analysis in an additional group of human donor eyes. Cellular localization of different complement components and regulators were determined in histologic sections of the retina using specific antibodies. Glaucomatous human retinas exhibited more prominent immunolabeling for the complement components C1q and C3b and the membrane attack complex that is made up of complement components C5b, C6, C7, C8, and multiple C9 molecules (Fig. 3). Increased complement immunolabeling of the glaucomatous human retina was most prominent in inner layers, including the RGCs and inner plexiform layers. Increased immunolabeling was detectable in all donor eyes examined; however, this increase was not uniform and exhibited regional as well as individual differences.

Cellular localization studies also analyzed retinal immunolabeling for complement regulators CD35 and CD59. Although

FIGURE 2. Differential regulation of CFH expression in human glaucoma. CFH expression was determined by Western blot analysis of retinal protein samples obtained from 10 human donor eyes with glaucoma and compared with 10 age-matched control eyes without glaucoma. After normalization to β -actin, average band intensities were compared between control and glaucomatous samples. This comparison detected a significant decrease in CFH expression in glaucomatous retinas compared with controls (Mann-Whitney rank sum test; $P < 0.001$). When the average normalized intensity value obtained from control samples was used to calculate the fold change in CFH expression, 7 of 10 glaucomatous samples exhibited a greater than two-fold decrease.

CD35 was detected in the human retina by LC-MS/MS analysis, CD59 (membrane attack complex inhibiting protein) was not detected by proteomic analysis. However, based on previous studies reporting the localization of this complement regulatory protein in the inner retina, including RGCs,^{24,25} double-immunofluorescence labeling used a specific antibody to CD59 to determine the immunolabeling pattern in human glaucoma. Faint immunolabeling was detectable for CD59 in human retinas. In addition to nonneuronal cells, some NeuN-positive neurons in the RGC layer exhibited CD59 immunolabeling. However, retinal CD35 immunolabeling was localized mainly to NeuN-negative non-neuronal cells (Fig. 4). Neither CD35 nor CD59 immunolabeling exhibited a prominent alteration between glaucomatous and nonglaucomatous eyes.

Thus, proteomic analysis and tissue immunolabeling collectively supported the expression and differential regulation of different complement components and regulators in human glaucoma. Bioinformatics analysis of the mass spectrometric data also established protein interaction networks associated with complement regulation. Figure 5 exemplifies a high-probability network that was populated by the Ingenuity Pathways Analysis System. By highlighting the molecular pathways, this extended network provides guidelines for ongoing research to better understand the regulation and dysregulation of complement activation in human glaucoma.

Downregulation of a Major Complement Regulator, Complement Factor H, by Oxidative Stress In Vitro

To determine whether CFH expression is regulated by oxidative stress, in vitro experiments were performed using H_2O_2 treatment. These experiments used cocultures of selected RGCs and macroglial cells to better simulate in vivo conditions and astrocyte-derived signals involved in complement regulation.⁸ Both macroglia (positive for GFAP) and RGCs (positive for NeuN and Brn-3) exhibited CFH immunolabeling in these cocultures (Fig. 6).

As shown in Figure 6, treatment of cocultures with H_2O_2 resulted in a prominent downregulation of CFH expression assessed by quantitative Western blot analysis and by a de-

crease in the number of surviving cells (Mann-Whitney rank sum test; $P = 0.01$). H_2O_2 -treated cells exhibited a significant decrease in CFH expression compared with untreated controls (Mann-Whitney rank sum test; $P < 0.01$). Downregulation of CFH expression was parallel to increased HNE immunolabeling in these cells, reflecting oxidative stress-induced secondary modification by lipid peroxidation products. To determine whether the CFH downregulation detected in H_2O_2 -treated cells was related to oxidative stress or simply represented a cellular response to cell death, additional cocultures were treated with staurosporine to induce cell death. Despite a similar rate of cell death (Mann-Whitney rank sum test; $P > 0.05$), no prominent downregulation in CFH expression was detectable in staurosporine-treated cells (Fig. 6).

To validate the relevance of these in vitro observations to human glaucoma, human retinal protein samples were also examined for oxidative stress. Western blot analysis detected increased HNE immunolabeling in glaucomatous samples compared with controls (Mann-Whitney rank sum test; $P < 0.001$), reflecting protein modification induced by byproducts of oxidative stress in the glaucomatous human retina (Fig. 7).

Thus, the findings of in vitro experiments supported that the exposure of retinal cells to H_2O_2 -induced oxidative stress leads to the downregulation of CFH expression. The oxidative stress detected in glaucomatous human retinas may similarly constitute a risk factor for the altered regulation of CFH expression in these eyes.

DISCUSSION

Expression of Complement Components in the Glaucomatous Human Retina Supporting the Classical and the Lectin Pathways of Complement Activation

Our proteomic analysis of human retinal protein samples coupled with the immunohistochemical analysis of human retinas revealed that the glaucomatous human retina can synthesize various complement components involved in the classical and the lectin pathways. Detected expression of a full lytic com-

FIGURE 3. Immunohistochemical analysis of the cellular localization of complement components in the human retina. Consistent with proteomic findings, histologic sections of the glaucomatous human retina exhibited prominent immunoperoxidase labeling for the complement components C1q and C3b and the membrane attack complex C5b-9. However, a decrease was detectable in immunolabeling of the glaucomatous retina for the complement regulatory protein, CFH. Immunolabeling for different complement components was most prominent in the inner retina, including primarily the RGCs and inner plexiform layers. The *bottom* panels show negative controls, in which the first antibody was replaced with serum. gc, retinal ganglion cells layer; in, inner nuclear layer; on, outer nuclear layer. Scale bar, 100 μ m.

plement system and complement receptors, along with the differential regulation of complement inhibition, strongly suggest that the complement system is activated during neurodegenerative injury in human glaucoma. However, it remains unclear whether complement activation reflects a physiological complement-mediated tissue clearance process or a complement-mediated component of the neurodegenerative injury.

The complement system is known to be a powerful part of the innate immune defense to eliminate infections. However, it has become clear that not only molecular patterns on invading pathogens but also unwanted host cells and cell debris can be recognized by the complement system to enhance phagocytosis. Different pathways of complement activation, including the classical, lectin, and alternative pathways, represent a cas-

FIGURE 4. Immunohistochemical analysis of the cellular localization of CFH expression in the human retina. Images show double-immunofluorescence labeling of the RGC layer in human retina sections. Used antibodies were a specific antibody to CFH (*red*) and antibodies to different cell markers (*green*), GFAP (an astrocyte marker), Brn-3 (a RGC marker), or NeuN (a nonspecific neuronal marker). (A) In the control retina, CFH immunolabeling was detectable in both GFAP-positive astrocytes and GFAP-negative neurons in the RGC layer. (B) In glaucomatous eyes, colocalization of CFH and GFAP was similar; however, neuronal CFH immunolabeling exhibited a prominent decrease. Note that the *red* corresponding to GFAP-negative neurons in the merged image in (A) decreased in (B). (C, D) CFH and Brn-3 double immunolabeling. Lower magnification images in these panels support a prominent decrease in CFH immunolabeling of multiple Brn-3-positive RGCs in glaucoma. Consistently, in the higher magnification image in (E), NeuN-positive neurons in the RGC layer (*arrow*) exhibit prominent immunolabeling for CFH in the control retina. However, no CFH immunolabeling is detectable in the NeuN-positive RGC (F), whereas NeuN-negative glial cells in the same glaucomatous retina still exhibit immunolabeling for CFH. (G, H) Double-immunofluorescence labeling of the RGC layer in glaucomatous eyes using antibodies to CD35 or CD59 (*red*) and a neuronal marker, NeuN (*green*). Although both neuronal and nonneuronal cells exhibited CD59 immunolabeling, CD35 immunolabeling was detectable primarily on NeuN-negative nonneuronal cells. Scale bars: 50 μ m (A, B, E-H); 150 μ m (C, D).

FIGURE 5. Bioinformatics analysis of complement regulation. Bioinformatics analysis of the mass spectrometric data (using the Ingenuity Pathways Analysis System) established extended networks of signaling molecules associated with complement regulation in glaucoma. In this extended high-probability network, *blue* shows the proteins out of thousands of proteins identified in the human retinal proteome. Detailed information for abbreviated proteins is available at <http://www.ncbi.nlm.nih.gov/protein>.

cade of proteolytic cleavages. C1q can start the classic complement activation pathway by binding to the Fc-region of immunoglobulins but can also bind to a wide variety of molecules, thereby initiating the complement cascade for their clearance.²⁶ Although complement opsonins promote phagocytosis by the glia bearing complement receptors, complement anaphylatoxins initiate local proinflammatory responses. Complement also participates in host defense by triggering the formation of the terminal membrane attack complex, which permits uncontrolled ion fluxes, cell swelling, and osmotic lysis. Interestingly, the terminal complement pathway can also result in apoptosis in a caspase-dependent pathway.²⁷ In addition, receptor-dependent and -independent signals transduced by complement components play important roles in immunoregulation by determining the activation thresholds of T and B lymphocytes.^{26,28,29} Thus, after sensing danger signals from dying RGCs, the complement system is able to respond directly and indirectly by activating innate and adaptive immune responses in glaucoma.

It has been increasingly reported in recent years that C1q produced by glia and neurons is particularly abundant in areas of tissue damage and is involved in the clearance of cell debris and toxic components in the CNS. Complement gene transcription and protein synthesis have also been detected in the retina and optic nerve.⁵⁰ Previous histopathologic studies of human tissues and *in vivo* studies using animal models have demonstrated that different complement components, including C1q and C3, are also synthesized in glaucomatous retinas.^{6,7} In addition, membrane attack complex has been shown to be formed in the RGC layer of both human and rat glaucoma.⁷ Data obtained from gene array analysis also support increased expression of complement components in the retina in monkey,³¹ rat,³² and mouse³³ glaucoma. It seems possible that increased autoantibodies detected in the serum^{34,35} and retinal parenchyma³⁶ of the patients with glaucoma can initiate the classical complement pathway through interaction with C1q.

In addition to the classical pathway of complement activation, our proteomic findings support the possibility of comple-

ment activation in human glaucoma through the lectin pathway. Although the classical pathway is initiated after C1q binding, the lectin pathway is initiated by binding of the recognition molecule mannan-binding lectin (MBL), which, through activation of the MBL-associated serine proteases MASP1 and MASP2, forms an enzyme leading to C3 cleavage similar to the classical pathway.³⁷ MBL that belongs to the collectins family of pattern recognition receptors containing C-type lectins^{38,39} recognizes carbohydrate patterns found on the surfaces of a large number of pathogenic microorganisms but also interacts with structures exposed on dying cells and promotes their noninflammatory elimination.⁴⁰ Different families of lectins, including C-type lectins and galectins, exhibit important links in the modulation of the immune response.^{41–43} Given that the lectin pathway is triggered by the recognition of particular carbohydrates, it would be interesting to determine whether the carbohydrate-containing macromolecules, such as glycosaminoglycans⁴⁴ and advanced glycation end products⁴⁵ (production of which is increased in glaucoma^{13,35}) activate this pathway for their clearance.

Apparently, dying RGCs during glaucomatous neurodegeneration can be targeted and removed through complement-mediated processes involving reactive glia. During apoptosis, several active processes ensure the fast removal of potentially toxic and proinflammatory content of the dying cell to prevent immunization with autoantigens. Among various alterations in the intracellular and extracellular composition of an apoptotic cell to enhance its uptake by phagocytes, one is their increased ability to bind complement-initiating molecules, such as C1q^{46–48} and MBL.⁴⁹ For example, phosphatidylserine is one of the C1q ligands on apoptotic cells.⁵⁰ Thus, complement activation, as a necessary step, can help tissue cleaning and remodeling, minimize the activation and duration of inflammation, and promote neuronal survival. However, complement activation to an inappropriate extent, because of either increased local biosynthesis or insufficient regulation, or both, has also been implicated as a factor in the exacerbation and propagation of tissue injury in neurodegenerative diseases.^{51–53} Similarly, although complement activation detected

FIGURE 6. In vitro experiments determining the regulation of CFH expression by oxidative stress. (A) Phase-contrast image of the cocultured RGCs and macroglia. (B) Both GFAP-positive macroglia and NeuN- and Brn-3-positive RGCs exhibited CFH immunolabeling in these cocultures. (C) Treatment of cocultures with staurosporine (100 nM) or H_2O_2 (50 μ M) for 24 hours resulted in a significant decrease in the number of surviving cells (Mann-Whitney rank sum test; $P = 0.003$ and $P = 0.01$, respectively). The survival rate was expressed as the percentage of the total cell number in control wells. (D) Quantitative Western blot analysis. Retinal cells exposed to H_2O_2 -induced oxidative stress exhibited a significant decrease in CFH expression (Mann-Whitney rank sum test; $P < 0.01$), which was parallel to a prominent increase in HNE adducts. However, CFH expression did not prominently change in staurosporine-treated cells that exhibited no prominent HNE modifications. Data represent at least three independent experiments and are presented as mean $\pm$ SD.

in human glaucoma may serve to support the removal of apoptotic cell debris, if uncontrolled, it may also damage surrounding RGCs through lytic or sublytic injury. On the other hand, with respect to the ability of antibodies to bind C1q, activation of the complement cascade may be associated with the internalization and potential destructive roles of autoantibodies proposed in glaucoma.⁵ Whether the complement system merely flags dying cells and cell debris for enhanced phagocytosis or also contributes to immune system dysregulation in glaucoma should be further studied.

Complement Regulatory Protein Expression in Human Glaucoma

In addition to different complement components, we detected complement regulatory proteins in the human retina. It is increasingly evident that because excess cytolytic activity of the complement system may lead to tissue injury and inflammation, intrinsic regulatory mechanisms promote safe disposal of cell debris and avoid collateral damage. Combined actions of cell surface and fluid phase complement inhibitors provide an intrinsic regulatory mechanism to protect self-cells from complement-mediated lysis.^{54,55} In humans, a number of membrane-bound complement inhibitors include decay-accelerating factor (CD55), membrane cofactor protein (CD46), CD59, and complement receptor 1 (CD35).^{24,25,56} Similar to other

brain⁵¹ and eye^{24,57} tissues, RGCs and axons acquire complement inhibitor molecules as a way to remain protected against excessive complement attack after injury.³⁰ However, there is evidence suggesting that in contrast to astrocytes and microglia, which express high levels of complement inhibitors and are well protected from complement-mediated damage, brain neurons express low levels of complement inhibitors and are highly susceptible to complement attack.^{58,59} Our LC-MS/MS analysis detected CD35 expression in the human retina, and tissue immunolabeling demonstrated its localization mainly to nonneuronal cells. CD59 was not detected by proteomic analysis; however, consistent with previous observations,^{24,25} double-immunolabeling studies indicated its localization to neuronal and nonneuronal cells. Neither proteomic nor immunohistochemical analysis detected a prominent alteration in the expression of these complement regulators in human glaucoma.

In addition to these membrane-bound complement inhibitors, proteomic analysis of the human retina detected fluid-phase complement inhibitors, including a CFH-related protein, C4 binding protein, and clusterin. These regulatory molecules, which have previously been shown to be present in the retina and the optic nerve,^{30,60} do not completely block complement activation but are sufficient to prevent massive cell lysis and inflammation.²⁶ Findings of Western blot analysis and immu-

FIGURE 7. Oxidative stress in the glaucomatous human retina. Western blot analysis of human retinal protein samples obtained from 10 donors with glaucoma and 10 age-matched controls without glaucoma detected a prominent increase in HNE immunolabeling of glaucomatous samples compared with controls (Mann-Whitney rank sum test, $P < 0.001$). When the average β -actin-normalized intensity value obtained from control samples was used to calculate the fold change in HNE immunolabeling, all glaucomatous samples exhibited an over two-fold increase.

nohistochemistry supported proteomic findings that CFH is constitutively expressed in retinal cells, including RGCs. Because of its affinity for C3b, this regulator protein prevents the assembly of the C3/C5-convertase (C3bBb/C3bC3bBb) of the alternative pathway by multiple mechanisms.^{61–64} In addition to serving as a predominant regulator in the alternative complement pathway, CFH may affect the complement activation initiated by the classical pathway because it inhibits the alternative pathway serving as an amplification loop.⁶⁵ By virtue of its cofactor activity for C3b degradation, CFH can also restrict the assembly of the C3/C5-convertase (C4bC2a/C3bC4bC2a) of the classical pathway.⁶⁶ CFH-related proteins (such as CFH-related 4 detected in our proteomic analysis) also function as inhibitors of the complement pathway by blocking C5 convertase activity and interfering with C5b surface deposition and MAC formation, thereby controlling complement activation in a sequential manner to CFH function.⁶⁷ An important observation of this study was a trend toward downregulation of CFH expression in the glaucomatous human retina. Although the expression of classical pathway components appears to represent a relatively nonspecific response to tissue injury in glaucoma, this alteration in complement regulation would leave RGCs more vulnerable to complement-mediated lysis because insufficient inhibition of the downstream portions of the complement cascade may lead to the formation of a cytolytic membrane attack complex, as also supported by immunohistochemical findings.

Endogenous ligands of C1q, including apoptotic cells, can also bind fluid-phase complement inhibitors to prevent excessive complement activation. Dying cells first downregulate

membrane-bound complement inhibitors to signal for phagocytosis. During overwhelming apoptosis or insufficient phagocytosis, apoptotic cells remaining in tissues for a longer time, so-called late apoptotic cells, may acquire the ability to bind complement activators, such as C1q and MBL, thereby initiating the complement cascade for rapid removal of cell debris.⁶⁸ Concomitant to this, cells acquire fluid-phase complement inhibitors, such as CFH, that compensate for the downregulation of membrane-bound complement regulatory proteins and allow enhanced complement-mediated recognition for phagocytosis while preventing overt inflammation because of the release of C5a and assembly of the membrane attack complex.⁶⁹ Therefore, the CFH downregulation that we detected in the glaucomatous human retina does not seem to be an expected component of the cell death-related tissue cleaning process but appears to facilitate collateral complement attack and inflammatory insult. This is supported by the findings of a recent study in which exogenous administration of CFH in an experimental model of autoimmune encephalomyelitis has resulted in a significant decrease in clinical score and inflammation by protecting neurons from complement opsonization, axonal injury, and leukocyte infiltration.⁷⁰

Thus, complement activation is under the tight control of complement inhibitors, and, depending on the balance between activation and regulatory inhibition, the final outcome may either be well-balanced complement activation necessary for tissue cleaning and healing or uncontrolled complement attack leading to collateral cell lysis, inflammation, and risk of autoimmunity.²⁶ Dysregulation of complement activation has been associated with multiple autoimmune diseases, and the

disease-associated mutations identified in several complement components and regulators include genomic variations of the CFH gene, as reported in patients with AMD.⁷¹⁻⁷⁴ The precise role of complement in the etiology of these diseases is unresolved, but it seems clear that excess complement attack and local inflammation can exacerbate neuronal loss and that complement inhibition often results in neuroprotection,⁵² as evident in RGC injuries.^{8,75} Our findings suggest that imbalances in complement regulation may also contribute to the progression of neurodegenerative injury in glaucoma.

Among the 20 donor eyes used in our proteomic analysis, four glaucomatous and four nonglaucomatous eyes (samples 7, 8, 9, 10; Figs. 2, 7) had AMD. However, we think this should not have affected our protein samples obtained from the peripheral retina of the macular region. In addition, AMD is not expected to cause any alteration in the inner retinal neurons, and AMD-related alterations in CFH expression have been associated primarily with the retinal pigment epithelium, which was not present in our samples. Concerning the possibility of genomic variations of the CFH gene in these donors, our Western blot data did not reveal a detectable difference in CFH protein expression between the donors with or without macular degeneration. However, it would be interesting to determine whether patients with glaucoma exhibit similar genomic variations of the CFH gene, although our present data support oxidative stress-related epigenetic factors in the altered regulation of CFH expression in glaucoma. It is also important to clarify how such coexisting conditions determine individual differences in susceptibility to glaucomatous injury. Because of the retrospective nature of our data collection, we considered that the assessment of a relationship between the proteomic findings and clinical variables would not be precisely informative. It should be recognized that despite their unique importance, studies using human donor tissues may be challenging because of the retrospective nature of data collection, difficulties excluding other disease conditions or treatment effects, and the possibility of perimortem tissue alterations. However, we were careful to minimize such confounding factors, and the utilized tissues of glaucomatous and nonglaucomatous donors were matched for donor age, cause of death, postmortem period, and clinical detection of macular degeneration. We hope that in vivo studies using transgenic models will be able to quantitatively assess neuronal damage and its relationship to complement regulation in glaucoma.

Role of Oxidative Stress in the Regulation of Complement Activation

Findings of this study support that although complement activation may serve as an intrinsic signal for the elimination of dying RGCs in glaucoma, oxidative stress-induced downregulation of CFH may lead to increased vulnerability of adjacent neurons to complement-mediated injury. The downregulated expression of CFH we detected in the glaucomatous human retina could be attributed to the decreasing number of CFH-expressing neurons in glaucoma. However, findings of immunohistochemical analysis also supported decreased CFH immunolabeling in surviving RGCs in glaucomatous eyes compared with controls. In addition, in vitro experiments provided supportive evidence that exposure to oxidative stress leads to the downregulation of CFH expression. The oxidative stress we detected in our glaucomatous human retinal samples further supports the relevance of these in vitro observations to human disease.

As discussed, among the human donor eyes used in our proteomic analysis, four glaucomatous and four nonglaucomatous eyes had AMD. It should be recognized that both glaucoma and AMD are age-related diseases and have been associ-

ated with oxidative stress. However, based on Western blot analysis, no prominent difference was detectable in the oxidative stress detected in donors with glaucoma with or without AMD.

Consistent with our findings, oxidative stress has been found to downregulate CFH expression in human brain neurons⁷⁶ and in retinal pigment epithelial cells.^{77,78} Interestingly, complement activation plays an important role in the inflammatory process after oxidative stress,⁷⁹ and both the classical⁸⁰ and the lectin pathways⁸¹ of complement activation have been implicated in mediating tissue injury after oxidative stress of endothelial cells. Thus, in addition to many other immunostimulatory consequences,³ oxidative stress may play a role as a risk factor contributing to the dysregulation of complement activation in glaucoma. Ongoing studies should further clarify how oxidative stress translates into a stimulus affecting complement regulation.

In conclusion, proteomic findings in the human retina have significantly expanded the list of complement components and regulators involved in glaucomatous neurodegeneration. Findings of this study also suggest that alterations in the physiological balance between complement activation and intrinsic regulation under glaucomatous stress conditions may have an important impact on the progression of neurodegenerative injury. Ongoing studies should further clarify the importance of the complement system in glaucoma and identify the specific mechanisms of interactions among complement proteins, immune system cells, and immune mediators so that manipulation of the balance between complement activation and inhibition can represent an additional therapeutic opportunity for glaucoma patients.

References

1. Tezel G, Wax MB. *Glaucoma Chem Immunol Allergy*. 2007;92:221-227.
2. Wax MB, Tezel G. Immunoregulation of retinal ganglion cell fate in glaucoma. *Exp Eye Res*. 2009;88:825-830.
3. Tezel G. The role of glia, mitochondria, and the immune system in glaucoma. *Invest Ophthalmol Vis Sci*. 2009;50:1001-1012.
4. Wax MB, Tezel G, Yang J, et al. Induced autoimmunity to heat shock proteins elicits glaucomatous loss of retinal ganglion cell neurons via activated T-cell-derived fas-ligand. *J Neurosci*. 2008;28:12085-12096.
5. Tezel G, Wax MB. The mechanisms of hsp27 antibody-mediated apoptosis in retinal neuronal cells. *J Neurosci*. 2000;20:3552-3562.
6. Stasi K, Nagel D, Yang X, et al. Complement component 1Q (C1Q) upregulation in retina of murine, primate, and human glaucomatous eyes. *Invest Ophthalmol Vis Sci*. 2006;47:1024-1029.
7. Kuehn MH, Kim CY, Ostojic J, et al. Retinal synthesis and deposition of complement components induced by ocular hypertension. *Exp Eye Res*. 2006;83:620-628.
8. Stevens B, Allen NJ, Vazquez LE, et al. The classical complement cascade mediates CNS synapse elimination. *Cell*. 2007;131:1164-1178.
9. Tezel G, Yang X, Luo C, Peng Y, Sun SL, Sun D. Mechanisms of immune system activation in glaucoma: oxidative stress-stimulated antigen presentation by the retina and optic nerve head glia. *Invest Ophthalmol Vis Sci*. 2007;48:705-714.
10. Tezel G, Yang X, Luo C, et al. Hemoglobin expression and regulation in glaucoma: insights into retinal ganglion cell oxygenation. *Invest Ophthalmol Vis Sci*. 2010;51:907-919.
11. Tezel G, Yang X, Cai J. Proteomic identification of oxidatively modified retinal proteins in a chronic pressure-induced rat model of glaucoma. *Invest Ophthalmol Vis Sci*. 2005;46:3177-3187.
12. Yang X, Luo C, Cai J, Pierce WM, Tezel G. Phosphorylation-dependent interaction with 14-3-3 in the regulation of bad trafficking in retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2008;49:2483-2494.

13. Tezel G, Luo C, Yang X. Accelerated aging in glaucoma: immuno-histochemical assessment of advanced glycation end products in the human retina and optic nerve head. *Invest Ophthalmol Vis Sci.* 2007;48:1201-1211.
14. Cummins TD, Barati MT, Coventry SC, Salyer SA, Klein JB, Powell DW. Quantitative mass spectrometry of diabetic kidney tubules identifies GRAP as a novel regulator of TGF-beta signaling. *Biochim Biophys Acta.* 2010;1804:653-661.
15. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem.* 2002;74:5383-5392.
16. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* 2003;75:4646-4658.
17. Powell DW, Weaver CM, Jennings JL, et al. Cluster analysis of mass spectrometry data reveals a novel component of SAGA. *Mol Cell Biol.* 2004;24:7249-7259.
18. 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;20:8693-8700.
19. Tezel G, Yang X. Caspase-independent component of retinal ganglion cell death, in vitro. *Invest Ophthalmol Vis Sci.* 2004;45:4049-4059.
20. Tezel G, Yang X. Comparative gene array analysis of TNF-alpha-induced MAPK and NF-kB signaling pathways between retinal ganglion cells and glial cells. *Exp Eye Res.* 2005;81:207-217.
21. Tezel G. Proteomics in defining pathogenic processes involved in glaucomatous neurodegeneration. In: Tombran-Tink J, Barnstable J, Shields MB, eds. *Ophthalmology Research Series, Glaucoma: Mechanisms of the Glaucomas: Disease Processes and Therapeutic Modalities.* Totowa, NJ: Humana Press Inc.; 2008;425-441.
22. Ullian EM, Sapperstein SK, Christopherson KS, Barres BA. Control of synapse number by glia. *Science.* 2001;291:657-661.
23. Tezel G, Wax MB. Hypoxia-inducible factor 1alpha in the glaucomatous retina and optic nerve head. *Arch Ophthalmol.* 2004;122:1348-1356.
24. Bora NS, Gobleman CL, Atkinson JP, Pepose JS, Kaplan HJ. Differential expression of the complement regulatory proteins in the human eye. *Invest Ophthalmol Vis Sci.* 1993;34:3579-3584.
25. Vogt SD, Barnum SR, Curcio CA, Read RW. Distribution of complement anaphylatoxin receptors and membrane-bound regulators in normal human retina. *Exp Eye Res.* 2006;83:834-840.
26. Sjoberg AP, Trouw LA, Blom AM. Complement activation and inhibition: a delicate balance. *Trends Immunol.* 2009;30:83-90.
27. Nauta AJ, Daha MR, Tijssma O, van de Water B, Tedesco F, Roos A. The membrane attack complex of complement induces caspase activation and apoptosis. *Eur J Immunol.* 2002;32:783-792.
28. Bohana-Kashtan O, Ziporen L, Donin N, Kraus S, Fishelson Z. Cell signals transduced by complement. *Mol Immunol.* 2004;41:583-597.
29. Kohl J. Self, non-self, and danger: a complementary view. *Adv Exp Med Biol.* 2006;586:71-94.
30. Ohlsson M, Bellander BM, Langmoen IA, Svensson M. Complement activation following optic nerve crush in the adult rat. *J Neurotrauma.* 2003;20:895-904.
31. Miyahara T, Kikuchi T, Akimoto M, Kurokawa T, Shibuki H, Yoshimura N. Gene microarray analysis of experimental glaucomatous retina from cynomolgus monkey. *Invest Ophthalmol Vis Sci.* 2003;44:4347-4356.
32. Ahmed F, Brown KM, Stephan DA, Morrison JC, Johnson EC, Tomarev SI. Microarray analysis of changes in mRNA levels in the rat retina after experimental elevation of intraocular pressure. *Invest Ophthalmol Vis Sci.* 2004;45:1247-1258.
33. Steele MR, Inman DM, Calkins DJ, Horner PJ, Vetter ML. Microarray analysis of retinal gene expression in the DBA/2J model of glaucoma. *Invest Ophthalmol Vis Sci.* 2006;47:977-985.
34. Tezel G, Seigel GM, Wax MB. Autoantibodies to small heat shock proteins in glaucoma. *Invest Ophthalmol Vis Sci.* 1998;39:2277-2287.
35. Tezel G, Edward DP, Wax MB. Serum autoantibodies to optic nerve head glycosaminoglycans in patients with glaucoma. *Arch Ophthalmol.* 1999;117:917-924.
36. Wax MB, Tezel G, Edward PD. Clinical and ocular histopathological findings in a patient with normal-pressure glaucoma [see comments]. *Arch Ophthalmol.* 1998;116:993-1001.
37. Petersen SV, Thiel S, Jensenius JC. The mannan-binding lectin pathway of complement activation: biology and disease association. *Mol Immunol.* 2001;38:133-149.
38. Hansen S, Holmskov U. Structural aspects of collectins and receptors for collectins. *Immunobiology.* 1998;199:165-189.
39. Sheriff S, Chang CY, Ezekowitz RA. Human mannose-binding protein carbohydrate recognition domain trimerizes through a triple alpha-helical coiled-coil. *Nat Struct Biol.* 1994;1:789-794.
40. Nauta AJ, Raaschou-Jensen N, Roos A, et al. Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur J Immunol.* 2003;33:2853-2863.
41. Avni O, Pur Z, Yefenof E, Baniyash M. Complement receptor 3 of macrophages is associated with galectin-1-like protein. *J Immunol.* 1998;160:6151-6158.
42. Vasta GR, Quesenberry M, Ahmed H, O'Leary N. C-type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. *Dev Comp Immunol.* 1999;23:401-420.
43. Kang YS, Do Y, Lee HK, et al. A dominant complement fixation pathway for pneumococcal polysaccharides initiated by SIGN-R1 interacting with C1q. *Cell.* 2006;125:47-58.
44. Hamad OA, Ekdahl KN, Nilsson PH, et al. Complement activation triggered by chondroitin sulfate released by thrombin receptor-activated platelets. *J Thromb Haemost.* 2008;6:1413-1421.
45. Uesugi N, Sakata N, Nangaku M, et al. Possible mechanism for medial smooth muscle cell injury in diabetic nephropathy: glycooxidation-mediated local complement activation. *Am J Kidney Dis.* 2004;44:224-238.
46. Korb LC, Ahearn JM. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J Immunol.* 1997;158:4525-4528.
47. Navratil JS, Watkins SC, Wisniewski JJ, Ahearn JM. The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. *J Immunol.* 2001;166:3231-3239.
48. Nauta AJ, Trouw LA, Daha MR, et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol.* 2002;32:1726-1736.
49. Ogden CA, deCathelineau A, Hoffmann PR, et al. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med.* 2001;194:781-795.
50. Paidassi H, Tacnet-Delorme P, Garlatti V, et al. C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. *J Immunol.* 2008;180:2329-2338.
51. Gasque P, Dean YD, McGreal EP, VanBeek J, Morgan BP. Complement components of the innate immune system in health and disease in the CNS. *Immunopharmacology.* 2000;49:171-186.
52. van Beek J, Elward K, Gasque P. Activation of complement in the central nervous system: roles in neurodegeneration and neuroprotection. *Ann N Y Acad Sci.* 2003;992:56-71.
53. Shen Y, Meri S. Yin and Yang: complement activation and regulation in Alzheimer's disease. *Prog Neurobiol.* 2003;70:463-472.
54. Liszewski MK, Farries TC, Lublin DM, Rooney IA, Atkinson JP. Control of the complement system. *Adv Immunol.* 1996;61:201-283.
55. Elward K, Gasque P. "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. *Mol Immunol.* 2003;40:85-94.
56. Kim DD, Song WC. Membrane complement regulatory proteins. *Clin Immunol.* 2006;118:127-136.
57. Sohn JH, Kaplan HJ, Suk HJ, Bora PS, Bora NS. Chronic low level complement activation within the eye is controlled by intraocular complement regulatory proteins. *Invest Ophthalmol Vis Sci.* 2000;41:3492-3502.
58. Singhrao SK, Neal JW, Rushmere NK, Morgan BP, Gasque P. Differential expression of individual complement regulators in the brain and choroid plexus. *Lab Invest.* 1999;79:1247-1259.

59. Singhrao SK, Neal JW, Rushmere NK, Morgan BP, Gasque P. Spontaneous classical pathway activation and deficiency of membrane regulators render human neurons susceptible to complement lysis. *Am J Pathol.* 2000;157:905-918.
60. Mandal MN, Ayyagari R. Complement factor H: spatial and temporal expression and localization in the eye. *Invest Ophthalmol Vis Sci.* 2006;47:4091-4097.
61. Weiler JM, Daha MR, Austen KF, Fearon DT. Control of the amplification convertase of complement by the plasma protein beta1H. *Proc Natl Acad Sci U S A.* 1976;73:3268-3272.
62. Pangburn MK, Schreiber RD, Muller-Eberhard HJ. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. *J Exp Med.* 1977;146:257-270.
63. Jokiranta TS, Zipfel PF, Hakulinen J, et al. Analysis of the recognition mechanism of the alternative pathway of complement by monoclonal anti-factor H antibodies: evidence for multiple interactions between H and surface bound C3b. *FEBS Lett.* 1996;393:297-302.
64. Kuhn S, Zipfel PF. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *Eur J Immunol.* 1996;26:2383-2387.
65. Harboe M, Ulvund G, Vien L, Fung M, Mollnes TE. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin Exp Immunol.* 2004;138:439-446.
66. Ollert MW, David K, Bredehorst R, Vogel CW. Classical complement pathway activation on nucleated cells: role of factor H in the control of deposited C3b. *J Immunol.* 1995;155:4955-4962.
67. Heinen S, Hartmann A, Lauer N, et al. Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. *Blood.* 2009;114:2439-2447.
68. Trouw LA, Blom AM, Gasque P. Role of complement and complement regulators in the removal of apoptotic cells. *Mol Immunol.* 2008;45:1199-1207.
69. Trouw LA, Bengtsson AA, Gelderman KA, Dahlback B, Sturfelt G, Blom AM. C4b-binding protein and factor H compensate for the loss of membrane-bound complement inhibitors to protect apoptotic cells against excessive complement attack. *J Biol Chem.* 2007;282:28540-28548.
70. Griffiths MR, Neal JW, Fontaine M, Das T, Gasque P. Complement factor H, a marker of self protects against experimental autoimmune encephalomyelitis. *J Immunol.* 2009;182:4368-4377.
71. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science.* 2005;308:421-424.
72. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2005;102:7227-7232.
73. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science.* 2005;308:419-421.
74. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science.* 2005;308:385-389.
75. Kuehn MH, Kim CY, Jiang B, Dumitrescu AV, Kwon YH. Disruption of the complement cascade delays retinal ganglion cell death following retinal ischemia-reperfusion. *Exp Eye Res.* 2008;87:89-95.
76. Lukiw WJ, Zhao Y, Cui JG. An NF- κ B-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. *J Biol Chem.* 2008;283:31315-31322.
77. Chen M, Forrester JV, Xu H. Synthesis of complement factor H by retinal pigment epithelial cells is down-regulated by oxidized photoreceptor outer segments. *Exp Eye Res.* 2007;84:635-645.
78. Wu Z, Lauer TW, Sick A, Hackett SF, Campochiaro PA. Oxidative stress modulates complement factor H expression in retinal pigmented epithelial cells by acetylation of FOXO3. *J Biol Chem.* 2007;282:22414-22425.
79. Collard CD, Lekowski R, Jordan JE, Agah A, Stahl GL. Complement activation following oxidative stress. *Mol Immunol.* 1999;36:941-948.
80. Collard CD, Vakeva A, Bukusoglu C, et al. Reoxygenation of hypoxic human umbilical vein endothelial cells activates the classic complement pathway. *Circulation.* 1997;96:326-333.
81. Collard CD, Vakeva A, Morrissey MA, et al. Complement activation after oxidative stress: role of the lectin complement pathway. *Am J Pathol.* 2000;156:1549-1556.