Retina

Complement Activation in the Vitreous of Patients With Proliferative Diabetic Retinopathy

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PURPOSE. A growing body of evidence points to complement dysregulation in diabetes. Early studies have indicated the presence of complement components inside the eye in patients with diabetic retinopathy, but these data have been confounded by leakage of proteins from the systemic circulation into the vitreous cavity.

METHODS. We took samples of plasma and vitreous from patients with and without proliferative diabetic retinopathy (PDR) and measured levels of 16 complement components as well as albumin. We employed a normalized ratio using local and systemic complement and albumin levels to control for vascular leakage into the vitreous cavity.

RESULTS. Before normalizing, we found significantly higher levels of 16 complement components we measured in PDR eyes compared to controls. After normalizing, levels of C4, factor B, and C5 were decreased compared to controls, while C3a and Ba levels were elevated compared to controls. We also found higher ratios of C3a/C3, C5a/C5, and Ba/factor B in PDR eyes compared to controls.

CONCLUSIONS. We found evidence of local, intraocular activation of C3, C5, and factor B. The normalized data suggest involvement of the alternative complement pathway. By showing activation of specific complement components in PDR, this study identifies targets for diagnostic and therapeutic potential.

Keywords: complement, diabetic retinopathy, vitreous

Diabetic retinopathy (DR), a serious ocular complication of diabetes, is present in more than 60% of patients with type 2 diabetes and is the leading cause of permanent visual impairment in the working-age population.[1,2] Diabetic retinopathy is classified into two broad categories: nonproliferative diabetic retinopathy (NPDR), which is less severe, and proliferative diabetic retinopathy (PDR), which confers the highest risk of severe vision loss.[3,4] Proliferative diabetic retinopathy is characterized by retinal neovascularization, the new growth of abnormal, permeable blood vessels resulting from ischemia.[5] Established risk factors for PDR include chronic hyperglycemia, increased duration of diabetes, and hypertension, as well as environmental and genetic factors that play contributing roles.[1,2,6]

The complement system normally functions as the first line of defense of the innate immune system. It is composed of three separate pathways, the classical, alternative, and mannose-binding lectin, that work in tandem to promote inflammation, opsonize pathogens, attack cell membranes, and enhance host immune response.[7] Complement activity

is balanced by free-circulating regulators and membrane-bound receptors to prevent damage to host tissues.[8] The complement system has been implicated in various diabetic disease states, including nephropathy, as well as ocular diseases such as age-related macular degeneration and uveitis.[5,9,10]

Despite the critical role of complement in the systemic inflammatory response, complement dysregulation in diabetic disease remains understudied on both a systemic and ocular level.[4,11] Moreover, there is a paucity of information on the role of this dysfunction in patients with PDR.[5,12–14] Early studies on complement in PDR eyes have indicated the presence of complement components in the vitreous cavity, but these studies have been limited by sample size and confounded by vascular leakage into the eye.[15,16] To the best of our knowledge, there are no existing studies comparing relative complement levels in both the plasma and vitreous in diabetic eyes. Furthermore, there are no studies that have controlled for leakage of circulating complement into the vitreous.

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We undertook this study to analyze samples of vitreous and compare complement levels in eyes with PDR to eyes without diabetic retinopathy. Our hypothesis was that by controlling for vascular leakage using paired vitreous and plasma samples, this study would be able to pinpoint specific complement components for diagnostic and therapeutic potential.

METHODS

This case-control study was approved by the Colorado Multiple Institutional Review Board and la Comité de Ética en Investigación de la Asociación para Evitar la Ceguera en Mexico, IAP. All samples were collected at the Asociación para Evitar la Ceguera en Mexico (APEC) in Mexico City. All research conformed to the tenets of the Declaration of Helsinki. Informed consent was obtained from the patients following explanation of the nature and possible consequences of the study. Each patient granted consent for medical chart review, collection of a plasma sample, and collection of a vitreous sample.

Data coordination, management, and statistical analysis were performed in the Division of Ophthalmic Epidemiology, Department of Ophthalmology, University of Colorado School of Medicine (UCSOM), and sample analysis was performed at Exsera BioLabs, Division of Rheumatology, UCSOM. Surgeries and sample collection were performed between July 2018 and February 2019.

Cases and Controls

All patients underwent a preoperative dilated fundus examination. Cases were diabetic patients undergoing pars plana vitrectomy (PPV) for tractional retinal detachment as a result of PDR. Controls were patients without clinical evidence of DR undergoing PPV for epiretinal membrane or macular hole.

Exclusion Criteria

Exclusion criteria were as follows: concurrent ocular procedures other than cataract surgery, comorbidities of the retina, NPDR, vitreous hemorrhage (identified before or during surgery), and treatment with laser or anti–vascular endothelial growth factor injections within the 3 months preceding surgery.

Collection and Processing of the EDTA Plasma Sample

Following phlebotomy, the EDTA tube was spun at 3000 revolutions per minute (rpm) in a cooled (4 deg Celsius [°C]) centrifuge for 10 minutes to isolate plasma. Aliquots of plasma were stored at −80°C within 30 minutes of collection. The mean ± SD time from phlebotomy to spin was 8.2 ± 2.8 minutes (range, 3–21 minutes).

Vitreous Collection

After the eye was prepped and draped in the normal sterile manner, vitrectomy trocars were placed through the pars plana in the standard fashion. The infusion cannula was left clamped to prevent sample dilution. Air was infused into the eye during core vitrectomy to maintain physiologic pressure.

During vitrectomy, at least 1 cc of undiluted vitreous was removed using a 3- or 5-cc syringe connected to the aspiration tubing of the vitrectomy probe. All samples were stored at −80°C.

Complement Factor Analysis

The frozen cryovials were transferred from APEC to Exsera BioLabs, a College of American Pathologists (CAP)/Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, for analysis. Complement factors C1q, C4, C2, MBL, C4b, C3, factor B, factor D, Properdin, C3a, C3b, Ba, factor H, factor I, C5, and C5a were measured, as well as albumin for normalization. Measurement of complement components and activation fragments was performed by two methods. The Ba and C3a levels were measured by ELISA (Quidel Corp, San Diego, CA, USA). The remaining measurements were performed by multiplex Luminex immunoassays (MilliporeSigma, Burlington, MA, USA). For both methods, Exsera had previously optimized the methods to measure the low-concentration vitreous. For both plasma and vitreous humor, the methods were optimized and validated to the level required for regulated laboratory analysis. All analyses were performed in duplicate with the resulting mean values used for analyses. For the multiplex Luminex data, the mean fluorescent intensity was the raw value, and for the ELISA analysis, the raw value was optical density. Six nonpoint, standard curves were fit with a four-parameter parametric curve fit to calculate the absolute quality in pg/mL, ng/mL, or µg/mL, as appropriate. Three quality controls (QCs) were included in each run, including at least one laboratory-developed and characterized QC. The QCs were monitored for performance. For all testing in the study, the values returned were within the required parameter of assay performance.

Statistical Analysis

Risk factors in the final analytic data set included the following: age, sex, diabetic status, body mass index (kg/m^2), history of tobacco use, and history of comorbid conditions, including treated hypertension, renal disease, and peripheral vascular disease. Descriptive statistics included basic frequencies for categorical variables and means, associated standard deviations, medians, and ranges for continuous variables. Median complement factor levels in the plasma and vitreous were compared between cases and controls. To adjust for overall leakage in the vitreous, the ratio of each complement factor in the vitreous and plasma was adjusted for albumin levels in both the vitreous and plasma, with the following normalized ratio: $\frac{Vitreous\ complement \div Vitreous\ albumin}{Plasma\ complement \div Plasma\ albumin}$. Relative complement activation ratios were also compared across groups for C3a, Ba, and C5a with the following ratio: Relative complement activation ratio = Complement factor/Parent complement (e.g., C5a/C5). Categorical variables were compared between cases and controls with the chi-square test. The Wilcoxon rank-sum test was used for all comparisons of continuous variables and complement ratios. Spearman correlation coefficients were used to assess correlations between complement levels and age. Due to the large number of statistical tests, the false discovery rate was used to adjust for multiple comparisons testing for complement comparisons. A *P* value of <0.05 was considered statistically significant throughout the analysis. SAS software (version

TABLE 1. Demographic Characteristics and Comorbid Conditions for Patient Cases With Prolific Diabetic Retinopathy (PDR) Versus Controls

Characteristic	PDR	Controls	*P* Value
Total No.	39	29	—
Age, y			
Median	54	66	<0.001
Range	40–70	53–77	
Sex			
Male	15 (38.5)	7 (24.1)	0.212
Female	24 (61.5)	22 (75.9)	
Type 2 diabetes			
Yes	39 (100)	10 (34.5)	<0.001
No	0	19 (65.5)	
Body mass index, kg/m²			
Median	28.9	28.8	0.674
Range	21.4–34.2	21.7–41.5	
Smoking			
Never	31 (79.5)	21 (72.4)	0.402
Current	3 (7.7)	1 (3.4)	
Former	5 (12.8)	7 (24.1)	
History of treated hypertension	21 (53.8)	15 (51.7)	0.862
History of renal disease	6 (15.4)	1 (3.4)	0.225
History of peripheral vascular disease	5 (12.8)	2 (6.9)	0.690

Values are presented as number (%) unless otherwise indicated.

9.4; SAS Institute, Cary, NC, USA) was used for merging laboratory and epidemiologic data and for statistical analyses.

RESULTS

Table 1 shows demographic characteristics for cases and controls. Cases were significantly younger than controls. Cases all had type 2 diabetes, whereas 34.5% of controls had diabetes without evidence of retinopathy. None of the other comorbid conditions in Table 1 were significantly different between cases and controls.

Analysis of complement levels in both the plasma and vitreous of cases and controls is shown in Table 2. In plasma samples, we found higher levels of C2 ($P = 0.048$), factor D ($P = 0.050$), Ba ($P < 0.001$), and C5 ($P = 0.045$) in cases compared with controls, whereas controls had higher plasma albumin levels ($P = 0.045$). In vitreous samples, all complement components and albumin were higher in cases compared to controls (all $P < 0.001$). Age was not correlated with any of the measured complement factors in the plasma or vitreous for cases or controls.

In Table 3, complement levels observed in the vitreous were adjusted using measured plasma albumin concentrations as described in the Methods. Following this normalization, levels of C4, factor B, and C5 in the vitreous of PDR eyes were decreased compared to controls, while C3a and Ba levels were elevated compared to controls. A subanalysis excluding diabetic controls was performed to compare cases to nondiabetic controls only, and it also showed significantly decreased C4, factor B, and C5 as well as significantly increased C3a and Ba.

Table 4 shows relative complement activation ratios in PDR cases versus controls using parent complement levels when applicable. This ratio demonstrates that PDR cases had significantly higher activation ratios of C3a/C3, Ba/B, and C5a/C5 relative to controls.

DISCUSSION

In this study, we analyzed vitreous samples from eyes with PDR and from controls without diabetic retinopathy. Using a normalized ratio to correct for vascular leakage, we identified local activation of complement factors C3a, C5a, and Ba, as well as local consumption of C4, C5, and factor B. These finding suggest activation of the alternative complement pathway in PDR.

In recent years, a growing body of literature has focused on complement inside the eye, primarily in macular degener-

TABLE 2. Complement Levels in Plasma and Vitreous for Cases With PDR Versus Controls

	Plasma			Vitreous		
Characteristic	PDR	Controls	FDR *P* Value	PDR	Controls	FDR *P* Value
Total with sample	38	27	—	33	27	—
Albumin (mg/dL)	3609 (629)/3760	4006 (295)/3980	0.045	168 (119)/127	67 (100)/18	<0.001
C1q (ng/mL)	113,142 (20,481)/111,200	110,249 (24,146)/115,820	0.963	527 (721)/312	146 (217)/91	<0.001
C4 (ng/mL)	134,761 (34,690)/133,859	127,784 (28,853)/130,216	0.698	4313 (2691)/3123	1672 (2182)/741	<0.001
C2 (ng/mL)	8419 (11,364)/5480	4461 (2443)/3942	0.048	2502 (2124)/1886	257 (414)/100	<0.001
MBL (ng/mL)	1417 (919)/1554	1112 (889)/1272	0.108	4.1 (8.1)/1.6	0.5 (0.3)/0.4	<0.001
C4b (ng/mL)	12,628 (3637)/12,062	10,958 (3193)/11,076	0.244	347 (357)/215	86 (125)/28	<0.001
C3 (ng/mL)	60,471 (17,719)/56,682	56,6904 (15,119)/57,642	0.549	10,737 (16298)/7151	3644 (7975)/436	<0.001
Factor B	149,545 (25,608)/145,694	155,232 (29,596)/149,804	0.576	3032 (1514)/2861	1393 (2069)/628	<0.001
Factor D (ng/mL)	2779 (2022)/1976	1967 (1675)/1558	0.050	388 (506)/278	97 (149)/28	<0.001
Properdin (ng/mL)	23,278 (4403)/22,757	23,210 (4022)/22,842	0.933	147 (237)/74	26 (38)/12	<0.001
C3a (ng/mL)	79 (47)/67	78 (68)/61	0.258	40 (37)/24	8.6 (17.3)/0.4	<0.001
C3b/iC3b (ng/mL)	1005 (666)/952	828 (553)/953	0.576	386 (422)/217	172 (351)/12	<0.001
Ba (ng/mL)	936 (502)/843	602 (188)/571	<0.001	212 (150)/188	59 (110)/8	<0.001
Factor H (ng/mL)	281,862 (52,412)/284,059	280,448 (54,217)/280,708	0.963	2244 (3245)/1213	537 (899)/217	<0.001
Factor I (ng/mL)	26,256 (4960)/25,538	27,139 (6321)/27,667	0.319	2148 (1394)/2135	709 (781)/374	<0.001
C5 (ng/mL)	46,987 (11,441)/45,938	41,050 (11,950)/38,429	0.045	358 (319)/284	134 (168)/75	<0.001
C5a (pg/mL)	593 (183)/500	567 (204)/510	0.602	32 (71)/18	8.7 (12.8)/2.5	<0.001

Values are presented as mean (SD)/median unless otherwise indicated. FDR, false discovery rate; MBL, mannose-binding lectin.

TABLE 3. Normalized Ratio for Cases With PDR Versus Controls Adjusted for Albumin in the Vitreous and Plasma

Characteristic	PDR Normalized Ratio	Controls Normalized Ratio	Mean Case Ratio Versus Mean Control Ratio	FDR P Value
Total with ratio	32	25	—	—
C1q	0.10 (0.10)/0.08	0.24 (0.32)/0.11	0.42	0.267
C4	0.84 (0.40)/0.74	1.90 (2.98)/1.24	0.44	0.036
C2	11.86 (9.84)/8.80	10.35 (15.01)/5.43	1.15	0.267
MBL	0.11 (0.15)/0.06	0.30 (0.61)/0.11	0.37	0.279
C4b	0.60 (0.42)/0.50	1.12 (2.29)/0.66	0.54	0.327
C3	4.30 (5.15)/3.01	3.89 (2.97)/2.71	1.11	0.618
Factor B	0.49 (0.19)/0.51	0.89 (0.53)/0.86	0.55	0.004
Factor D	4.15 (3.12)/3.14	4.13 (2.08)/4.00	1.00	0.455
Properdin	0.12 (0.12)/0.08	0.12 (0.10)/0.09	1.00	0.682
C3a	15.84 (14.12)/10.08	4.06 (4.20)/2.13	3.90	<0.001
C3b/iC3b	11.21 (13.30)/8.08	27.89 (45.18)/10.90	0.40	0.333
Ba	6.25 (3.24)/5.54	3.64 (3.05)/3.17	1.72	0.008
Factor H	0.16 (0.16)/0.12	0.17 (0.11)/0.14	0.94	0.333
Factor I	2.21 (1.61)/1.79	3.32 (2.90)/2.40	0.67	0.155
C5	0.18 (0.15)/0.16	0.72 (0.93)/0.51	0.25	0.011
C5a	1.37 (2.56)/0.90	1.73 (1.65)/1.31	0.79	0.088

Values are presented as mean (SD)/median unless otherwise indicated. Normalized ratio = (CV/AV)/(CP/AP), where
CV = each complement in the vitreous, AV = albumin in the vitreous, CP = each complement in plasma, and AP = albumin in plasma.

TABLE 4. Relative Activation Ratios for Vitreous Complement in Cases Versus Controls

Characteristic	Relative Activation Ratio* for PDR Cases	Relative Activation Ratio* for Controls	Ratio/FDR P Value,† Cases Versus Controls
Total	33	27	—
C3a/C3	0.010 (0.013)/0.004	0.002 (0.002)/0.001	5.0/<0.001
Ba/B	0.095 (0.129)/0.063	0.028 (0.039)/0.013	3.4/<0.001
C5a/C5	0.097 (0.097)/0.051	0.066 (0.082)/0.033	1.47/0.002

* Relative activation ratio = (C5a/C5) or (Ba/factor B) or (C3a/C3).
† P value of Wilcoxon rank-sum test compared to control group.

ation.[12] Despite this attention, only a few studies have examined the role of complement in one of the most common causes of blindness—diabetic retinopathy. Muramatsu et al.[15] found significantly elevated levels of C5a in the vitreous of PDR eyes, while Manoharan et al.[16] found elevated levels of factor D and C9. Cadaveric studies in diabetic eyes have shown higher levels of C3 and factor B, as well as lower levels of the complement inhibitors CD55 and CD59 in diabetic eyes.[17,18] These studies provided early evidence of complement dysregulation in PDR, but they were severely limited by small sample sizes and, more important, interference due to vascular leakage into the eye.

It is well known that patients with PDR, and those with diabetes in general, have higher serum levels of various complement components.[19–22] These elevated levels, however, are no more specific to diabetic retinopathy than they are to other systemic complications of diabetes. Because vascular leakage is a defining feature of PDR, these circulating complement components gain entry into the eye. A simple measurement of vitreous complement levels is therefore insufficient to determine whether complement inside the eye is locally produced or if it entered the vitreous cavity though leaking vasculature or by way of vitreous hemorrhage. Any sampling of vitreous complement in PDR eyes is likely to be confounded by leakage from the systemic circulation.

In collecting vitreous samples, we sought to solve this problem in two ways. First, we excluded patients with vitreous hemorrhage. Second, we corrected for vascular leakage

using a normalized ratio: $\frac{Vitreous\ complement \div Vitreous\ albumin}{Plasma\ complement \div Plasma\ albumin}$. This ratio is a simple adaptation of the Goldmann-Witmer coefficient that has long been used to differentiate between local and systemic production of antibodies found in intraocular fluid.[23] Because albumin is normally only present in the vitreous in low concentrations, higher levels indicate increased retinal vascular leakage. Using the ratio, higher levels of vitreous albumin would result in a lower corrected value of complement inside the eye. Before employing the albumin-normalized ratio, we found significant elevations in PDR eyes of every complement component we tested. After normalizing, we found that the ratios of activated fragments to their parent complement components (i.e., C5a/C5) were significantly higher in PDR eyes versus controls for C3a, C5a, and Ba. These normalized data, corrected for vascular leakage, clearly indicate local complement activation independent of systemic circulating complement levels. Taken together, the data suggest activation of the alternative pathway.

While these results clearly indicate complement activation in PDR eyes, the ultimate role of complement in the pathogenesis of PDR is less clear. Our data do not indicate whether complement plays a causative role or whether it occurs as a reaction to tissue damage from other mechanisms. Similarly, complement may simply be a byproduct of neovascularization rather than the cause of it. Because the half-life of C3a is approximately 30 minutes, the detection of local C3a in our data indicates ongoing complement activation, but it does not indicate past activity, a fact

that further limits our ability to directly attribute previous vascular damage to complement activity.[24]

We are therefore left to hypothesize about the role of complement inside the diabetic eye. By understanding the role that complement plays in causing vascular damage in other organs, it is possible to infer a similar effect inside the eye. In the glomerulus, for instance, complement has been shown to directly mediate vasculopathy via formation of the membrane attack complex (MAC).[25] Glycation of CD59, a MAC inhibitor, results in unchecked MAC activity and is thought to be a causative factor in complement-mediated vascular injury in the glomerular capillaries.[26] It is plausible that a similar MAC-mediated mechanism underlies damage to retinal vasculature in diabetes, leading to ischemia and proliferative disease. Indeed, deposition of C5b-9, the components comprising the MAC, has been observed within the vessel walls of cadaveric diabetic eyes, although it has not yet been shown to result in cell death.[27] Despite the suggestive evidence, further study is needed to determine the actual function of complement in PDR.

This study has limitations. First, the sample size, while significantly larger than any previously published data, still comes from a patient population at a single institution. The results therefore should be seen as a preliminary step toward a greater understanding of the subject. Second, while we looked specifically at samples from eyes with PDR, there is substantial clinical heterogeneity within any PDR population: some eyes may have mild neovascularization, while others may have extensive tractional retinal detachments or rubeosis. Beyond excluding vitreous hemorrhage, we did not differentiate between mild and severe PDR in our patients. Third, we included diabetic patients in our control group. While a subanalysis of the nondiabetic control eyes showed decreased C4, factor B, and C5 as well as significantly increased C3a and Ba—the same as the combined control group—it is possible that subclinical, or even nonvascular, diabetic damage occurred in the control eyes and may have altered measured complement levels. Finally, our results do not indicate whether complement activation is a cause or an effect of diabetic retinopathy and angiogenesis, and additional research is required to determine causality. Despite these limitations, this study is distinguished by a comparatively large data set that has been controlled for vascular leakage. Future study should be directed toward targeting of specific complement components for therapeutic potential.

In this study, we identified local activation of complement in the vitreous of patients with PDR. Using normalized ratios, we demonstrated that this local activation is ongoing and separate from systemic complement activity.

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