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
Complement Activation in Diabetic Retinopathy

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 the 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/m²), 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: 

| Plasma complement – Plasma albumin | 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
Complement Activation in Diabetic Retinopathy

**Table 1.** Demographic Characteristics and Comorbid Conditions for Patient Cases With Proliferative Diabetic Retinopathy (PDR) Versus Controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PDR</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No.</td>
<td>39</td>
<td>29</td>
<td>—</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>54</td>
<td>66</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Range</td>
<td>40-70</td>
<td>53-77</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 (38.5)</td>
<td>7 (24.1)</td>
<td>0.212</td>
</tr>
<tr>
<td>Female</td>
<td>24 (61.5)</td>
<td>22 (75.9)</td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>39 (100)</td>
<td>10 (34.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>19 (65.5)</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>28.9</td>
<td>28.8</td>
<td>0.674</td>
</tr>
<tr>
<td>Range</td>
<td>21.4–34.2</td>
<td>21.7–41.5</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>31 (79.5)</td>
<td>21 (72.4)</td>
<td>0.402</td>
</tr>
<tr>
<td>Current</td>
<td>3 (7.7)</td>
<td>1 (3.4)</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>5 (12.8)</td>
<td>7 (24.1)</td>
<td></td>
</tr>
<tr>
<td>History of treated hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of renal disease</td>
<td>6 (15.4)</td>
<td>1 (3.4)</td>
<td>0.225</td>
</tr>
<tr>
<td>History of peripheral vascular disease</td>
<td>5 (12.8)</td>
<td>2 (6.9)</td>
<td>0.600</td>
</tr>
</tbody>
</table>

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, factor B, and C5. These findings 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-
C5a/C5 0.097 (0.097)/0.051 0.066 (0.082)/0.033 1.47/0.002

Second, we corrected for vascular leakage in two ways. First, we excluded patients with vitreous hemorrhage. Any sampling of vitreous complement in PDR eyes is locally produced or if it entered the vitreous circulation. A simple measurement of vitreous complement levels can be 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. These elevated levels, however, are no more specific to diabetic retinopathy than 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 through 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: 

\[
\frac{\text{Vitreous complement}}{\text{Vitreous albumin}} \div \frac{\text{Plasma complement}}{\text{Plasma albumin}}
\]

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. 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 Bα. 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 by-product 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. 21

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). 22 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. 23 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. 24 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 ruberosis. 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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