Identification of VEGF-Independent Cytokines in Proliferative Diabetic Retinopathy Vitreous

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PURPOSE. To identify inflammatory cytokines significantly elevated and independent of VEGF levels in the vitreous of proliferative diabetic retinopathy (PDR) patients that may serve as novel diagnostic factors or therapeutic targets.

METHODS. Thirty-nine cytokines and chemokines were measured from the vitreous of 72 patients undergoing vitrectomy (29 controls and 43 PDR) via a magnetic bead-based immunoassay. Patient information, including sex, age, history of smoking, cancer diagnosis and treatment, and presence of diabetes and hypertension were also collected. Univariate and multivariate logistic regression analyses were performed to assess the association of cytokine concentrations and patient demographics with disease.

RESULTS. Nineteen cytokines were significantly elevated in the vitreous of PDR patients compared with controls, including five novel cytokines that have not previously been associated with PDR: sCD40L, GM-CSF, IFNα2, IL-12p40, and MCP-3. Sixteen cytokines were found to be statistically independent of VEGF. Of these, 14 show a statistically significant interaction with VEGF while two do not. With regards to patient demographics, age and hypertension were statistically significant risk factors with the odds of disease decreasing with increasing age and increasing 3-fold for hypertensive patients.

CONCLUSIONS. This is the first report of a comprehensive multiplex analysis to identify novel VEGF-independent cytokines associated with PDR. Of the 39 inflammatory cytokines tested, 16 are predictive of disease risk, independent of VEGF levels. These PDR-associated cytokines represent potential targets in the treatment of PDR, both in conjunction with anti-VEGF therapy, as well as for patients that are nonresponders to such therapy.

Keywords: diabetic retinopathy, VEGF, cytokine, vitreous

Damage to the retinal vasculature can cause leakage of blood and fluid (diabetic macular edema) or abnormal growth of new vessels resulting in traction retinal detachment or vitreous hemorrhage. Although laser retinal photoocoagulation has been the standard of care for proliferative diabetic retinopathy (PDR; reviewed in Giulian1), many patients still develop vitreous hemorrhage or traction retinal detachment despite laser surgery.2 As high levels of VEGF have been detected in vitreous of patients with PDR,3 development and use of anti-VEGF therapies (ranibizumab, bevacizumab, aflibercept) has become an area of intense study in the treatment of these diseases (reviewed in Giulian4).

Although information on the long-term efficacy and response of anti-VEGF agents for PDR is lacking, studies indicate short-term benefits of anti-VEGF therapy for the majority of patients.4–7 However, a high proportion of these patients showed recurrence beginning at 12 weeks,5–7,8 indicating the need for repeated anti-VEGF treatment. Additional concerns regarding adverse systemic side effects (reviewed in Simo and Hernandez9) and the possible association with tractional retinal detachment6,10 have been reported. Thus, it is desirable to develop alternative therapeutic strategies that avoid long-term anti-VEGF treatment.

While VEGF has been the major focus of treatment modalities for retinal neovascular disease, other factors are likely to play a role in the development and progression of these diseases, including PDR. We propose other inflammatory cytokines or chemokines may act in a fashion similar to VEGF to drive endothelial cell proliferation and motility. As such, these cytokines represent potential therapeutic targets for treatment of PDR.

As a first step to test our hypothesis, we performed a 72-patient clinical study to identify novel inflammatory cytokines and chemokines with elevated levels in vitrectomy samples from patients diagnosed with PDR. Using a multiplexed magnetic bead-based immunoassay, we identified 19 cytokines that are significantly elevated in PDR patient vitreous, with five of these not having been associated with PDR previously. Of note, 2-variable logistic regression (LR) indicates the expression of 16 of these cytokines is statistically independent of vitreal VEGF levels, implicating these factors as potential diagnostic and therapeutic targets for DR, particularly for patients who fail to respond to anti-VEGF therapy.
Table 1. Patient Demographics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MH, n = 16</th>
<th>MP, n = 9</th>
<th>ERM, n = 4</th>
<th>PDR, n = 43</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>72 ± 7</td>
<td>70 ± 8</td>
<td>70 ± 8</td>
<td>58 ± 11</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex, M:F</td>
<td>3:13</td>
<td>6:3</td>
<td>1:3</td>
<td>22:21</td>
<td>0.0740</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5/16</td>
<td>2/9</td>
<td>2/4</td>
<td>43/43</td>
<td>&lt;1 × 10^{-10}</td>
</tr>
<tr>
<td>Ever smoker</td>
<td>3/16</td>
<td>1/9</td>
<td>0/4</td>
<td>10/40‡</td>
<td>0.3655</td>
</tr>
<tr>
<td>Hypertension</td>
<td>10/16</td>
<td>5/9</td>
<td>3/4</td>
<td>36/42§</td>
<td>0.0270</td>
</tr>
<tr>
<td>History of cancer</td>
<td>3/16</td>
<td>3/9</td>
<td>0/4</td>
<td>4/42</td>
<td></td>
</tr>
<tr>
<td>Received laser treatment</td>
<td>1/16</td>
<td>1/9</td>
<td>0/4</td>
<td>37/43</td>
<td>1 × 10^{-8}</td>
</tr>
</tbody>
</table>

* These demographics include three distinct control groups: MH, MP, and ERM.
† Fisher’s exact test.
‡ 3/43 unknown smoking status.
§ 1/43 unknown hypertensive status.
|| 1/43 unknown cancer history.

METHODS

Study Population

The study was approved by the institutional review boards of St. Mary’s Hospital and the Van Andel Institute. The research was in compliance with the tenets of the Declaration of Helsinki. The study consisted of 72 patients requiring pars plana vitrectomy. Informed consent was obtained prior to entering the study. Patients aged less than 21 years were excluded. Patient backgrounds and demographics are summarized in Table 1. Forty-three patients diagnosed with PDR had vitreomies for vitreous hemorrhage, while 29 control patients had vitreomies for conditions not associated with retinal vascular diseases: macular hole (MH; n = 16); macular pucker (MP; n = 9); and epiretinal membrane (ERM; n = 4). All 43 PDR patients were diabetic, while 10 of 29 non-PDR patients had diabetes with no evidence of retinopathy. The majority of patients have had diabetes for over 10 years, and very few had been recently diagnosed with diabetes mellitus. One nondiabetic control patient (MP) also had branched retinal vein occlusion (BRVO). With regards to sex, smoking, and a history of cancer, no significant differences were observed between non-PDR and PDR groups. Age was significantly different between control and PDR cases (P < 0.0001), with an average age of 71 ± 7 years (range, 60–88) for control patients and 58 ± 11 years (range, 38–88) for PDR patients. None of the 72 patients had evidence of retinal detachment, nor did any patients receive anti-VEGF therapy prior to vitrectomy. Although a history of receiving laser treatment was significantly different between the control and PDR groups (P < 1 × 10^{-8}), only 2 control patients (1 MH, 1 MP) had received laser therapy while the majority of PDR patients (37/43) had previous laser treatment.

Sample Collection

Undiluted vitreous samples were obtained during a standard three-port pars plana vitrectomy; the infusion was set to off during acquisition of samples. Approximately 1 mL vitreous fluid was collected and immediately chilled on ice. Within 1 hour of collection, the samples were centrifuged at 13,000 rpm for 15 minutes at 4°C, aliquoted, and stored at −70°C until assayed. Total protein concentration for each vitreous sample was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL).

Measurement of Cytokines Using Multiplex Analysis

Multiplexed bead-based immunoassay was used for the simultaneous measurement of 39 cytokines and chemokines within each vitreous sample using MAGPIX instrumentation with xMAP detection technology (Luminex Corporation, Austin, TX). This technology utilizes internally color-coded magnetic microspheres coupled to analyte-specific antibodies, allowing for the simultaneous measurement of up to 50 analytes within each sample. The bead panel kit used—which measures 39 cytokines and chemokines per sample in a 96-well format (Supplementary Table S1)—was purchased from EMD Millipore (Milliplex Human Cytokine/Chemokine Magnetic Bead Panel I kit, Cat. # HCYTMAG-60K-PX39; Billerica, MA) and the assay was performed according to manufacturer specifications. Undiluted vitreous sample (25 µL neat per well) was assayed in duplicate. Standard curves for each cytokine were generated from reference standards supplied with the kit. Data were analyzed using xPONENT software (Luminex Corp., Austin, TX). For instances where the measured values fell below the limit of detection, we set the recorded concentration at the limit of detection. Cytokines for which the majority of levels were at or below the limit of detection were deemed nonmeasurable.

Statistical Analysis

Values of vitreous cytokine concentrations are reported as mean (pg/mL) ± standard deviation. Heat maps and dendrograms from unsupervised hierarchal clustering were generated using the heatmap.2 function in the GPlots 2.11.0 library for R version 2.15.0 (available in the public domain at http://cran.us.r-project.org; Comprehensive R Archive Network) on log2-transformed values of cytokine concentrations for all samples in each of the four patient groups (MH, MP, ERM, PDR) to provide a global view of changes in cytokine values for all patients. Clustering was performed using default parameters to heatmap.2 (row centering, Euclidian distance, and complete linkage). Logistic regression analysis was performed using PROC LOGISTIC with commercial software (SAS 9.2; SAS Institute, Cary, NC). Univariate analysis with individual logistic regressions was performed to identify individual cytokines and significantly associated with PDR, while Fisher’s exact tests were performed to identify significant differences in patient demographics between the control and PDR groups. Multivariate (2-variable) logistic regressions were performed to identify significant statistical interactions between VEGF and other cytokines, and between patient demographics and individual cytokines. Spearman rank-order correlations were performed.
RESULTS

Multiple Inflammatory Cytokines and Chemokines Are Associated With PDR

Using a multiplexed magnetic bead immunoassay (Luminex), we measured the levels of 39 cytokines and chemokines in vitreous samples of PDR patients (Supplementary Table S1). A complete dataset of cytokine expression is included in the supplementary data (Supplementary Fig. S1), and is summarized in Table 2. Univariate logistic regression was performed to evaluate the appropriateness of grouping all nonretinopathy diagnoses (MH, MP, ERM) as non-PDR controls (outcome = MH, MP, ERM, respectively) and including the diabetic non-PDR samples (outcome = diabetes). There were no significant differences in cytokine expression noted between vitreous samples from non-PDR patients who were or were not diagnosed with diabetes (data not shown). Furthermore, data from the non-PDR, nondiabetic patient with associated retinopathy (BRVO) had no effect on the overall analysis and results. Therefore, in subsequent analyses, these 29 samples were grouped together as non-PDR controls.

Table 2. Vitreal Levels of Cytokines in Patients

<table>
<thead>
<tr>
<th>Cytokine*</th>
<th>Control†</th>
<th>PDR‡</th>
<th>Univariate P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD40L</td>
<td>2.3 ± 1.07 (1.58–4.36)</td>
<td>3.6 ± 1.82 (1.58–9.55)</td>
<td>0.0032</td>
</tr>
<tr>
<td>Eotaxin16,27</td>
<td>3.2 ± 2.96 (0.98–17.54)</td>
<td>4.9 ± 2.14 (2.52–11.26)</td>
<td>0.0089</td>
</tr>
<tr>
<td>FGF-212,13</td>
<td>3.8 ± 2.93 (1.19–17.28)</td>
<td>8.0 ± 5.31 (1.57–31.67)</td>
<td>0.0009</td>
</tr>
<tr>
<td>FLT3L16</td>
<td>8.0 ± 6.48 (2.47–53.13)</td>
<td>19.3 ± 8.62 (5.98–40.11)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1.6 ± 0.71 (0.95–3.18)</td>
<td>2.5 ± 1.42 (0.95–6.15)</td>
<td>0.0030</td>
</tr>
<tr>
<td>GROα16,27</td>
<td>14.4 ± 21.12 (1.78–109)</td>
<td>31.6 ± 24.46 (4.1–149.5)</td>
<td>0.0039</td>
</tr>
<tr>
<td>IFN-α2</td>
<td>1.6 ± 0.69 (0.77–4.41)</td>
<td>2.2 ± 0.87 (0.87–4.56)</td>
<td>0.0075</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>0.8 ± 0.55 (0.38–2.67)</td>
<td>1.8 ± 1.56 (0.38–7.84)</td>
<td>0.0026</td>
</tr>
<tr>
<td>IL-16–18,51</td>
<td>6.9 ± 16.19 (0.41–77.99)</td>
<td>45.2 ± 73.68 (1.14–435)</td>
<td>0.0005</td>
</tr>
<tr>
<td>IL-714</td>
<td>1.7 ± 0.93 (0.65–4.87)</td>
<td>2.9 ± 1.79 (0.92–7.02)</td>
<td>0.0070</td>
</tr>
<tr>
<td>IL-816–19,51</td>
<td>12.4 ± 30.49 (1.49–52.34)</td>
<td>96.2 ± 173.82 (6.87–1082.5)</td>
<td>0.0003</td>
</tr>
<tr>
<td>IL-1051</td>
<td>1.7 ± 1.09 (1.06–6.11)</td>
<td>4.2 ± 3.05 (1.06–15.21)</td>
<td>0.0003</td>
</tr>
<tr>
<td>IP-1016,51</td>
<td>196.1 ± 388.18 (22.88–2023)</td>
<td>769.8 ± 532.20 (172–2467)</td>
<td>0.0002</td>
</tr>
<tr>
<td>MCP-117–20,51</td>
<td>1217.7 ± 971.81 (504–5698)</td>
<td>2839.8 ± 1743.21 (825–8327)</td>
<td>0.0008</td>
</tr>
<tr>
<td>MCP-3</td>
<td>3.1 ± 1.57 (1.67–10.59)</td>
<td>5.2 ± 3.22 (2.05–15.04)</td>
<td>0.0122</td>
</tr>
<tr>
<td>MDC16</td>
<td>23.5 ± 39.38 (2.27–152)</td>
<td>86.4 ± 55.22 (4.79–274)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MIP-115</td>
<td>2.1 ± 0.79 (0.85–10.88)</td>
<td>4.5 ± 1.82 (1.0–26.3)</td>
<td>0.0186</td>
</tr>
<tr>
<td>VEGF16–20,51</td>
<td>18.9 ± 63.62 (0.39–343)</td>
<td>422.6 ± 655.77 (0.39–3165)</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

* Previously published reports associating increased levels of these cytokines in PDR.
† Cytokine levels in pg/mL ± SD (range in cytokine values).
‡ PDR samples (outcome = PDR). Values in parentheses represent mean (SD).

All cytokines are significant at P < 0.05. Values in parentheses represent mean (SD). The majority of vitreous sample concentrations were at or below our limit of detection. The 27 cytokines with measurable levels, univariate logistic regression analysis identified 19 cytokines with a mean vitreous concentration significantly higher in PDR patients compared with non-PDR controls (Table 2). Five of these cytokines have not been previously associated with PDR. These include GM-CSF, IL-12p40, sCD40L, MCP-3, and IFN-α2. The remaining 14 cytokines including eotaxin, FGF-2, MIP-1β, VEGF, FLT3L, GRO, interleukins 1α, 6, 7, 8, and 10, IP-10, MCP-1, and MDC were previously observed to be elevated in vitreous of patients with PDR. Similar results were obtained by Mann-Whitney analysis (Supplementary Table S2, left column), except that Mann-Whitney analysis identified two additional cytokines, GM-CSF and sIL-2ra, as significantly elevated in PDR patient vitreous (Supplementary Table S2, right column).

Cytokine levels were markedly different between patient samples. For example, whereas samples from patients with PDR on average contained more VEGF (in pg/mL) than samples from non-PDR patients (423 ± 656 vs. 18 ± 64) there was a wide range of expression within PDR samples (<1–3165 pg/mL; Fig. 1, x-axis, Fig. 2a). These differences may be caused by confounding factors such as vitreous hemorrhage.21 However, when we compared the total vitreous protein levels between control and PDR vitreous samples, we failed to observe a significant difference (Fig. 2a).

Figure 1. The relationship between VEGF expression and total vitreous protein. Scatterplot of VEGF concentrations in pg/mL of control vitreous samples (includes MH, MP, and ERM; closed circles) and PDR samples (open circles) compared with total vitreous protein concentrations (in mg/mL). VEGF expression was not correlated with total vitreous protein. R² (controls) = 0.0654; R² (PDR) = 0.0683; R² (all) = 0.0450.
FIGURE 2. Expression levels of cytokines in patient vitreous. Cytokine levels were measured by a magnetic bead-based immunoassay. (a) VEGF and IL-6 levels (pg/mL) and (b) levels for all cytokines for each patient samples are shown. To visualize all the data, the values in (a) are plotted using a log scaled y-axis. For comparison, patient samples in the upper panels are organized in a manner identical to that used in the lower panel where the log2-transformed values for each cytokine (ordinate) in each patient sample (abscissa) are plotted using a heat map. This format is useful to facilitate
comparison of expression levels across the data set. The heat map in (b) was generated using unsupervised hierarchical clustering. The accompanying dendrograms show how cytokines and patient samples grouped according to similar patterns of expression. Patients’ diagnoses of macular holes (green), macular puckers (blue), epiretinal membranes (red), and proliferative diabetic retinopathy (purple) are indicated at the top of the heat map. Color codes in each panel refer to the key used in (b) with red for low expression and white for the highest expression levels.

statistically significant difference between any of the control groups and PDR (Supplementary Fig. S2a). We also found no correlation between vitreous VEGF levels and total vitreous protein levels ($R^2 = 0.0430$; Fig. 1). Finally, we found no statistical difference in total vitreous protein levels when comparing samples with low VEGF concentrations (≤100 pg/mL) to those with high VEGF concentrations (>100 pg/mL) (Supplementary Fig. S2b).

We observed a wide range in concentrations within the PDR sample group for multiple cytokines, including FLT3L, GRO, IL-6, IL-8, IP-10, MCP-1, and MDC (Supplementary Fig. S1). Consequently, it was difficult to differentiate patients based on the expression of each cytokine. To determine whether patients could be better distinguished by global patterns of cytokine expression, unsupervised clustering of the cytokine and sample data was performed (Fig. 2). This analysis successfully segregated 42/45 (98%) of PDR samples and 26/29 (90%) of non-PDR samples. The cytokines themselves broadly clustered in three groups, as seen in the y-axis dendrogram in Figure 2b, based on overall cytokine levels among all patients. One of these—consisting of GRO, FLT3L, Fractalkine, IL-6, MDC, IL-8, and VEGF—was markedly elevated in samples from patients diagnosed with PDR. A second group had low to moderate levels of expression for all samples. This cluster could be further broken down into subsets, with one subset containing MCP-3, sCD40L, eotaxin, FGF-2, sILRa, IL-10, MIP-1b, and G-CSF having higher levels in the PDR samples compared with controls. The third cluster of cytokines had high levels of expression for all samples (MCP-1 and IP-10), although the levels in PDR patients remained higher than controls. The single PDR sample clustering among the non-PDR samples expressed elevated VEGF levels comparable to those observed in many patients with PDR. In contrast, two of the non-PDR samples (both MP patients) clustering among the PDR samples expressed low levels of VEGF more similar to those observed in non-PDR samples. Neither of these MP patients had diabetes. One had retinopathy (BRVO), while the other had no history of neovascular issues. The third non-PDR sample (MH) grouping with the majority of PDR patient samples expressed elevated levels of VEGF and other cytokines similar to PDR samples. This patient did not have a history of retinopathy or other neovascular disease.

Inflammatory Cytokines and Chemokines Are Independent Predictors of Disease

To identify cytokines that were associated with PDR independent of VEGF, 2-variable logistic regression analysis was performed with VEGF as the second predictor. This analysis revealed that 16 of the 18 cytokines other than VEGF that were significantly elevated in PDR remained significantly associated with disease when VEGF levels are accounted for (Table 3). The two remaining cytokines (FGF-2 and MIP-1b) are no longer significant when adjusting for VEGF concentration, indicating they are not independent predictors of disease (Supplementary Table S3). Based on global cytokine levels, we found no pattern in clustering of the VEGF-independent cytokines (Fig. 2b). Of the 16 cytokines that remain associated with disease after adjusting for VEGF concentration, 14 (eotaxin, FLT3L, GM-CSF, GRO, IFNα2, IL-1α, IL-6, IL-7, IL-8, IL-10, IL-12p40, IP-10, MCP-1, and MDC) were found to significantly interact with VEGF such that the ability of these cytokines to predict PDR depends on VEGF levels (Table 3). Soluble CD40L (sCD40L) and MCP-3 were statistically independent and not interacting with VEGF, indicating that these two factors have predictive ability independent of VEGF levels (Table 3, top). All of these cytokines positively correlated with VEGF levels (i.e., their levels were increased when VEGF levels were increased; Supplementary Table S4, right column).

Age, Medical History, and Hypertension Are Associated With PDR but Not With the Expression of Inflammatory Cytokines

The current standard of care for PDR remains panretinal photocoagulation laser treatment. Consequently, in our study a history of laser treatment was significantly associated with disease ($P = 1 \times 10^{-8}$). Laser treatment has been reported to decrease VEGF levels, and while analysis of our data showed a decrease in the levels of VEGF that did not reach statistical significance, levels of sCD40L and IP-10 increased significantly among patients who received laser surgery compared with those who did not (Supplementary Table S5). Comparison of control samples to PDR samples of patients who did not receive laser treatment revealed that sCD40L levels were not significantly different between these two groups by either univariate or Mann-Whitney analyses, while IP-10 was significantly different by Mann-Whitney test only ($P < 0.02$). Both of these cytokines had elevated levels in PDR samples following laser treatment compared with samples of patients who did not receive laser treatment, which implies that laser treatment may play a role in elevating these cytokines in the vitreous of PDR patients.

To rule out the time since laser treatment as a confounding factor in the significance of elevated cytokines, we compared the time between laser treatment and vitrectomy among PDR patients. Of the 43 PDR patients, 18 had vitrectomies performed within 1 year of laser treatment (median time = 4 months), 17 had vitrectomies more than 1 year after laser treatment; (median time = 48 months); and 6 patients never received laser treatment (2 patients received laser treatment at an unknown date and were not included in this analysis). We found no statistically significant difference in age between PDR patients who had vitrectomies within 1 year of laser treatment compared with those who had over a year between surgeries (Fisher’s exact test; $P = 0.305$). When comparing the 35 PDR patients who had laser surgery prior to vitrectomy, we found no statistically significant difference in cytokine levels between those within a year of laser treatment compared to those who had more than a year between laser and vitrectomy (data not shown). These data suggest that the time since laser treatment did not correlate with cytokine levels in PDR patients.

Of the five remaining patient demographics (age, sex, smoking, hypertension, history of cancer), only age and hypertension were significant predictors of disease by Fisher’s exact test ($P < 0.0001$ and $P = 0.027$, respectively). Increasing age was associated with lower odds of disease (odds ratio [OR] = 0.8364; confidence interval [CI] 0.761–0.919), while hypertensive patients had a greater than 3-fold increased odds of disease (OR = 3.67; CI 1.168–11.515).

As age was significantly different between non-PDR and PDR groups, multivariate logistic regression was performed for each of the 19 significant cytokines using age as a second predictor for PDR. FGF-2, FLT-3L, IL-12, IL-6, IL-10, MCP-3, and
VEGF were still significant predictors of disease \( (P < 0.05) \) after adjusting for age. All 19 cytokines negatively correlated with age (Supplementary Table S4, left column), indicating that their levels were increased for younger patients. To determine if PDR patients had elevated cytokine levels because they are younger than controls, we performed two additional analyses. First, to determine if age influences cytokine levels in disease samples, PDR patients were divided between those aged less than 60 years (20 patients) and those aged 60 years or older (23 patients). Univariate logistic regression (with age greater than or equal to 60 set as the outcome) and Mann-Whitney statistical analyses were performed to identify any cytokines that were significantly different between younger and older patients. Mann-Whitney analysis showed only IL-6 was significantly different between these two groups \( (P < 0.04) \). This difference may be attributed to the disparate range in values for the older group \((<60 \text{ range, } 1.14–435)\) and the younger group \( \geq 60 \text{ range, } 5.78–88.53 \). In the second analysis, we compared cytokine levels in the subset of 60–70-year-old age-matched patients \( (Fisher's \text{ exact test for age; } P = 0.173) \) in both the control and PDR \( (15 \text{ control, } 20 \text{ PDR, Supplementary Table S6}) \). Mann-Whitney analysis indicated that the majority \( (17/19) \) of cytokines remained significant. Univariate analysis similarly showed 11/19 cytokines remained significantly associated with disease. Collectively, our analyses indicate any correlation with age likely reflects that PDR occurs earlier in life, and as a consequence, PDR patients require vitrectomies at a younger age.

As the majority of patients with diabetes have had the disease for over 10 years (Supplementary Fig. S1), we were unable to determine if duration of diabetes has an impact on cytokine levels among PDR patients. Furthermore, upon comparing the 10 diabetic control patient–cytokine levels to those of PDR patients, we found no change in the statistical significance of the 19 cytokines found to be statistically elevated in PDR compared with controls.

All 19 cytokines significantly elevated in PDR patient vitreous remained significant after adjusting for smoking history and hypertensive status. Furthermore, as smoking history was not a significant predictor of disease on its own, it remained a poor predictor of disease even when other patient variable were taken into account, such as age and hyperten-

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>2-Variable LR, no int*</th>
<th>2-Variable LR, int*</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD40L</td>
<td>0.0291</td>
<td>Insig</td>
</tr>
<tr>
<td>MCP-3</td>
<td>0.0367</td>
<td>Insig</td>
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<tr>
<td>Eotaxin</td>
<td>N/A</td>
<td>0.0075</td>
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<td>FLT3L</td>
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<td>0.0009</td>
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<td>GM-CSF</td>
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<td>IL-10</td>
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<td>IL-12p40</td>
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<tr>
<td>MCP-1</td>
<td>N/A</td>
<td>0.0013</td>
</tr>
<tr>
<td>MDC</td>
<td>N/A</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

* 2-variable logistic regression using VEGF as the second predictor.

**DISCUSSION**

VEGF is a potent proangiogenic cytokine that is highly expressed in many angiogenesis-dependent diseases.\(^5\) Since its first detection in the vitreous of patients with proliferative diabetic retinopathy,\(^22,24–26\) VEGF has been recognized as one of the primary factors driving retinal neovascularization and macular edema. In support of this, several therapies targeting VEGF have shown short-term benefit for patients with PDR.\(^4,6,7\)

Despite recent progress made with the use of anti-VEGF therapies, there are still critical gaps in the knowledge of how to best treat patients with diabetic retinopathy. Adverse effects, particularly for diabetic patients,\(^9\) and the possible association with tractional retinal detachment,\(^8,10\) have been reported. And while the majority of PDR patients do respond well to anti-VEGF therapy, at least in the short-term, we still do not understand why some patients respond well and others do not. Moreover, we do not yet know the long-term response and toxicities to anti-VEGF therapy. These concerns provide a strong rationale for the development of other antiangiogenic therapeutics as standalone drugs or for use in combination with anti-VEGF therapy.

Approximately 30% of PDR patients fail to respond to initial anti-VEGF treatment, and the majority of those who do respond will require multiple rounds of intravitreal injections.\(^5,7,8\) One possible explanation for these results is that elevated VEGF levels in some patients may be sufficient to overcome the anti-VEGF therapy. Indeed, our results demonstrate VEGF levels vary between patients by three orders of magnitude. This extreme variability suggests VEGF expression is highly unregulated or alternatively regulated in PDR. In the latter scenario, the retina may even respond to anti-VEGF therapies by relieving transcriptional or translation repression of its own production and increasing VEGF production. As an alternative, we propose that in addition to VEGF-dependent retinal neovascularization, one or more alternative cytokine-mediated signaling pathways may drive retinal neovascularization. There is ample precedence for this with several studies demonstrating cytokines other than VEGF can drive angiogenesis in a variety of in vitro and in vivo models (reviewed by Folkman\(^25\)). These reports provide a strong rationale for the present study.

The question of whom we should use for control patients was of some concern to us. To ensure that all 29 samples resulting from vitrectomy for non-neovascular indications could be grouped together as “non-PDR” controls, we performed univariate logistic regression for each of the 27 measurable cytokines, separating out each control condition as an outcome in the analysis. We found no significant differences for any of the 27 cytokines for any of the control group comparisons \( (ERM \text{ to MH/MP, MH to ERM/MP, MP to ERM/MH}) \). Furthermore, as eight of the non-PDR controls were diabetic, we performed a similar analysis setting diabetes as the outcome in univariate logistic regression analysis. Again, we found that none of the 27 measurable cytokines were significantly different between the diabetic and non-diabetic control samples, indicating that, for this analysis, these 29 control cases can be grouped together as “non-PDR” controls.
It was interesting to note that statistical analysis suggested the odds of disease decreased with age in PDR patients. However, this result is misleading and likely reflects the early onset and progression of this disease. In fact, it is not surprising to find a greater than 10-year age difference between these two populations.\textsuperscript{27,28} Similarly, statistical analyses suggesting levels of some cytokines are increased in younger patients may also be misleading. As age had no bearing on cytokine levels among PDR patients, and a smaller age-matched cohort reconfirmed the majority of significant cytokines between control and PDR patients, it seems clear that the cytokines identified in this study are significantly elevated in PDR patients due to disease status of these patients and not their younger age.

Our results demonstrate the vitreous of PDR patients contain a complex mix of cytokines and chemokines that has the potential to influence disease pathology and therapeutic response. While we expected to see some differences in cytokine expression levels, we were surprised to find so many to be significantly upregulated in the vitreous of PDR patients. Of the 39 cytokines and chemokines tested, we identified 19 that were significantly elevated in PDR patient vitreous. Five of these have not been associated with PDR previously. Moreover, it is very likely the PDR vitreous is more complex than we have shown here, since we have only tested a small fraction of the many inflammatory cytokines and chemokines that may be present.

The origins of these cytokines and chemokines remain an important unanswered question. They may be secreted by retinal cells including tissue macrophages responding to retinal inflammation or released from the extracellular matrix by the actions of matrix metalloproteases that are upregulated in PDR.\textsuperscript{29,30} In addition, since the retinal-blood barrier may be compromised in PDR patients,\textsuperscript{31,32} these factors may have systemic origins. Regardless of their source, any of these cytokines and chemokines may contribute to pathology either by directly promoting neovascularization or indirectly by causing changes in the retinal microenvironment that facilitate disease progression (e.g., by causing neuronal cell death).\textsuperscript{33}

Many of the cytokines and chemokines we identified have been previously reported to play a role in neovascular disease. Several—such as eotaxin, IL-1, IL-6, IL-7, IL-8, IL-10, MCP-1, MCP-3, GRO, sCD40L, and FGF—have been reported to promote angiogenesis.\textsuperscript{34–44} While others such as MDC and FLT3L have not been evaluated in this context. Interestingly, three of the factors shown to be VEGF-independent, GM-CSF, IFN\textgamma{}2, and IP-10, have been reported to play antiangiogenic roles in a variety of conditions.\textsuperscript{45–51} These cytokines have well documented proinflammatory activities, and while IP-10 has been reported to be elevated in diabetic retinopathy,\textsuperscript{52} GM-CSF levels were reported to be decreased in the aqueous of DR patients.\textsuperscript{52} IFN\textgamma{}2 has been tested as a treatment for AMD, due to its antiangiogenic and anticcitot morbility properties (reviewed in Lindner\textsuperscript{46}). However, the results were inconclusive, and IFN\textgamma{}2 therapy was classed as ineffective and unsafe.\textsuperscript{53–55} Whether these proinflammatory and antiangiogenic factors are elevated in response to inflammation associated with diabetic retinopathy or are involved in its pathogenesis is unclear and requires further analysis.

Although the majority of control patients had undetectable levels of VEGF, approximately one quarter (8/29) had elevated levels of VEGF (Fig. 2, Supplementary Fig. S1). Why these patients have elevated vitreal VEGF levels is unclear. Half of these patients were diabetic, so elevated VEGF levels may indicate these patients are at a higher risk of developing diabetic retinopathy. However, the remaining patients had no diabetes diagnosis. In these cases, patients may have another underlying, undiagnosed vascular complication, such as cancer. Long-term follow-up of these patients may shed light on this discrepancy.

From a clinical perspective, any of the cytokines and chemokines we identified are potential therapeutic targets for the treatment of PDR or other retinal neovascularization. However, the VEGF-independent, interacting vitreal factors are particularly interesting. This is not only because they are associated with disease, but also because several of these factors including GRO, FLT3L, IL6, IL8, and MDC were markedly elevated in samples from patients diagnosed with PDR and as a consequence, may be better therapeutic targets than some of the less abundant factors. However, it is important to note that our analyses cannot distinguish whether any of these vitreal factors play a causative role in PDR. Additional experiments are required to determine whether any of these factors is necessary and sufficient to induce retinal neovascularization. These same cytokines may also serve as biomarkers that predict response to therapy. In this scenario, an ophthalmologist could sample vitreous and tailor therapy to match a patient’s disease. An expanded evaluation of cytokines before and after anti-VEGF treatment in responders and nonresponders is a logical next step to this study. However, collection of these samples will require a long-term effort since the majority of PDR patients respond favorably to anti-VEGF treatment in the short term (<6 months)\textsuperscript{55–58} and nonresponder samples are consequently less available.

In summary, while other studies, including multiplex bead and proteomic analyses, have analyzed multiple proteins simultaneously in the vitreous (reviewed in Simo-Servat et al.),\textsuperscript{21} this is the first report to our knowledge utilizing a comprehensive multiplexed immunobead-based assay to identify novel VEGF-independent cytokines and chemokines associated with PDR. These PDR-associated cytokines represent potential targets in the treatment of PDR, both in conjunction with anti-VEGF therapy, as well as for patients that are nonresponders to such therapy.

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**References**


