Alterations in the Intraocular Cytokine Milieu after Intravitreal Bevacizumab

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PURPOSE. Several complications after intravitreal bevacizumab (IVB) treatment have been described including tears of the retinal pigment epithelium and tractional retinal detachment. The etiology of these complications remains unclear. The purpose of this study was to characterize changes in the intraocular levels of inflammatory cytokines after IVB as a possible explanation for these complications.

METHODS. Twenty-nine patients with proliferative diabetic retinopathy (PDR) undergoing pars plana vitrectomy (PPV) for vitreous hemorrhage (VH) with IVB pretreatment were prospectively enrolled. Aqueous humor samples were taken at the time of IVB pretreatment and approximately 1 week later at the time of PPV. Multiplex cytokine arrays were used to assay 20 different cytokines. Multivariate general linear regression was performed to determine differences in cytokine levels between the two study visits. Proportional hazards regression was performed to determine the relationship between cytokine levels at PPV and postoperative outcomes.

RESULTS. After treatment with IVB, vascular endothelial growth factor (VEGF) concentrations in the aqueous humor decreased (P = 0.0003), whereas the concentrations of IL-8 and transforming growth factor (TGF)β increased after IVB (P < 0.03). The level of IL-8 at the time of PPV was associated with the occurrence of recurrent VH after surgery (hazard ratio, 1.52; P = 0.02).

CONCLUSIONS. Alterations in the intraocular inflammatory cytokine milieu occur after IVB injection, possibly as a compensatory mechanism in response to VEGF inhibition. The increased concentrations of inflammatory cytokines after IVB may be clinically significant and may be responsible for some of the complications after IVB. (Invest Ophthalmol Vis Sci. 2010;51:2388–2392) DOI:10.1167/iovs.09-4065

The discovery of elevated vitreous levels of vascular endothelial growth factor (VEGF) in eyes of patients with diabetic retinopathy (DR) heralded an era of improved understanding of the molecular pathophysiology of this disease.1,2 Since then, numerous other chemical mediators and cytokines have been identified in the vitreous of patients with DR, enhancing our understanding of the ocular biochemical milieu of this condition. Elevated levels of angiopoietin (Ang)-2,3 leptin,4 interleukin (IL)-1β,5 IL-2,6 IL-8,7 platelet-derived growth factor (PDGF),8 monocyte chemoattractant protein (MCP)-1,9 transforming growth factor (TGF)β,9 placental growth factor (PIGF),10,11 fibroblast growth factor (FGF) basic,12 and tumor necrosis factor (TNF)-α13 have been described in the vitreous of eyes with DR. Furthermore, vitreous levels of interleukin (IL)-6, MCP-1, intercellular adhesion molecule (ICAM)-1, and pigment epithelium-derived factor (PEDF) have been shown to correlate with severity of DR.13–15 Although measurement of vitreous cytokines has proven useful to our understanding of DR, obtaining vitreous samples has inherent risks and complications. Aqueous humor is more easily and safely obtainable, and aqueous humor levels of cytokines have been shown to correlate with their corresponding vitreous levels.15–16

Intravitreal bevacizumab (Avastin; Genentech, Inc., San Francisco, CA) is commonly used for the treatment of DR and neovascular age-related macular degeneration (AMD).17,18 As the use of intravitreal bevacizumab (IVB) has increased over the past few years, so has an understanding of its potential complications and limitations. Development and progression of tractional retinal detachment (TRD) in patients with proliferative diabetic retinopathy (PDR) has been described after IVB.19,20 In the setting of neovascular AMD with pigment epithelial detachments (PEDs), numerous reports have emerged describing tears of the retinal pigment epithelium (RPE) after IVB.21–23 The etiology of these complications after IVB remains unclear. One hypothesis is that acute alterations in the intraocular cytokine profile may contribute to these effects by promoting inflammation and fibrosis. The purpose of this study was to determine whether acute changes in the intraocular cytokine milieu occur after IVB. Patients with PDR undergoing pars plana vitrectomy (PPV) with IVB pretreatment were recruited, to allow aqueous humor sampling at two different time points approximately 1 week apart, without significant added risk to the patients.

METHODS

Patients and Procedures

Eligibility criteria for this prospective 1-year study included PDR with nonclearing vitreous hemorrhage (VH) requiring PPV. Exclusion criteria included VH secondary to ocular disease other than diabetes, intravitreal injection of bevacizumab within the past 3 months, and intravitreal injection of triamcinolone during the past 6 months. As per our routine treatment protocol, approximately 1 week before the planned surgery, the patients were injected with intravitreal bevacizumab (1.25 mg, 0.05 mL) as a pretreatment, to reduce the rate of intraoperative hemorrhage.24–26 Immediately before the intravitreal injection, an anterior chamber paracentesis was performed and 150 μL of aqueous humor was withdrawn and stored at −20°C. At the scheduled time of surgery, another anterior chamber paracentesis was performed immediately before commencement of PPV and another 150 μL of aqueous humor was obtained and stored at −20°C. To prevent dilution of aqueous humor, the infusion cannula was left clamped after
placement. After this, the anterior chamber paracentesis was performed, the infusion cannula was unclamped, the other working ports were created, and PPV was started. Anterior chamber paracentesis was performed under sterile conditions with a 30-gauge needle attached to an insulin syringe. PPV was performed by one of two surgeons (PJK, KTE), using standard three-port 23-gauge techniques followed by air-fluid exchange. In some cases, intravitreal triamcinolone (IVT, 4 mg) was injected at the conclusion of the procedure. The use of IVT was based on surgeon preference; one surgeon routinely used IVT at the end of the procedure, whereas the other never used it. After surgery, the patients were observed as per routine care. At the conclusion of the study, the patients’ charts were retrospectively reviewed to collect demographic information (age, sex, diabetes type, hemoglobin A1c) and postoperative outcomes (Snellen visual acuity and occurrence of recurrent VH). Furthermore, the presence of TRD noted either before or during surgery was recorded. The study was performed with the patient’s informed consent and conducted under a protocol approved by the institutional review board (IRB) at Sunnybrook Health Sciences Centre (Toronto, ON, Canada) and in accordance with the ethical standards of the 1964 Declaration of Helsinki.

Cytokine Analysis

Preliminary screening of 20 different cytokines was performed on samples from the first five patients, to select candidate cytokines for further study. Initial cytokine analysis included Ang-2, leptin, IL-1β, IL-2, IL-6, IL-8, IL-10, PDGF-AA, PDGF-AB, PDGF-BB, MCP-1, IFN-γ, TGF-β1, TGF-β2, VEGF, PGF, PDEF, FGF basic, TNF-α, and ICAM-1. Only cytokines that showed a difference between study visits at a significance level of 0.50 during the preliminary screening were assayed for in future patient samples. Cytokines were assayed with a sandwich-ELISA multiplex system (SearchLight; Aushon Biosystems, Billerica, MA) in a CLIA (Clinical Laboratory Improvement Amendments)-certified laboratory. Briefly, undiluted or diluted aqueous humor samples were assayed in 96-well microplates coated with capture antibodies. Detection of analytes was performed with chemiluminescent secondary antibodies. Cytokine levels were quantified with a charged-coupled device (CCD) camera and imaging system. Standard curves were generated with known amounts of cytokine proteins, and analyte concentrations were calculated from the standard calibration curves.

Statistics

Analysis of the screening samples from the first five patients was performed with a two-tailed paired t-test with a significance level (α) of 0.50. The analysis on cytokine levels chosen for further analysis based on the preliminary screening was performed with general linear models (SAS System 9.2, SAS Institute, Inc., Cary, NC). For each cytokine, multivariate general linear models were performed to compare the preoperative adjusted means of the cytokines by diabetes type, and, separately, by the presence of TRD. The covariates considered for adjustment were: age, sex, and hemoglobin A1c. General linear models were also used to evaluate whether there were any significant changes in cytokine levels between the pretreatment sample and the sample obtained immediately before PPV. In addition to the previously mentioned covariates (age, sex, and hemoglobin A1c, diabetes type, and presence of TRD), we considered the number of days elapsed between pretreatment and PPV as a possible covariate. Because we were adjusting for multiple covariates, we selected the best-fitting model by choosing the one with the lowest Akaikes information criterion (AIC) as the best model.

Proportional hazards regression analysis was used for the analyses of the events of postoperative recurrent VH and visual acuity. Snellen visual acuities were converted to logMAR equivalents for this analysis. We evaluated the effects of the cytokine levels at PPV by dichotomizing the levels into the top 50th percentile versus the lower 50th percentile. Since all the patients experiencing recurrent VH were in the top 50th percentile of IL-8 concentration, we analyzed IL-8 concentration as a continuous variable. We considered the following covariates in the analyses: age, sex, hemoglobin A1c, diabetes type, TRD, and IVT. We chose the covariates that were significant in a univariate analysis for each cytokine. We then added in a model those that were significant at the 0.10 level. Life-table analyses were used to graphically demonstrate the time-to-event (VH) in the top 50th percentile versus the lower 50th percentile of cytokine concentration.

RESULTS

A total of 29 patients were enrolled. Basic demographic and laboratory information on this cohort is displayed in Table 1. The mean postoperative follow-up time of the patient cohort was 15 weeks (range, 1–53). The cytokines PDGF-BB, IFN-γ, TGF-β1, IL-1β, TNF-α, and FGF basic were undetectable in our aqueous humor samples with the multiplex assay that we used. After preliminary screening, the cytokines chosen for further analysis were VEGF, PI GF, IL-2, IL-6, IL-8, MCP-1, and TGF-β2.

Multivariate analyses for difference in baseline in cytokine levels demonstrated that patients with TRD had an adjusted mean VEGF level of 457 pg/mL, whereas those without TRD had mean VEGF level of 1411 pg/mL, a difference that was statistically significant (P = 0.048). Patients with type 1 diabetes had an adjusted mean IL-6 level of 47.8 pg/mL, whereas those with type 2 diabetes had an adjusted mean IL-6 level of 113.8 pg/mL, a difference that reached borderline statistical significance (P = 0.059).

Multivariate analyses of cytokine levels demonstrated changes in the concentrations of several cytokines at a mean time of 10 days after IVB (Table 2, Fig. 1). Both VEGF and PI GF concentrations decreased after IVB; however, only the change in VEGF levels was statistically significant (P = 0.0003). Of note, the concentrations of all the inflammatory mediators we tested increased after IVB. The increase in IL-6 concentration was not statistically significant, and the increase in IL-2 and MCP-1 only reached borderline statistical significance (P = 0.065 and 0.068, respectively). Concentrations of IL-8 and TGF-β2 were significantly elevated after IVB (P = 0.0204 and 0.0009, respectively).

A total of five (17%) of our patient cohort developed recurrent VH during the follow-up period. Recurrent VH occurred at mean time of 15 weeks (range, 0.7–38) after PPV. As the mean follow-up of our cohort was also 15 weeks (range, 1–53), our proportional hazards analysis (which is a time-to-event analysis), adjusted for the variable follow-up and prevented improper conclusions due to insufficient follow-up. All patients with recurrent VH were in the upper 50th percentile of IL-8 concentration at the time of PPV. Proportional hazards analysis demonstrated that IL-8 levels at vitrectomy were associated with a statistically significant (P = 0.02) increased risk of recurrent VH (Table 3). A graphic life table analysis of the IL-8 level in the top 50th percentile versus the lower 50th percentile is shown in Figure 2. No other cytokines were found to be
Intravitreal Injection of Bevacizumab has been shown to cause tumor regression in animal models. It is likely that our study was statistically underpowered to detect significant mechanisms. The TGF-β family of cytokines plays an important role in mediating fibrosis and scar contraction. Human vitreous from patients with PDR and PVR has been shown to cause significantly larger contraction of collagen gels compared with nonproliferative controls, an effect that correlates with the

**DISCUSSION**

We observed an expected decrease in the aqueous humor concentration of VEGF after IVB, which is consistent with previous observations. Although we also observed a decrease in PlGF, this result was not statistically significant. A statistically significant decrease in PlGF mediated by IVB would have been very interesting from a therapeutic standpoint. We observed statistically significant increases in the aqueous humor concentrations of IL-8 after IVB. The putative role of TGF-β in complications after IVB may be discerned based on its known biological functions. The TGF-β family of cytokines plays an important role in mediating fibrosis and scar contraction. Human vitreous from patients with PDR and PVR has been shown to cause significantly larger contraction of collagen gels compared with nonproliferative controls, an effect that correlates with the

**TABLE 2. Adjusted Change in Cytokine Concentration after Intravitreal Injection of Bevacizumab**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Pre-bevacizumab (pg/mL) (95% CI)</th>
<th>Post-bevacizumab (pg/mL) (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>1044 (583–1505)</td>
<td>116 (50–182)</td>
<td>0.0003*</td>
</tr>
<tr>
<td>PlGF</td>
<td>27.7 (14.3–41.2)</td>
<td>17.2 (12.0–22.5)</td>
<td>0.0887</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.71 (1.27–2.16)</td>
<td>2.12 (1.63–2.62)</td>
<td>0.00652</td>
</tr>
<tr>
<td>IL-6</td>
<td>81 (8–171)</td>
<td>1065 (72.5–2406)</td>
<td>0.151</td>
</tr>
<tr>
<td>IL-8</td>
<td>42.3 (26.2–58.5)</td>
<td>59.3 (42.8–75.8)</td>
<td>0.0204*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2246 (1798–2693)</td>
<td>3514 (2152–4476)</td>
<td>0.0075</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>5554 (2770–8358)</td>
<td>8256 (5478–11034)</td>
<td>0.0009*</td>
</tr>
</tbody>
</table>

The data are adjusted for any of the following covariates based on the best-fitting model: age, sex, hemoglobin A1c, presence of tractional retinal detachment, and number of days between the pre- and post-bevacizumab samples. The mean (±SD) number of days between when the baseline and the vitrectomy samples were obtained was 10 (±5). The pre-bevacizumab cytokine levels were determined from aqueous samples taken immediately before intravitreal administration of bevacizumab, the post-bevacizumab levels were determined immediately before vitrectomy.

* Indicates statistically significant result (P < 0.05).

**TABLE 3. Adjusted Risk of Recurrent Vitreous Hemorrhage after Intravitreal Injection of Bevacizumab**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Hazard Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>0.61 (0.04–9.83)</td>
<td>0.73</td>
</tr>
<tr>
<td>PlGF</td>
<td>0.74 (0.11–4.80)</td>
<td>0.75</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.57 (0.08–3.96)</td>
<td>0.57</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.86 (0.13–5.74)</td>
<td>0.88</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.32 (1.05–1.65)</td>
<td>0.02*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.84 (0.13–5.62)</td>
<td>0.86</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>0.91 (0.81–1.02)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

For IL-8 no covariates were included. For TGF-β2, age was the only covariate. For all other cytokines, TRD was the only covariate. The cytokine levels in aqueous humor were measured at the time of vitrectomy. For all but IL-8, the hazard ratio compares the upper 50% levels versus the lower 50% levels. For IL-8, it denotes the increase in risk for an increase of 10 pg/mL in the concentration of IL-8.

* Indicates statistically significant result (P < 0.05).

associated with recurrent VH. Final mean visual acuity in our cohort was 20/230 (range, 20/40–no light perception). We found no associations between cytokine levels at PPV and postoperative visual acuity.

**FIGURE 1.** Adjusted mean aqueous humor concentrations of inflammatory cytokines after IVB. Error bars, SEM. *Statistically significant result (P < 0.05). Adjusted for any of the following covariates based on the best-fitting model: age, sex, hemoglobin A1c, presence of TRD, and number of days between the pre and post-bevacizumab injection samples. The mean (± SD) number of days between the date of the pre- and postbevacizumab injection samples was 10 (±5).

For IL-8 no covariates were included. For TGF-β2, age was the only covariate. For all other cytokines, TRD was the only covariate. The cytokine levels in aqueous humor were measured at the time of vitrectomy. For all but IL-8, the hazard ratio compares the upper 50% levels versus the lower 50% levels. For IL-8, it denotes the increase in risk for an increase of 10 pg/mL in the concentration of IL-8.

* Indicates statistically significant result (P < 0.05).

We observed statistically significant higher concentrations of VEGF in patients with TRD compared with those without TRD. This difference suggests that the intraocular cytokine milieu is important in the development of tractional changes. Differences in vitreous levels of TNF-α have been reported between patients with PDR and proliferative vitreoretinopathy (PVR), supporting the idea that the development of tractional membranes may require a specific intraocular cytokine profile.

Statistically significant increases in the aqueous humor concentrations of IL-8 after IVB were observed in our study. The association of IL-8 with the risk of recurrent VH suggests that the observed increase in inflammatory mediators in this study is clinically significant. The association of IL-8 with postoperative VH may be explained by its role in angiogenesis. IL-8 mediates angiogenesis via both VEGF-dependent and independent mechanisms.

We also observed a statistically significant increase in aqueous humor TGF-β2 concentrations after IVB. The putative role of TGF-β2 in complications after IVB may be discerned based on its known biological functions. The TGF-β family of cytokines plays an important role in mediating fibrosis and scar contraction.
Altered Intraocular Milieu after Bevacizumab

The results of this study demonstrate that alterations in the intraocular cytokine milieu occur after IVB injection and that these alterations may be clinically significant. The angiogenic process is mediated by numerous growth factors and cytokines. The selective antagonism of one component of this process is likely to result in compensatory increases in other components, which may have deleterious effects in certain cases. Compensatory elevations of alternative angiogenic and inflammatory factors after VEGF inhibition are well described in animal and human studies. Compensatory angiogenic pathways after VEGF blockade that involve upregulation of FGF, PIGF, and erythropoietin have been described. Serum VEGF and PIGF levels have been shown to increase more than 10-fold in colorectal patients receiving systemic bevacizumab. A better understanding of these compensatory changes in the intraocular milieu after IVB will not only lead to strategies (e.g., combination therapy) to prevent the development of postinjection complications, but also a better ability to treat patients with DR and neovascular AMD.

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