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_2 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, 1.32; $P = 0.02$).

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,[7] transforming growth factor (TGF)-β,[9] placental growth factor (PlGF),[10,11] fibroblast growth factor (FGF) basic,[12] and tumor necrosis factor (TNF)-α[5] 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

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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 analytes 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-β_1, TGF-β_2, VEGF, PlGF, PEDF, 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 Akaike 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-β_1, 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, PlGF, IL-2, IL-6, IL-8, MCP-1, and TGF-β_2. 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 PlGF 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-β_2 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

Table 1. Demographics of Patient Cohort

Characteristic	Mean (±SD)
Age, y	57 (14)
Males, %	69
Type 1 diabetes, %	24
Tractional retinal detachment, %	34
Intravitreal triamcinolone during vitrectomy, %	83
Hemoglobin A1c, %*	8.2 (1.5)
Days between IVB pretreatment and vitrectomy, *n*	10 (5)

$n = 29$.

* All hemoglobin Alc values were obtained within 1 month of the date of vitrectomy.

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

Cytokine	Pre-bevacizumab (pg/mL) (95% CI)	Post-bevacizumab (pg/mL) (95% CI)	*P*
VEGF	1044 (583–1505)	116 (50–182)	0.0003*
PlGF	27.7 (14.3–41.2)	17.2 (12.0–22.5)	0.0887
IL-2	1.71 (1.27–2.16)	2.12 (1.63–2.62)	0.0652
IL-6	81 (−8–171)	1065 (−275–2406)	0.151
IL-8	42.3 (26.2–58.5)	59.3 (42.8–75.8)	0.0204*
MCP-1	2246 (1798–2693)	3314 (2152–4476)	0.0675
TGF-β_2	5554 (2770–8338)	8256 (5478–11034)	0.0009*

The data are adjusted for any of the following covariates based on the best-fitting model: age, sex, hemoglobin Alc, 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

Cytokine	Hazard Ratio (95% CI)	*P*
VEGF	0.61 (0.04–9.83)	0.73
PlGF	0.74 (0.11–4.80)	0.75
IL-2	0.57 (0.08–3.96)	0.57
IL-6	0.86 (0.13–5.74)	0.88
IL-8	1.32 (1.05–1.65)	0.02*
MCP-1	0.84 (0.13–5.62)	0.86
TGF-β_2	0.91 (0.81–1.02)	0.09

For IL-8 no covariates were included. For TGF-β_2, 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.

Discussion

We observed an expected decrease in the aqueous humor concentration of VEGF after IVB, which is consistent with previous observations.[27–29] 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. A novel anti-VEGF therapy that is currently in clinical trials for the treatment of DR and neovascular AMD is aflibercept (VEGF-Trap Eye; Regeneron Inc., Tarrytown, NY). In addition to binding VEGF, aflibercept is able to bind PlGF.[30] The ability to bind VEGF and PlGF may offer increased efficacy in the treatment of DR and neovascular AMD. PlGF plays a vital role in pathogenic angiogenesis, and antibodies targeting PlGF have been shown to cause tumor regression in animal models.[31,32] It is likely that our study was statistically underpowered to detect differences in PlGF levels after IVB. However, given the important role of PlGF in angiogenesis, the potential effect of IVB on PlGF that we observed deserves further investigation.

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),[33] 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.[34,35]

We also observed a statistically significant increase in aqueous humor TGF-β_2 concentrations after IVB. The putative role of TGF-β_2 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.[36–38] 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

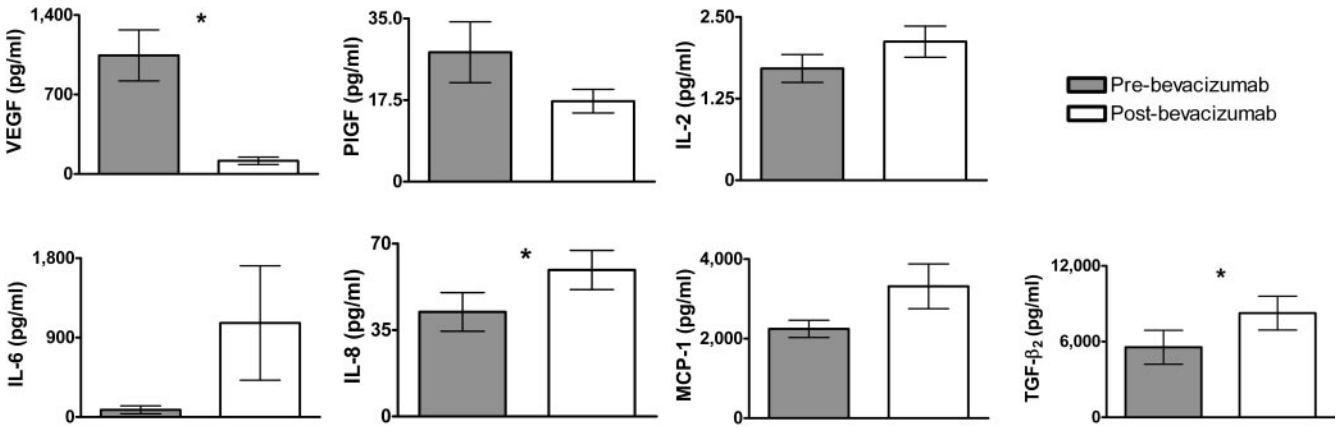

Figure 1. Adjusted mean aqueous humor concentrations of inflammatory cytokines after IVB. Error bars, SEM. *Statistically significant result ($P < 0.05$). Adjusted for any of the following covariates based on the best-fitting model: age, sex, hemoglobin A1c, presence of TRD, and number of days between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

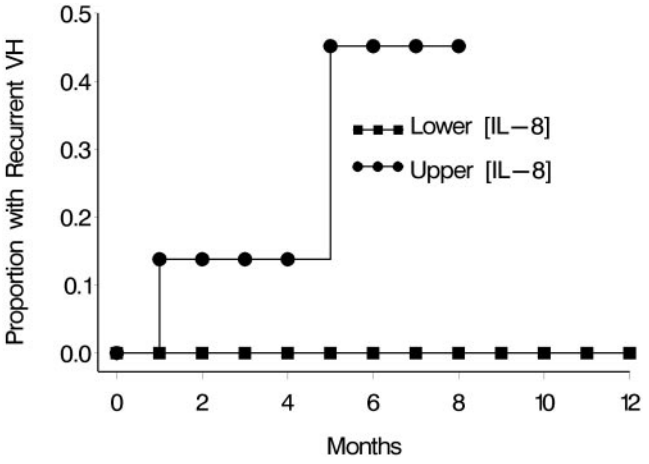

FIGURE 2. Proportion of patients with recurrent VH after pars plana vitrectomy. Life-table analysis showing proportion of patients with recurrent VH in the upper/lower 50th percentiles of IL-8 concentration at pars plana vitrectomy.

vitreous concentration of TGF-β_2.[39,40] Rapid contraction of the fibrovascular tissue within choroidal neovascular membranes and retinal neovascular membranes has been proposed as the underlying pathogenic mechanism of RPE tears and TRDs occurring after IVB.[19–23,41,42] An acute intraocular elevation of TGF-β_2 after IVB may thus mediate a rapid contraction of these membranes, thereby leading to excessive force on the RPE or retina and causing tears and detachments. Although we also observed an elevation in the intraocular concentrations of IL-2, IL-6, and MCP-1 after IVB, these results were not statistically significant. Our study was underpowered to detect statistically significant changes in these cytokines. Given the results of this study, future investigations into the levels of these cytokines after IVB are warranted.

A recent report of vitreous levels in eyes of patients with PDR who received pretreatment IVB has suggested that IVB influences intraocular mediators beyond VEGF.[29] This report was based on differences in vitreous cytokine levels at the time of PPV in eyes with PDR and IVB pretreatment compared with controls (eyes without IVB pretreatment). In addition to VEGF, these investigators found decreased levels of stromal cell-derived factor (SDF)-1α in eyes with IVB pretreatment. They did not find any significant differences in other inflammatory cytokines. Our paired study design of obtaining pretreatment and intraoperative aqueous samples from the same patients may have contributed to our ability to detect differences in cytokine concentrations that have not as yet been reported. Other papers have recently been published describing the use of multiplex assay technology to quantify cytokine levels in aqueous humor of patients with retinal vein occlusion,[28] exudative AMD,[43] and diabetic macular edema.[27] In two of these reports,[27,28] aqueous cytokine levels after IVB were measured, and no differences were found between post-IVB and baseline levels. However, the post-IVB sample in these reports was taken 1 to 3 months after the initial IVB injection, which may have been too long to detect the acute changes that we saw. Furthermore, concurrent intravitreal steroid, which may have diminished the increase in inflammatory cytokines, was administered in one of these studies.[27] Of interest, these investigators found a negative correlation between the levels of VEGF and those of IL-6 and MCP-1 in eyes after IVB injection (but not before IVB injection), supporting our observation of increased inflammatory mediators after a reduction in VEGF levels.

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.[44] 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.[45,46] Compensatory angiogenic pathways after VEGF blockade that involve upregulation of FGF,[47] PlGF,[48,49] and erythropoietin have been described.[50] Serum VEGF and PlGF levels have been shown to increase more than 10-fold in colorectal patients receiving systemic bevacizumab.[51] 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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