Levels of Erythropoietin and Vascular Endothelial Growth Factor in Surgery-Required Advanced Neovascular Glaucoma Eyes Before and After Intravitreal Injection of Bevacizumab

Minwen Zhou, Shida Chen, Wei Wang, Wenbin Huang, Bing Cheng, Xiaoyan Ding, and Xiulan Zhang

Zhongshan Ophthalmic Center, State Key Laboratory of Ophthalmology, Sun Yat-sen University, Guangzhou, People’s Republic of China

Correspondence: Xiulan Zhang, Professor of Ophthalmology, Vice Director of Glaucoma Department, Director of Clinical Research Center, Director of Institution of Drug Clinical Trials, Zhongshan Ophthalmic Center, State Key Laboratory of Ophthalmology, Sun Yat-sen University, 54 South Xianlie Road, Guangzhou, China 510060; zhangxl2@mail.sysu.edu.cn.

PURPOSE. To evaluate changes of levels of erythropoietin (EPO) and VEGF in aqueous humor before and after an intravitreal injection of bevacizumab (IVB) and determine the underlying correlation between them.

METHODS. This prospective study involved 21 eyes of 21 patients with surgery-required advanced neovascular glaucoma (NVG) and 20 control subjects from October 2011 through November 2012. The NVG eyes received the IVB treatment before antiglaucomatous surgery. Aqueous humor was collected at the time of the IVB injection (pre-IVB) and at the time of antiglaucomatous surgery (post-IVB). Aqueous humor and plasma VEGF and EPO levels were measured with ELISA and chemiluminescence methods, respectively.

RESULTS. The mean aqueous humor EPO and VEGF concentrations in pre-IVB eyes were significantly higher than those of the control subjects (P < 0.001), whereas plasma levels showed no significant difference. There was a statistically significant correlation between the aqueous humor EPO and the VEGF concentration (r = 0.612; P = 0.003). The mean aqueous humor VEGF in post-IVB eyes dramatically decreased from 1704.83 ± 757.82 to 19.02 ± 14.65 pg/mL (P < 0.001). However, EPO remained almost the same: 326.60 ± 104.28 compared with 312.67 ± 103.23 mU/mL (P = 0.675).

CONCLUSIONS. The NVG eyes showed high aqueous EPO and VEGF levels, and there was a positive correlation between them. However, levels of EPO did not change after post-IVB, whereas those of VEGF decreased.

Keywords: erythropoietin, vascular endothelial growth factor, neovascular glaucoma, intravitreal injection of bevacizumab

As is well known, VEGF is a primary angiogenic factor in the pathogenesis of ocular neovascular and neovascular glaucoma (NVG), which is induced by hypoxia. Studies have shown that the concentration of VEGF in the eye is greatly increased in proliferative diabetic retinopathy, central retinal vein occlusion, and NVG. Others have demonstrated that factors such as erythropoietin (EPO) and platelet-derived growth factor-C are also involved in neovascularization and that they play an important role in the pathogenesis of NVG.

EPO shows angiogenic activity in vascular endothelial cells, stimulating proliferation, migration, and angiogenesis in vitro, probably by means of the EPO receptor expressed in those cells. Such angiogenic activity involves several signal transduction cascades including extracellular signal-regulated kinase, Janus kinase 2 (JAK2), and the signal transducer and activator of transcription 5 (STAT5). Previous studies confirmed that the level of EPO in the aqueous humor is increased in eyes with POAG and pseudoexfoliative glaucoma (PXG). The cause of the elevated aqueous EPO concentration in eyes with POAG and PXG may be related to hypoxia, ischemia, or elevated reactive oxygen species caused by glaucomatous damage.

EPO and VEGF have been found to share a common pathway, with their expression induced by Hif1α in a hypoxia-dependent fashion. However, the correlation between EPO and VEGF is still controversial.

This study is the first to evaluate VEGF and EPO levels in the aqueous humor and the plasma of patients with NVG and compare these before and after an intravitreal injection of bevacizumab (pre-IVB and post-IVB, respectively).

PATIENTS AND METHODS

Subjects and Enrollment Criteria

This was a prospective, comparative study. It was approved by the Ethical Review Committee of Zhongshan Ophthalmic Center and adhered to the provisions of the Declaration of Helsinki for research involving human subjects. Written informed consent was obtained from all the participants involved in the study. All subjects were from a Chinese Han population. All NVG patients...
received IVB treatment first. Approximately 1 week later, only those with uncontrolled IOP with maximum antiglaucomatous medications were recruited for this study, prospectively and consecutively, and scheduled for antiglaucomatous surgery (FP-7 Ahmed glaucoma valve implantation [AGVI]) from October 2011 through November 2012 at the Glaucoma Department of Zhongshan Ophthalmic Center. The medications for decreasing IOP were applied as follows: topical β-blockers, topical carbonic anhydrase inhibitors, and topical α2-adrenergic agonists. Systemic medications to decrease IOP were applied if necessary.

A total of 21 eyes (21 patients) with NVG were recruited in this study, which included 1 case of secondary Eales’ disease, 1 case of secondary Coats’ disease, 8 cases of secondary diabetic retinopathy, and 11 cases of secondary ischemic central retinal vein occlusion. All patients underwent complete ocular examinations including best-corrected visual acuity, IOP, slit-lamp biomicroscopy, and funduscopic.

In the control group, the exclusion criteria included prior intraocular surgery and the presence of any other ocular diseases. All patients in the two groups were relatively healthy, with no systemic disease known to affect the plasma EPO concentration, such as kidney disease, arteriosclerotic disease (e.g., history of myocardial infarction, coronary artery disease), hematologic diseases (e.g., polycythemia vera, anemia), immune disease, history of drug usage (chemotherapeutic agents, iron preparations, granulocyte colony-stimulating factor, zidovudine), or diabetes mellitus (except the NVG group). The only systemic medications allowed were those used for arterial hypertension in all the groups and for diabetes mellitus in the NVG group.

**Anti-VEGF Injection**

All NVG patients received 0.05 mL of 1.25 mg IVB via an intravitreal injection using a 30-gauge needle in the inferior-temporal quadrant, 3.5 mm to 4 mm posterior to the limbus. The antiglaucomatous medications (introduced above) and anti-inflammatory eye drops (prednisolone acetate ophthalmic suspension, United States Pharmacopoeia; Allergan, Inc., Irvine, CA) were applied for controlling the IOP and the inflammatory response.

**Aqueous Humor Sampling**

Aqueous humor samples were obtained at two time points: (1) at the time of IVB administration (pre-IVB), and (2) at the time when sequential AGVI was performed (post-IVB). Aqueous humor was collected by means of anterior chamber paracentesis performed with a 30-gauge needle. Each aqueous humor sample was then placed into an Eppendorf tub and rapidly frozen at −80°C and was protected from light until the levels of VEGF and EPO were measured.

Aqueous humor samples were also collected from 20 eyes (20 patients) of healthy controls at the time of cataract surgery (phacoemulsification plus intraocular lens implantation) at the Glaucoma Department of Zhongshan Ophthalmic Center, from October 2011 to September 2012. All samples were obtained at the beginning of the surgery prior to any conjunctival or intraocular manipulation to avoid breakdown of the blood-aqueous barrier associated with surgical trauma.

**Blood Sampling**

Venous blood samples (5 mL) to test for plasma VEGF and EPO were collected in the fasting state, just before the patients were prepared for cataract surgery or received IVB treatment. The samples were centrifuged at 1000g for 15 minutes to obtain plasma, and then divided into aliquots and stored at −80°C until they were assayed.

**VEGF and EPO Analyses**

The levels of VEGF and EPO in the aqueous humor and the venous blood samples taken from the two groups were measured by a VEGF ELISA kit (MultiSciences, Hangzhou, China) and an EPO ELISA kit (eBioscience, San Diego, CA) according to the manufacturer’s protocol as previously described. In brief, 50 μL aqueous humor and 50 μL plasma sample were pipetted into 96-well plates precoated with a monoclonal antibody, specific for human VEGF and EPO, which does not cross-react with other isoforms or species. After 3 hours of incubation at room temperature, the wells were emptied and washed several times with the washing buffer provided. A second antibody was then added to the wells and incubated at 37°C for another 3 hours. After thoroughly washing off any excess antibody, a substrate was added to the wells and incubated at room temperature for 30 minutes in the dark. After the incubation, the reaction was terminated by adding a 100L stop solution to each well. The intensity of the color compound was measured using a multi-well plate reader (Multiskan Ascent; Thermo Fisher Scientific GmbH, Schwerte, Germany). To determine and correct for variability between the used ELISA plates, all samples were prepared and measured on the same day with the same standard preparation. Each experiment was performed in triplicate, and mean values of the determinations were used for the final results.

**Data Analysis**

The data were processed and statistically analyzed using SPSS for Windows XP (Version 13.0; SPSS, Chicago, IL). All data are expressed as mean ± SD. Categorical covariates were assessed individually with the χ² test. For comparison between the two different groups, an independent sample t-test was used to evaluate differences in the average between normal distributed data, while the Mann-Whitney U test was used when abnormal distributed data existed. Wilcoxon-signed rank test was used to detect changes pre-IVB and post-IVB within groups. Spearman’s rank-order correlation analysis was used to analyze the correlation between the aqueous humor EPO and the VEGF in the NVG patients, the correlation between the IOP and the aqueous humor EPO and the VEGF concentrations in the NVG group, and the correlation between the aqueous humor and plasma EPO and the VEGF concentrations in both groups. For all tests, P < 0.05 was considered significant.

**RESULTS**

**Patients’ Demographic Data**

Twenty-one NVG patients (21 eyes) and a normal control group of 20 subjects (20 eyes) who fulfilled the inclusion criteria were included in the study. All measurements were performed successfully, and there were no failures resulting from insufficient sampling in determining the VEGF or EPO. The mean ages of the NVG patients and the normal control individuals were 59.19 ± 10.95 (mean ± SD) and 62.40 ± 6.21 years, respectively. All data are summarized in Table 1. There were no significant differences in sex, mean age, and incidence of hypertension between the two groups.

**VEGF Levels at Pre-IVB in Aqueous and Plasma Samples**

Pre-IVB VEGF aqueous concentrations in the NVG and control subjects were 1704.83 ± 757.82 pg/mL (median, 1678.51 pg/
mL; range, 154.91–2714.95 pg/mL) and 199.68 ± 97.04 pg/mL (median, 172.36 pg/mL; range, 35.45–466.19 pg/mL), respectively. The level in the NVG subjects was higher than that of the control subjects, and the differences in the two groups were statistically significant (P < 0.001, by Mann-Whitney U test). However, there was no significant difference of the plasma VEGF concentrations between the NVG (10.08 ± 9.14 pg/mL: median, 7.77 pg/mL; range, 1.62–29.75 pg/mL) and the control group (12.61 ± 7.10 pg/mL: median, 11.76 pg/mL; range, 2.17–29.75 pg/mL) (P = 0.192, by Mann-Whitney U test) (Fig. 1).

EPO Levels at Pre-IVB in Aqueous and Plasma Samples

The mean pre-IVB aqueous humor level of EPO was 326.60 ± 104.28 mU/mL (median, 295.36 mU/mL; range, 189.57–511.01 mU/mL) in the NVG patients and 14.29 ± 4.39 mU/mL (median, 12.44 mU/mL; range, 8.21–25.27 mU/mL) in the control subjects. The EPO levels in the NVG group were significantly higher than those in the control group (P < 0.001, by Mann-Whitney U test). The differences in the plasma EPO concentrations between the NVG (10.08 ± 9.14 pg/mL: median, 7.77 pg/mL; range, 1.62–29.75 pg/mL) and the control group (12.61 ± 7.10 pg/mL: median, 11.76 pg/mL; range, 2.17–29.75 pg/mL) (P = 0.192, by Mann-Whitney U test) (Fig. 2).

Correlation Between the Aqueous Humor EPO and VEGF in the NVG Patients

The NVG group showed a statistically significant positive correlation between the VEGF and the EPO aqueous humor concentration (r = 0.513; P = 0.017) (Fig. 3).

Correlation Between the IOP and Aqueous Humor EPO and VEGF in the NVG Group

Neither the level of VEGF in the aqueous humor nor the EPO concentration was correlated with the IOP in the NVG group (all P > 0.05) (Figs. 4, 5).

VEGF and EPO Levels at Pre- and Post-IVB in the NVG Patients

Compared with pre-IVB, the mean aqueous humor VEGF concentration (1704.83 ± 757.82 pg/mL) significantly decreased post-IVB in the NVG eyes (19.02 ± 14.65 pg/mL, P < 0.001) (Fig. 6). The mean aqueous humor EPO concentration pre-IVB in the NVG group was 326.60 ± 104.28 mU/mL. Post-IVB, the level remained almost the same: 312.67 ± 103.23 mU/mL. The difference in the aqueous humor EPO concentration pre- and post-IVB did not reach statistical significance (P = 0.903) (Fig. 7).

Correlations of Aqueous Humor and Plasma VEGF and EPO Levels in the Study Groups

There were no significant correlations between the aqueous humor and the plasma VEGF levels in the two groups (all P > 0.05). The correlations between the aqueous humor and the plasma EPO also did not reach statistical significance in the groups (all P > 0.05) (Table 2).

DISCUSSION

In the present study, we found that the VEGF concentration showed a marked increase in the aqueous humor of the NVG eyes, a finding consistent with previous studies. This
confirms once again that VEGF plays an important role during angiogenesis in NVG. We also found that the VEGF concentration decreased to a dramatically low level after IVB treatment. Sawada and colleagues also reported similar results from diabetic retinopathy following IVB.20,21 Of importance, our study is the first to demonstrate that the EPO level in the aqueous humor did not respond to IVB treatment, although the EPO level was significantly increased in the NVG eyes pre-IVB. This finding seems to contrast with a report by Tam et al.18 who found that erythropoiesis is negatively regulated by endogenous VEGF in the animal models. When VEGF was blocked by the systemic blocking agent, it induced hepatic expression of EPO. Furthermore, VEGF inhibition could lead to an EPO increase through enhanced Hif1α feedback.22 The difference in our study, which showed that levels of EPO did not change after post-IVB, may be due to the topically applied IVB injected into the vitreous cavity, and there was only a topical effect.

This study confirmed that the levels of aqueous EPO in NVG were significantly higher than in control eyes. One small sample study (four cases of NVG) also found similar results.7 The increased concentrations of EPO in the NVG patients provide support for the idea that EPO is an important factor in the initiation and control of the ocular neovascular response. Hypoxia-induced retinal EPO expression and entry into the anterior chamber may be the main cause of the high concentrations.

**FIGURE 3.** The aqueous humor level of VEGF was significantly correlated with the level of EPO ($r = 0.513; P = 0.017$).

**FIGURE 4.** No correlation was observed between the IOP and the aqueous humor VEGF concentration in the NVG group ($r = 0.285; P = 0.211$). Each point represents a measurement from a single patient.

**FIGURE 5.** There was no correlation between the IOP and the aqueous humor EPO concentration in the NVG group ($r = 0.025; P = 0.913$). Each point represents a measurement from a single patient.

**FIGURE 6.** Compared with pre-IVB, the mean aqueous humor VEGF concentration significantly decreased post-IVB in the NVG eyes ($*P < 0.001$). Each point represents a measurement from a single patient.

**FIGURE 7.** There were no statistically significant differences pre- and post-IVB in the EPO concentration in the aqueous humor ($P = 0.903$). Each point represents a measurement from a single patient.
In addition to the remarkable angiogenic potential of EPO, several studies\textsuperscript{23,24} have verified that EPO has self-regulated neuroprotective effects by demonstrating an antiapoptotic role against ischemia-reperfusion injury. The increased level of EPO in glaucoma has been attributed to a compensatory mechanism, which results in an increase in glutamate, nitric oxide, and free radicals after glaucomatous damage.\textsuperscript{25} In short, the increased EPO in the NVG patients might result not only from retinal ischemia itself but also from a high IOP-induced self-regulated neuroprotective mechanism. Further research is required to shed light on this mechanism.

The present study found that IVB treatment does not directly affect EPO, although there was a positive correlation between the VEGF and EPO in the aqueous humor of the NVG patients. Thus, whether EPO acts independently of VEGF is still unclear. In clinical practice, we have found that ocular neovascularization of the iris does not disappear completely after anti-VEGF therapy. Therefore, we hypothesize that combination therapy,\textsuperscript{5} such as adjunctive EPO with VEGF blockade, is likely to be beneficial for the treatment of NVG. However, concerning the double mechanism mentioned above, EPO blockade may be hazardous for retinal diseases and NVG because of its role in neurologic protection.\textsuperscript{26} Further studies, including an analysis of neuronal side effects, will be necessary to determine whether EPO blockade is a practical and safe approach to the treatment of NVG.

This study took specific steps to eliminate potential inaccuracy. Thus, it measured the plasma levels of VEGF and EPO in the two study groups. It showed that the concentrations of plasma VEGF and EPO had no significance between the two groups. In addition, the correlation analysis revealed no correlation between the aqueous levels and plasma levels of VEGF and EPO in either group. These findings indicate that the increased aqueous levels of VEGF and EPO were not due to the systemic disease. Instead, they were likely the result of increased local production in the retina, followed by leakage into the anterior chamber.

Several limitations apply to this study. First, this study only considered a small size sample. Second, the antiglaucomatous drugs used in the treatment of NVG might have affected the level of EPO and VEGF concentrations in the aqueous humor. Third, the findings of the aqueous humor levels of EPO and VEGF do not allow us to draw conclusions about a causative relationship between these cytokines. To determine the pathogenesis of EPO in NVG, further studies are warranted of the local presence and intraretinal expression of EPO in eyes.

**Conclusions**

In conclusion, the present study indicated that EPO and VEGF are elevated in eyes with NVG. Anti-VEGF treatment did not act on EPO, and EPO may act independently of VEGF in NVG. Further studies are needed to determine whether combination therapy aimed at inhibiting VEGF and EPO simultaneously might provide better efficacy in inhibiting undesired blood vessel growth. However, we should be conscious that EPO remains a double-edged sword, with potentially both beneficial neuroprotective properties and nonbeneficial angiogenic activity.

**Acknowledgments**

Supported by the National Natural Science Foundation of China (Grant 81170849), Research Fund for the Doctoral Program of Higher Education of China (RFDP 20100171110077), and the Fundamental Research Funds of State Key Laboratory of Ophthalmology (2011C02). The authors alone are responsible for the content and writing of the paper.

Disclosure: M. Zhou, None; S. Chen, None; W. Wang, None; W. Huang, None; B. Cheng, None; X. Ding, None; X. Zhang, None

**References**

12. Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna J. Erythropoietin can promote erythroid progenitor...
survival by repressing apoptosis through Bcl-XL and Bcl-2.


