Assessment of Plasma Cytokine Profile Changes in Bevacizumab-Treated Retinopathy of Prematurity Infants

Lingkun Kong,1 Ann B. Demny,2 Ahmar Sajjad,1 Amit R. Bhatt,1 and Sridevi Devaraj3

1Department of Ophthalmology, Baylor College of Medicine, Houston, Texas, United States
2Department of Pediatrics, Baylor College of Medicine, Houston, Texas, United States
3Department of Clinical Pathology, Baylor College of Medicine, Houston, Texas, United States

Correspondence: Lingkun Kong, Department of Ophthalmology, Baylor College of Medicine, 6701 Fannin Street, Suite 610.25, Houston, TX 77030, USA; lkong@bcm.edu.
Submitted: October 28, 2015
Accepted: February 21, 2016
Citation: Kong L, Demny AB, Sajjad A, Bhatt AR, Devaraj S. Assessment of plasma cytokine profile changes in bevacizumab-treated retinopathy of prematurity infants. Invest Ophthalmol Vis Sci. 2016;57:1649–1654. DOI:10.1167/iovs.15-18528

Purpose. We compared changes of plasma angiogenesis cytokine profiles in infants who were treated with intravitreal injection of bevacizumab (IVB) for type 1 retinopathy of prematurity (ROP) with age-matched preterm non-ROP infants.

Methods. Thirteen infants with type 1 ROP and 13 age-matched preterm non-ROP infants were included. Blood samples were collected prior to treatment (time 0) and 6 weeks after the treatment (time 42). Plasma levels of nine cytokines from the angiogenesis growth factor panel and seven soluble cytokine receptors were measured using a magnetic multiplex assay.

Results. Plasma cytokine profiles changed from time 0 to time 42 in both groups. In bevacizumab-treated ROP infants, the following plasma angiogenesis growth factor and soluble cytokine receptor levels decreased significantly: soluble VEGF-A (sVEGF-A; \( P = 0.0001 \)), sVEGF-D (\( P = 0.04 \)), angiopoietin-2 (Ang-2; \( P = 0.002 \)), sVEGF receptor 1 (R1) and R2 (\( P = 0.005 \)), soluble IL-6 receptor (sIL-6R; \( P = 0.002 \)), soluble glycoprotein 130 (spg130; \( P = 0.0001 \)), and soluble TNF receptor (sTNFR) I and II (\( P = 0.0001 \)). The following factors and receptors increased significantly: sVEGF-C (\( P = 0.05 \)), placental growth factor (PIGF; \( P = 0.02 \)), endothelin-1 (ET-1; \( P = 0.0001 \)), and FGF-1 (\( P = 0.02 \)). At time 42, sVEGF-A, spg130, sIL-6R, sTNFR I, and sTNFR II were lower, and ET-1 level was higher, in bevacizumab-treated ROP infants compared to age-matched non-ROP infants.

Conclusions. The results suggest that bevacizumab treatment resulted in significant angiogenic cytokine profile changes in infants with severe ROP. The long-term clinical impact of these changes should be studied carefully.

Keywords: retinopathy of prematurity, intravitreal injection, Bevacizumab, cytokine, angiogenesis

Retinopathy of prematurity (ROP) is a vasoproliferative disorder that occurs in the developing retina of preterm infants. It is the leading cause of blindness in children,1 with an incidence rate of approximately 30% in preterm infants born at or earlier than 32 weeks gestation.2 Normal retinal vasculogenesis begins during the 14th week of gestation. This process consists of two stages: vasculogenesis followed by angiogenesis. In the vasculogenesis stage, precursor cells of mesenchymal origin enter the retina through the optic disc. These cells are responsible for the growth of the main retinal vessels. In the angiogenesis stage, capillary vessels increase in number and extend out from the optic disc into the peripheral retina.3 This process is tightly regulated by a complex network of angiogenic cytokines, extracellular matrix components, and growth factors. Vascular endothelial growth factor (VEGF) is an important component in this network and a major factor for the pathogenesis of ROP.4 Therefore, the goals of treatment are to reduce the production of VEGF by the immature retina and to eliminate the abnormal growth of new vessels. Laser photocoagulation of the peripheral avascularized retina is the treatment standard for type 1 ROP, with a failure rate of approximately 9.1%.5 Recently, intravitreal injection of the anti-VEGF antibody bevacizumab has been used as an off-label alternative therapy.6–11 However, there are concerns regarding the pharmacokinetics and the short- and long-term safety to ocular and neurologic development. Vascular endothelial growth factor is necessary for the development of systemic neurons and blood vessels in premature infants.12,13 and blockage of VEGF could potentially inhibit these processes. In adults, dose-dependent adverse effects from systemic bevacizumab-induced VEGF blockade have been reported, which include gastrointestinal perforations, wound-healing complications, hemorrhage, stroke or myocardial infarction, hypertension, proteinuria, and congestive heart failure.14

In our previous study, we described the pharmacokinetics of bevacizumab in premature infants and showed that intravitreal injection of bevacizumab reduced serum-soluble VEGF-A (sVEGF-A) levels15; however, the clinical significance of the reduction of sVEGF-A and its impact on other cytokines are unknown. We hypothesized that the reduction of sVEGF-A regulates the expression of other angiogenic cytokines secreted locally and systemically. To test this hypothesis, we assessed plasma cytokine profile changes in infants who were treated with bevacizumab and compared these profiles to age-matched non-ROP preterm infants. Our goal was to evaluate the potential negative effect of systemic bevacizumab treatment.
Plasma Cytokine Changes in Bevacizumab-Treated ROP

**Methods and Materials**

**Study Subjects**

The study followed the tenets of the Declaration of Helsinki, and informed consent was obtained from the parent or legal guardian of infants with ROP after explaining the study and its possible consequences. The informed consent was waived for non-ROP infants, and the research was approved by the institutional review board of Baylor College of Medicine.

Whole blood samples from 13 infants who were diagnosed as type I ROP were collected just prior to treatment (time 0) and 6 weeks after treatment (time 42). Plasma from 13 age-matched premature infants who did not develop ROP (non-ROP) and were not treated with anti-VEGF-related medications were collected between 32 and 34 weeks post menstrual age (PMA) (time 0) and 38 and 40 PMA (time 42) from salvaged blood at the Texas Children's Hospital clinical pathology laboratory. For plasma collection, whole blood was collected in a blood collection tube with 1.0 mg K2 EDTA (Microtainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Blood cells were removed by centrifuging at 3000g for 15 minutes at room temperature. The supernatant was aliquoted into clean polypropylene tubes and stored at -80°C until analysis.

The following clinical data were collected: ROP status at the time of treatment, gestational age (GA), birth weight (BW), gender, and time at first blood collection, wk 34.9.

**Measurement of Plasma Cytokines and Their Receptors**

A multiplex assay was used to measure plasma cytokines and their receptor levels. The protocols of the manufacturer were followed. The human angiogenesis/growth factor cytokine panel included the following factors: angiopoietin-2 (Ang-2), fibroblast growth factor (FGF)-1, FGF-2, sVEGF-A, sVEGF-C, sVEGF-D, endothelin-1 (ET-1), bone morphogenetic protein (BMP)-9, and placental growth factor (PIGF; Catalog # HAGP1MAG-12K, Millipore, Billerica, MA, USA). The human soluble cytokine receptor magnetic bead panel included s-interleukin (IL) 6R, soluble tumor necrosis growth factor receptor (sTNFR)I, sTNFRII, VEGF receptor (R)1, R2, R3, and soluble glycoprotein (sgp)130 (Catalog # HSRMAG32KPX14; Millipore, Billerica, MA, USA) (Table 1).

**Statistics**

Statistical analyses were performed using software SPSS version 21 (IBM, Software, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). Generalized estimating equation (GEE) models were used to analyze the relationships among variables. One-way analysis of variance (ANOVA) with a post-hoc multicomparison test was used to compare the three groups of data. Comparisons among variables were adjusted for GA and BW. Fisher's exact test was used for proportions. The confidence interval level was 95%. A P value less than 0.05 was considered statistically significant.

**Results**

Table 2 shows the demographic data of the subjects. Thirteen infants with type I ROP and 13 age-matched preterm non-ROP infants were included in the study. Infants with type I ROP were on average younger than non-ROP infants (P = 0.01). The BW of ROP infants was lower than non-ROP infants but was not statistically significant (P = 0.15). Post menstrual ages at the time blood samples were collected were similar in both groups.

**Changes of Plasma Cytokines With Time**

Nine soluble cytokines from the angiogenesis growth factor panel and seven soluble cytokine receptors were quantitated. In infants with ROP treated with bevacizumab, from time 0 to time 42, the following plasma angiogenesis growth factors and soluble cytokine receptor levels decreased significantly: sVEGF-A, sVEGF-D, Ang-2, sVEGF R1 and R2, sIL-6R, sgp130, and sTNFR I and II (P values are indicated in Fig. 1 and Fig. 2). The following factors and receptors increased significantly: sVEGF-C, PIGF, ET-1, and FGF-1 as shown in Figure 1 (growth factors) and Figure 2 (receptors). In non-ROP infants, a significant decrease was seen over the time course in levels of sVEGF-A, Ang-2, sVEGF R3, sIL-6R, and sTNFR I, and an increase was seen in PIGF levels. All other cytokines remained at similar levels from time 0 to time 42.

**Comparison of Plasma Cytokine Profiles in Bevacizumab-Treated Type I ROP Infants Versus Non-ROP Infants**

The plasma cytokine levels at pre- and posttreatment in both type I ROP and non-ROP infants are shown in Figure 1 (growth factors) and Figure 2 (receptors). In bevacizumab-treated ROP infants, plasma levels of VEGF-A (P = 0.001), sIL-6R (P = 0.0001), and sTNFR II (P = 0.0001) were significantly lower at time 42 compared to those in age-matched non-ROP infants. The plasma ET-1 level was significantly higher in bevacizumab-treated ROP infants (P = 0.004). The following plasma cytokine levels were similar in both groups at time 42: VEGF-C (P = 0.5), VEGF-D (P = 0.1), PIGF (P = 0.6), BMP-9 (P = 0.1), FGF-1 and FGF-2 (P = 0.4 and P = 0.2, respectively), Ang-2 (P = 0.1), and VEGF R1, VEGF R2, and VEGF R3 (P = 0.5, 0.2, and 0.3, respectively).

**Table 1. Plasma Cytokines Tested**

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Range of Measurement, pg/mL</th>
<th>Receptor</th>
<th>Range of Measurement, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>14–10,000</td>
<td>VEGF R1</td>
<td>122–500,000</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>7–5,000</td>
<td>VEGF R2</td>
<td>122–500,000</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>7–5,000</td>
<td>VEGF R3</td>
<td>122–500,000</td>
</tr>
<tr>
<td>Ang-2</td>
<td>14–10,000</td>
<td>sIL-6R</td>
<td>12–50,000</td>
</tr>
<tr>
<td>ET-1</td>
<td>3–2,000</td>
<td>sgp130</td>
<td>24–100,000</td>
</tr>
<tr>
<td>BMP-9</td>
<td>3–2,000</td>
<td>sTNFR I</td>
<td>12–50,000</td>
</tr>
<tr>
<td>PIGF</td>
<td>2–1,000</td>
<td>sTNFR II</td>
<td>12–50,000</td>
</tr>
<tr>
<td>FGF-1 and –2</td>
<td>14–10,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Characteristics of Preterm Infants With and Without Retinopathy of Prematurity**

<table>
<thead>
<tr>
<th>Type I ROP</th>
<th>Non-ROP</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight, g</td>
<td>778 ± 66</td>
<td>941 ± 96</td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>25.6 ± 0.6</td>
<td>28.0 ± 0.6</td>
</tr>
<tr>
<td>Gender, male (%)</td>
<td>7 (54)</td>
<td>6 (46)</td>
</tr>
<tr>
<td>PMA at first blood collection, wk</td>
<td>34.9 ± 1.7</td>
<td>34.9 ± 0.5</td>
</tr>
</tbody>
</table>
DISCUSSION

Bevacizumab is a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A.\textsuperscript{17} In our previous study, we showed that serum sVEGF-A levels were significantly reduced after bevacizumab treatment in severe ROP patients and remained at low levels.\textsuperscript{15} The reduction of sVEGF levels by bevacizumab could interrupt angiogenesis in developing organs, raising major safety concerns regarding the use of bevacizumab in premature infants. To address this issue, we evaluated the levels of plasma angiogenesis factors and soluble cytokine receptors in infants with ROP before and after bevacizumab treatment and compared these to premature infants who did not develop ROP as a control group. Our hypothesis was that developmental stages are a more significant source of variation in angiogenic cytokine levels than are ROP and bevacizumab treatment.

Angiogenesis is a complex process that is regulated precisely by many angiogenic cytokines. Vascular endothelial growth factors are the major growth factors in this process. The mammalian family of VEGF ligands consists of VEGF-A, -B, -C, -D, and -E, and PlGF.\textsuperscript{18} Our first objective was to determine whether the circulating bevacizumab reduced plasma levels of other VEGF ligands. We evaluated four VEGF family members, sVEGF-A, sVEGF-C, sVEGF-D, and PlGF (VEGF-B and -E were not measured in this study due to the availability of testing beads), and their soluble receptors R1, R2, and R3. As shown in Figures 1 and 2, after bevacizumab treatment, the plasma levels of sVEGF-A, sVEGF-D, VEGF R1, and VEGF R2 were significantly reduced; however, only the sVEGF-A level was significantly lower than that of the control group ($P = 0.0001$). Plasma levels of VEGF-C, VEGF-D, and PlGF were slightly higher in bevacizumab-treated ROP infants compared with non-ROP infants. The levels of all three soluble receptors were similar between these two groups. These results suggest that the changes of VEGF and its receptor level in the plasma are mainly related to the changes of ROP status (in ROP infants) and developmental status, except sVEGF-A in bevacizumab-treated ROP infants. Our next step was to analyze the differences in total plasma VEGF levels between the two groups using the GEE statistical model. The results showed that plasma levels of total VEGFs were similar in bevacizumab-

![Figure 1. The changes of angiogenesis growth factors in ROP and non-ROP infants. $P$ values are indicated on the top of each paired column. Black solid column is for ROP group, and the white column is for non-ROP group.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/935164/)
treated ROP infants 6 weeks after treatment as they were in the control group: 1897.7 ± 363.9 pg/mL versus 2226.1 ± 224.0 pg/mL, respectively (P = 0.29). These results suggest that systemic absorption of bevacizumab reduces plasma levels of sVEGF-A; however, the total sVEGF level remained similar to that in the controls.

Vascular endothelial growth factors act within a complex regulatory network, binding selectively to different receptors and activating networks that regulate angiogenesis, cell growth, and survival. Vascular endothelial growth factor-A is the most prominent and founding member, and it binds to both VEGF R1 and R2. Placental growth factor binds exclusively to VEGF R1 and could have comparable activity to VEGF. Vascular endothelial growth factor-C and VEGF-D bind to VEGF R3, but can be proteolytically processed to allow binding to VEGF R2. We hypothesize that the activity of other VEGF family members could compensate for the loss of sVEGF-A function and therefore that bevacizumab-treated infants could have an angiogenic process similar to that of the control group.

In this study, we hypothesized that reduction VEGF-A regulates the expression of other angiogenic cytokines. To test this hypothesis, we measured plasma levels of nine soluble growth factors and seven soluble cytokine receptors from the human angiogenesis/growth factor panel using the magnetic multiplex assay. The results showed that, in contrast to the reduction of sVEGF-A levels, plasma ET-1 levels increased significantly in infants with type I ROP at 42 days posttreatment when compared with the control group. Endothelin-1 is produced by both endothelial cells and vascular smooth
Plasma Cytokine Changes in Bevacizumab-Treated ROP

muscle cells, and its concentration is elevated in many tumors. The relationship between VEGF-A and ET-1 has been controversial. One study reported that ET-1 stimulates vaso-proliferative processes, and at maximally effective concentrations its effects were additive to that of VEGF by functioning through independent signaling mechanisms.

Other studies reported that VEGF enhanced ET-1 mRNA expression and ET-1 secretion in endothelial cells and vascular smooth muscle cells. This suggests that VEGF and ET-1 have reciprocal secretion in endothelial cells and vascular smooth muscle cells. However, one study also reported that ET-1 and VEGF have a regulatory relationship. Our results indicate that the reduction of sVEGF-A upregulated the expression of ET-1 in the plasma. The elevation of plasma ET-1 levels could be beneficial to infants by compensating for the loss of VEGF-A. However, this elevation itself could raise other concerns related to the overexpression of ET-1, such as increased smooth muscle cell proliferation, which is seen in persistent pulmonary hypertension of newborn infants. The long-term clinical impacts therefore need to be studied carefully.

A limitation of this study is the small sample size. A multicenter clinical placebo-controlled trial with a large sample is therefore necessary to study the safety of bevacizumab, both clinically and at the molecular level.

This work has direct clinical implications for the treatment of ROP. The findings indicate that levels of plasma angiogenesis growth factors and soluble cytokine receptors differ in ROP infants following bevacizumab treatment compared to those in age-matched control infants. Some of these differences (SPG130, H6R, and sTNFR I and sTNFR II) could be related to systemic health problems such as decreased humoral immune responses in preterm neonates and chronic lung disease. In general, ROP infants are not as healthy as non-ROP infants. However, it is clear that sVEGF-A levels decreased and ET-1 levels increased in bevacizumab-treated ROP infants. The reduction of sVEGF-A confirms the specific binding activity of bevacizumab. This raises another question: whether monotherapy with bevacizumab is sufficient for the treatment of severe ROP. The impact on lung and cardiac function of the changes in growth factor levels that we have described here should be monitored carefully.

Acknowledgments

The authors thank the medical staff at the Texas Children’s Hospital neonatal intensive care unit and Baylor College of Medicine for their invaluable assistance with the study patients. Supported by a grant from the Knights Templar Eye Foundation (1401).

Disclosure: L. Kong, None; A.B. Demny, None; A. Sajjad, None; A.R. Bhatt, None; S. Devaraj, None

References


