PLASMA CONCENTRATIONS OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN RETINOPATHY OF PREMATURITY AFTER INTRAVITREAL BEVACIZUMAB INJECTION

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Purpose: To investigate the changes in plasma concentrations of vascular endothelial growth factor (VEGF), insulin growth factor (IGF-1), erythropoietin, pigment epithelium–derived factor, and IgG1 after bevacizumab intravitreal injection in infants with retinopathy of prematurity.

Methods: Eleven eyes from six infants who received intravitreal injection of bevacizumab were enrolled in this study. At preinjection and postinjection 1, 2, 3, 4, 5, 6, 7, and 8 weeks, 0.5 mL of blood was collected from each infant. The plasma concentrations of VEGF, insulin growth factor, erythropoietin, pigment epithelium–derived factor, and IgG1 were measured by enzyme-linked immunosorbent assay. Five patients received simultaneous bilateral bevacizumab injection; one patient received unilateral injection.

Results: Of the infants who received intravitreal bevacizumab injection, two were males and four were females. The mean gestational age was 26 ± 2 weeks. The mean birth weight was 870 g. The mean plasma VEGF concentration before bevacizumab injection was 2.05 ± 3.00 ng/mL; plasma level decreased significantly to 0.16 ± 0.10 ng/mL and to 0.14 ± 0.14 ng/mL ($P = 0.028$) after 1 week and 2 weeks, respectively. Moreover, the plasma concentrations of VEGF did not return to the original level in any of the samples until 8 weeks after the injection. However, mean plasma IgG1, erythropoietin, insulin growth factor, and PEDF concentrations did not change significantly during the interval between preinjection and any other follow-up time points.

Conclusion: Intravitreal bevacizumab injections significantly reduce plasma VEGF concentration in infants with retinopathy of prematurity over a 7-week period.

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Retinopathy of prematurity (ROP)[1] is a developmental disease of low-birth-weight infants that potentially leads to blindness; ROP is the leading cause of infant blindness in developing countries.[2] The immature peripheral avascularized retinas in preterm infants cause vascular proliferation from the vascularized central retina. A multicenter trial of cryotherapy for ROP showed that cryoablation of the peripheral retina was effective in reversing retinopathies,[3] and their 15-year follow-up study confirmed the long-term benefits of this therapy.[4] Until recently, the peripheral avascular retina was ablated by laser photocoagulation, instead of cryotherapy, with similar therapeutic success.[5] A recent study demonstrated the success of anti–vascular endothelial growth factor (VEGF) intravitreal injections[1] with bevacizumab (Avastin, Genentech Inc., South San Francisco, CA) in reversing ROP. The rationale for this therapy is that the high concentration of VEGF in the vitreous,

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caused by hypoxia of the avascularized peripheral retina after preterm birth, leads to retinal vascular proliferation seen in ROP.[1]

Vascular endothelial growth factor is a critical cytokine that becomes elevated in the vitreous at roughly 31-week to 41-week postconception age and is involved in the pathogenesis of ROP.[7] Early treatment with cryoablation or laser ablation can reduce the level of intravitreal VEGF and stop the progression of ROP. However, this treatment could also damage the peripheral retina and can cause severe myopia and loss of visual field in the future, which would be a worse outcome for patients who start treatment with a mild case of ROP (i.e., Zone 1 ROP). These side effects could be reduced if anti-VEGF intravitreal injection is effective for reversing the pathologies of ROP. However, VEGF is well established as a key molecule involved with development of organs in infants, especially during vasculogenesis.[8] Another challenge to treating ROP is that processes of angiogenesis are also associated with neurogenesis processes[9]; therefore, the impacts of anti-VEGF intravitreal injection on systemic VEGF levels and other related cytokines in infants should be evaluated carefully.

A current study on ROP shows that systemic VEGF levels were suppressed for 2 weeks after bevacizumab intravitreal injection.[10] In adults, the reduction was detected for at least 1 month after bevacizumab intravitreal injection, and their systemic adverse events also have been reported such as higher risk of myocardial infarction.[11–13] Another study of ranibizumab injection therapy found that VEGF suppression decreases and levels return to preinjection status after 4 weeks.[14] However, there have been no studies about whether suppression lasts longer after bevacizumab intravitreal injections. The reduction of systemic VEGF in rapidly developing infants with low weight and early gestational age raises concerns about the side effects of these injections. Here, we show that avastin intravitreal injection (AVI) in infants could suppress plasma VEGF, at least for 7 weeks, while causing no significant changes to the levels of other secreted proteins, such as pigment epithelium–derived factor (PEDF), insulin growth factor (IGF)-1, and erythropoietin.

Methods

This study complies with the tenets of the Declaration of Helsinki. All study protocols were approved by the Gospel Hospital Institutional Review Board. Informed consent was obtained from the parents of all patients.

Routine fundus examination for ROP was conducted by indirect ophthalmoscopy with sclera indentation using topical anesthesia. Patients' ROP stage was determined using the International Classification of Prematurity System while under general anesthesia.[15] We included 11 eyes from 6 patients who were classified as having threshold ROP with plus disease in Zone 1 or Zone 2.

After general anesthesia with sedation and irrigation with povidone–iodine, 0.6 mg (0.025 mL) of bevacizumab (Avastin 100 mg/4 mL) patients were intravitreally injected with a sharp 30-gauge needle into the eye, 1.5 mm posterior to the limbus under sterile conditions in an operating room. A sterile cotton tip was applied to the injection site to avoid reflux after the needle was removed. After the injection procedure, topical antibiotics, steroid, and antiglaucoma medication were prescribed.

Any blood remaining after routine blood tests was collected in sterile tubes with EDTA and centrifuged at 5,000 rpm for 10 minutes until a clear separation between plasma and cell components was observable. After centrifugation, blood plasma was stored at −80°C until analysis before AVI and 1, 2, 3, 4, 5, 6, 7, and 8 weeks after AVI.

Enzyme-Linked Immunosorbent Assay

Nunc-Immuno microplates (MaxSorp F96 Lot #0890; Nalge Nunc International, Penfield, NY) were used for the solid phase for the enzyme-linked immunosorbent assay. Assays were conducted at room temperature with gentle shaking, except during the color reaction when shaking was vigorous. Wells were coated with capture proteins by exposure to 100 ul of recombinant human VEGF (1 μg) in phosphate-buffered saline (pH = 7.4) for 16 hours (or overnight). Unbound protein was poured out, and wells were treated with 150 μL of 5 g/L bovine serum albumin (BSA) solution in phosphate-buffered saline for 1 hour to reduce any nonspecific binding by blocking any free space on the microplate well walls after coating. After washing the wells 4 times with washing buffer (PBST: phosphate-buffered saline containing 5 g/L of Tween-20) and shaking them until dry, we added to each well 100 μL of VEGF standard, control, or unknown bevacizumab solutions (diluted in PBST/BSA: phosphate-buffered saline containing 5 g/L of BSA and 5 g/L of Tween-20) and incubated the mixture for 2 hours. The control and bevacizumab samples were diluted to a concentration of 1:1,000. The plates were washed 5 times with washing buffer; we then added to each well 100 μL of rabbit anti-human IgG diluted with PBST/BSA. These were incubated for 2 hours and washed again 5 times with washing buffer and then dried by shaking. To quantitate the rabbit antibodies during the

solid phase after the last step, 100 μL of goat anti-rabbit IgG–HRP was added to each well (1:5,000, diluted with PBST/BSA) and incubated for 1.5 hours. The plate was washed five times with washing buffer and shaken dry. The amount of the solid phase conjugate was quantitated by the enzymatic activity of HRP. The absorbance of the orange-colored product was measured at 450 nm within 10 minutes in the vector III microplate reader with computer control.

Patient VEGF, IGF-1, PEDF, erythropoietin, and IgG concentrations were measured with the Human VEGF-A Quantikine kits (R&D Systems, Minneapolis, MN). Working standards were prepared according to manufacturer's instructions. Assay Diluent RD1W (100 μL; R&D Systems) was added to the wells of a 96-well plate coated with monoclonal anti-VEGF/VEGFR1–2 antibodies. Samples, standards, and controls were subsequently dispensed in duplicate and incubated for 2 hours at room temperature. The plate was washed three times in a wash buffer. Next, VEGF/VEGFR1–2 conjugate (200 μL) was added to each well and incubated for 2 hours; washings were repeated. Under dark conditions, 200 μL of substrate solution (provided with the kit) was added, and the reaction was stopped after 25 minutes of incubation. Bound VEGF/VEGFR1–2 was detected by measuring absorbance at 450 nm in a photometer, and concentration was determined from a standard curve.

Statistics

Statistical analyses were performed using SPSS software version 18.0 (IBM, Armonk, NY). Data are presented as means and standard deviations. The statistic significant difference is $P < 0.05$, using Wilcoxon test or Mann–Whitney U test.

Results

A total of 11 eyes from 6 infants (2 males and 4 females) were injected with bevacizumab. Average body weight and gestational age at birth were 870 ± 103.87 g and 26.2 ± 2.6 weeks. The average week postconception age and body weight at injection were 34.6 ± 1.9 weeks and 1518.5 ± 369.32 g. Bilateral injection was performed on five pairs of eyes (Patients 1–5) and unilateral injection on one eye of Patient 6. The number of plasma samples was 6, 6, 6, 3, 2, 2, 3, 2, and 1 samples at 1 day and 1, 2, 3, 4, 5, 6, and 7 weeks after the injection, respectively (Table 1).

Plasma VEGF concentrations before injection, 1 week after injection, and then again at 2, 3, 4, 5, 6, and 7 weeks were 2.05 ± 3.00 ng/mL, 0.16 ± 0.09 ng/mL, 0.14 ± 0.13 ng/mL, 0.13 ± 0.01 ng/mL, 0.10 ± 0.02 ng/mL, 0.06 ± 0.02 ng/mL, 0.07 ± 0.01 ng/mL, and 0.06 ± 0.01 ng/mL, respectively (Figure 1A). The reduction of plasma VEGF concentrations after AVI was significantly decreased from the preinjection level ($P < 0.05$). In contrast, plasma IgG1, erythropoietin, IGF-1, and PEDF concentrations showed no significant change from preinjection levels at any of the time points after AVI. The IgG1 plasma concentrations before injection, 1 week after injection, and then again at 2, 3, 4, 5, 6, and 7 weeks were 8.62 ± 0.56 μg/mL, 8.34 ± 0.35 μg/mL, 8.29 ± 0.52 μg/mL, 8.08 ± 0.47 μg/mL, 8.01 ± 0.42 μg/mL, 7.83 ± 0.15 μg/mL, 8.05 ± 0.23 μg/mL, and 7.83 ± 0.14 μg/mL, respectively (Figure 1B). The erythropoietin plasma concentrations before injection, 1 week after injection, and then again at 2, 3, 4, 5, 6, and 7 weeks were 22.54 ± 16.35 μg/mL, 26.92 ± 27.62 μg/mL, 35.36 ± 36.46 μg/mL, 16.35 ± 11.43 μg/mL, 32.80 ± 21.44 μg/mL, 9.66 ± 2.83 μg/mL, 26.21 ± 19.68 μg/mL, and 11.24 ± 3.06 μg/mL, respectively (Figure 1C). The IGF-1 plasma concentrations before injection, 1 week after injection, and then again at 2, 3, 4, 5, 6, and 7 weeks were 57.46 ± 98.16 μg/mL, 22.49 ± 12.46 μg/mL, 25.15 ± 11.91 μg/mL, 16.89 ± 9.66 μg/mL, 28.71 ± 6.48 μg/mL, 31.67 ± 5.61 μg/mL, 40.71 ± 14.35 μg/mL, and 42.18 ± 13.58 μg/mL, respectively (Figure 1D). The PEDF plasma concentrations before injection, 1 week after injection, and then again at 2, 3, 4, 5, 6, and 7 weeks were 154.91 ± 73.67 μg/mL, 141.04 ± 51.00 μg/mL, 131.49 ± 42.18 μg/mL, 193.90 ± 13.19 μg/mL, 179.67 ± 6.36 μg/mL, 180.59 ± 10.23 μg/mL, 195.47 ± 4.98 μg/mL, and 192.85 ± 18.68 μg/mL, respectively (Figure 1E).

Discussion

Our study shows that plasma VEGF concentrations from 1 week to 7 weeks were significantly lower after intravitreal bevacizumab injection compared with the concentration of preinjection; however, concentrations of other secreted proteins, including erythropoietin, IGF-1, and PEDF showed no significant changes during the same period. Systemic absorption of intravitreal bevacizumab has an impact on VEGF plasma levels specifically, and the impacts for infants with ROP last up to 7 weeks after the injection.

Systemic absorption of bevacizumab (1.25 mg) after intravitreal injection in patients with diabetes and exudative age-related macular degeneration has been shown to reduce VEGF and PEDF plasma concentrations for up to 1 month (VEGF) and 7 days (PEDF) after the injection.[11,16] Another recent study found that reduced plasma VEGF after the injection began to

Table 1. Demographic Characteristics of Infants who Received Intravitreal Bevacizumab Injection

Patient	Gender	Gestational Age (Weeks + Days)	Birth Weight (g)	Age at Treatment (Gestational Age), Weeks + Days	Weight at Treatment (g)
1	M	26 + 3	955	33 + 5	1365
2	F	25	730	33 + 2	1325
3	F	26 + 3	920	35 + 1	1833
4	M	25 + 1	840	34 + 5	1955
5	F	25	780	32	963
6	F	31 + 5	995	37 + 6	1670
Mean		26 + 2	870	34 + 4	1519

increase after 1 month but never reached baseline preinjection concentration in diabetic adults.[6] These data provide strong evidence that bevacizumab intravitreal injection has a significant reducing effect on systemic VEGF and PEDF, at least for 1 month in adults.

For infants, information regarding changes in systemic cytokine levels after injection is very limited. Sato et al[10] recruited 11 infants and collected plasma samples at baseline and then again after injection with 0.5 mg to 1.0 mg of bevacizumab at 1 day after injection, 1 week, and then 2 weeks; they found that plasma VEGF at 1 week and 2 weeks after injection was significantly decreased compared with preinjection levels. This study includes no data beyond 2 weeks after injection. Another case report that measured plasma VEGF concentration after ranibizumab (0.2 mg) intravitreal injection in a single infant found that VEGF concentration was lowest 3 weeks after injection, but by postinjection Week 4, the concentration recovered to preinjection levels.[14] However, there is no recovery of plasma VEGF concentration until 7 weeks after bevacizumab intravitreal injection in our study. This difference could be explained by different clearance rate in vitreous and systemic absorption of

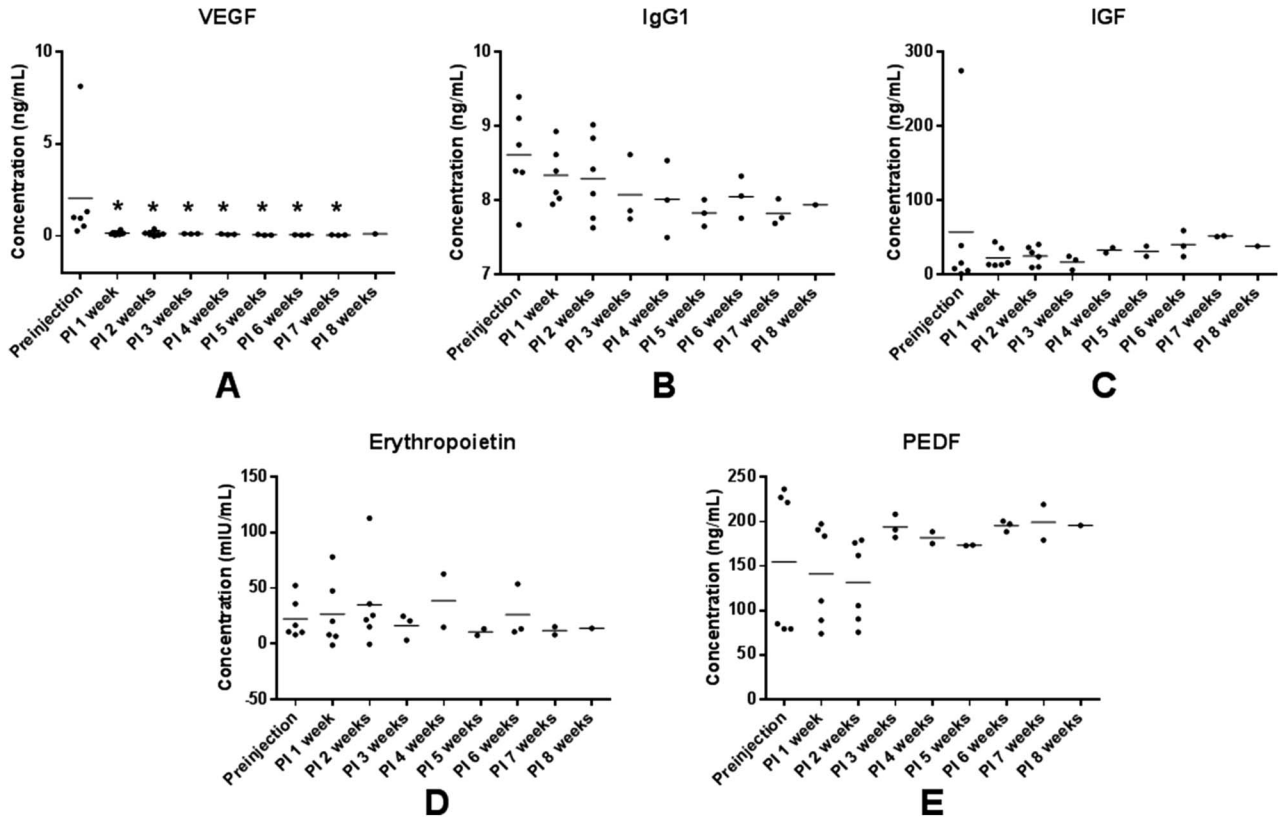

Fig. 1. Plasma concentration of VEGF (**A**), IgG1 (**B**), IGF (**C**), erythropoietin (**D**), and PEDF (**E**) at preinjection and postinjection 1, 2, 3, 4, 5, 6, and 7 weeks after bevacizumab intravitreal injection.[6] The concentrations of plasma VEGF after AVI significantly decreased over time. The plasma concentrations of other cytokines and IgG1 did not show any significant changes after AVI (*$P < 0.05$).

ranibizumab and bevacizumab.[17–19] In addition, four cases of bilateral VEGF suppression effect after unilateral bevacizumab intravitreal injection in ROP showed clearly bevacizumab entrance into systemic circulation.[20]

Vascular endothelial growth factor is a critical cytokine during organ development.[8] Studies of the effects of systemic VEGF inhibitors in mice have been shown that loss of fenestration of the organs such as pancreatic islets, thyroid, adrenal cortex, pituitary, villi of small intestine, kidney, and choroid plexus as a result of VEGF suppression.[21–23] The magnitude of capillary loss increased in case of younger age of mice after 10 days of anti-VEGF treatment.[24] Especially, the choroid capillary and plexus composed of fenestrated vessels need VEGF for their development and maintenance.[25,26] These developing organs of premature infant having fenestrated vessels can be exposed to the potential risks of systemic and intraocular VEGF suppression caused by intravitreal bevacizumab injection.

Based on our study's data, the reduction over a 7-week period of plasma VEGF could have negative developmental side effects, including systemic crisis in the injected infant. Therefore, bevacizumab injection should be considered with great caution for treatment of ROP in infants.

Key words: retinopathy of prematurity, VEGF, bevacizumab, IGF, PEDF, erythropoietin.

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