

Original Investigation

Serum Levels of Vascular Endothelial Growth Factor and Related Factors After Intravitreal Bevacizumab Injection for Retinopathy of Prematurity

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IMPORTANCE Intravitreal injections of bevacizumab (IVB) have been found to be effective for the treatment of retinopathy of prematurity (ROP). However, serum levels of vascular endothelial growth factor (VEGF) have been found to be suppressed for 2 weeks after IVB in patients with ROP. Changes in serum VEGF levels after IVB in patients with ROP may be important because VEGF also plays a role in the neurodevelopment of newborns.

OBJECTIVE To investigate the correlation of levels of VEGF and related growth factors with bevacizumab levels in the systemic circulation after IVB in patients with type 1 ROP.

DESIGN, SETTING, AND PARTICIPANTS We studied a prospective case series at an institutional referral center from December 1, 2011, through February 28, 2013. We enrolled patients with type 1 ROP who received IVB. We collected blood samples before and for as long as 8 weeks after IVB. The samples were tested for serum levels of bevacizumab and growth factors, including VEGF, VEGF receptor 1 (VEGFR1), VEGFR2, Tie2, erythropoietin, transforming growth factor β 1, insulinlike growth factor type 1, angiotensin 1, angiotensin 2, angiotensinlike 3, and angiotensin 4. The serum concentrations of these factors were measured using enzyme-linked immunosorbent assays.

MAIN OUTCOMES AND MEASURES Serum levels of VEGF, bevacizumab, and the other growth factors before and for as long as 8 weeks after IVB.

RESULTS We enrolled 8 patients with type 1 ROP. Bevacizumab levels were elevated 1 day after IVB in the 3 patients for whom measurements were available (mean [SD], 1425 [1010 (95% CI, 0-3934)] ng/mL; $P = .13$) and remained detectable in the serum as long as 8 weeks after IVB (285 ng/mL for the 1 patient with a measurement available). Serum VEGF levels were suppressed for the same period (mean [SD] level at 1 day after IVB, 379 [226 (95% CI, 190-568)] pg/mL for the 3 patients with measurements available; at 8 weeks, 216 pg/mL for the 1 patient with a measurement available). We found a negative correlation between the serum levels of bevacizumab and VEGF in the patients with ROP who received IVB ($r = -0.43$ [95% CI, -0.67 to -0.10]; $P = .01$). No changes were identified in the serum levels of any of the other factors after IVB. Bevacizumab may interfere with the actual level of VEGF in the serum, and the total VEGF level in the serum cannot be determined when bevacizumab is present. Wide CIs were noted in the measurement of these factors, probably owing to the small number of patients enrolled in this study.

CONCLUSIONS AND RELEVANCE Serum VEGF levels were suppressed for 2 months after IVB in patients with type 1 ROP owing to the leakage of bevacizumab into the systemic circulation.

JAMA Ophthalmol. 2015;133(4):391-397. doi:10.1001/jamaophthalmol.2014.5373
Published online January 8, 2015.

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Retinopathy of prematurity (ROP) is one of the leading causes of blindness in very low-birth-weight premature infants. Previous studies¹ have verified that the main pathologic growth factor mediating neovascularization and the development of ROP is vascular endothelial growth factor (VEGF). Identification of angiogenesis regulators has enabled the development of novel therapeutic approaches involving the use of anti-VEGF medications for the treatment of patients with stage 3 (proliferative) ROP.^{2,3}

Bevacizumab (Avastin) is a recombinant humanized monoclonal antibody that is directed against VEGF. Intravitreal injections of bevacizumab (IVB) have been found to be effective for stage 3 ROP or type 1 ROP as defined in the Early Treatment for Retinopathy of Prematurity (ETROP) study.⁴ However, Sato et al⁵ found that serum VEGF levels were suppressed for as long as 2 weeks after IVB, and the use of IVB for ROP remains uncertain because VEGF is considered to be an important neurodevelopmental growth factor in the early newborn period.⁶ The safety of anti-VEGF medications in the treatment of ROP remains to be elucidated.⁷ To better understand the systemic suppression of VEGF after IVB treatment in patients with ROP, we investigated the serum bevacizumab concentrations after IVB and the serum concentrations of VEGF and associated growth factors in the systemic circulation before and for as long as 8 weeks after IVB in patients with high-risk ROP. The assay used only measured free and not total VEGF levels.⁸ That is, the VEGF bound to bevacizumab would not be detected.

Methods

Patients

This study was approved by the institutional review board of Chang Gung Memorial Hospital in Taoyuan, Taiwan (contracts IRB100-3476B and IRB100-4294A3), and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from each parent for the enrollment of his or her child in the study. In addition, all parents signed informed consent before the administration of IVB.

This prospective cohort study assessed the serum levels of bevacizumab, VEGF, and other ROP-associated growth factors in patients with type 1 ROP before and after IVB. We enrolled patients with type 1 ROP as defined by the ETROP study⁴ who received IVB. We excluded patients who underwent prior laser treatment, laser treatment after IVB, or transfusions of whole blood before or after IVB. This study was conducted from December 1, 2011, through February 28, 2013, at Chang Gung Memorial Hospital. The status of the off-label use of IVB for ROP treatment was explained to the parents of the patients in detail. The choice of laser or IVB treatment was made by the parents. The parents were well informed about the efficacy of and possible complications from both forms of treatment, including the risks of using general anesthesia, possible decreased visual field and higher refractive errors associated with laser treatment, and the risks for retinal detachment, endophthalmitis, systemic VEGF suppression, and possible neurodevelopmental effects after IVB.

Measurement of Bevacizumab and Growth Factor Levels After IVB

Blood samples were collected before, at 1 day, and at 1, 2, 3, 4, 5, 6, and 8 weeks after IVB. Baseline blood samples were drawn 1 to 2 days before IVB. The tested serum targets included bevacizumab and growth factors related to ROP, including VEGF, VEGF receptor 1 (VEGFR1), VEGFR2, Tie2, erythropoietin, transforming growth factor β 1, insulinlike growth factor 1, angiopoietin 1, angiopoietin 2, angiopoietinlike 3, and angiopoietin 4.⁹⁻¹⁵

The serum concentration of bevacizumab was measured with an enzyme-linked immunosorbent assay (ELISA). The procedures were performed according to a prior study⁶ with some modifications. The blood samples were collected in sterile tubes and centrifuged at 3000 rpm for 10 minutes until a clear separation between the serum and the cell components was seen. The serum was then transferred to sterile tubes and stored at -20°C until the assay. Microwell plates (Nunc-Immuno MicroWell 96; Thermo Fisher Scientific) were coated with the 165 isoform of recombinant human VEGF (R&D Systems) at a concentration of 0.1 $\mu\text{g}/\text{mL}$ overnight at 4°C (100 $\mu\text{L}/\text{well}$). After blocking the wells to reduce nonspecific binding, 100 μL of each sample (after proper dilution) and different concentrations of the standard were added to the plates. A standard curve was prepared with bevacizumab concentrations ranging from 39 to 5000 pg/mL . Serum samples and standards were incubated for 2 hours at room temperature. After washing with phosphate-buffered saline with a 0.05% concentration of polysorbate 20 (Tween 20; Sigma-Aldrich), we added goat antihuman IgG (Abcam) conjugated with biotin. The samples were then incubated for 2 hours. After washing with phosphate-buffered saline with a 0.05% concentration of polysorbate 20, we added streptavidin-horseradish for 20 minutes, followed by washing. Horseradish substrate was then incubated for 20 minutes. The reaction was stopped by adding hydrogen sulfate (J. T. Baker), and the results were applied to a microplate reader at a wavelength of 450 nm with background subtraction at a wavelength of 570 nm for all values. The assay measured free bevacizumab levels, and all measurements were performed twice to obtain a mean value.

The serum concentration of VEGF was measured with an ELISA kit for human anti-VEGF (R&D Systems) that was able to detect the 121 and 165 isoforms of VEGF according to the manufacturer's protocol. The minimum detectable level of the test was 9.0 pg/mL . Tests for the other growth factors were performed using ELISA kits (Human Erythropoietin Platinum ELISA and Human/Mouse Transforming Growth Factor β 1 ELISA Ready-SET-Go; Bioscience, Inc) for human soluble VEGFR1/Flt-1, human soluble VEGFR2, human Tie2, human insulinlike growth factor 1, human angiopoietin 1, human angiopoietin 2, human angiopoietinlike 3, and human angiopoietin 4 (R&D Systems).

Statistical Analysis

The data are presented as mean (SD). We compared the data obtained before IVB (baseline) with those obtained after IVB.

Table 1. Demographic Characteristics of the Infants With ROP and IVB

Patient No./ Sex/GA, wk	Birth Weight, g	Study Eye	ROP Stage ^a	TVL	Risks Factors Present ^b									PMA at IVB, wk	ROP Regression
					IVH	PDA	RDS	PPHT	BPD	NEC	Sepsis	Anemia	Pneumonia		
1/M/28.6	780	Both	3	-	1	1	1	0	1	0	1	1	1	34	Yes
2/F/29.4	1170	Both	3	-	1	1	1	0	1	0	1	1	1	34	Yes
3/M/25.4	755	Both	3	+	1	0	1	0	1	0	1	1	0	34	Yes
4/M/25.3	810	Both	2	+	1	1	1	1	1	0	1	1	1	41	Yes
5/M/26.1	794	Both	1	-	1	0	1	0	1	0	0	1	0	37	Yes
6/F/25.4	785	Both	3	-	1	0	1	0	1	0	1	1	1	37	Yes
7/M/25.3	815	Both	3	+	1	0	1	0	1	0	1	1	1	38	Yes
8/F/34.3	1380	Right	3	+	1	0	1	0	0	0	0	1	0	41	Yes

Abbreviations: BPD, bronchopulmonary dysplasia; GA, gestational age; IVB, intravitreal injection of bevacizumab; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus; PMA, postmenstrual age; PPHT, primary pulmonary hypertension; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity;

TVL, tunica vasculosa lentis; -, absent; +, present.

^a All treated eyes had zone II ROP with plus disease.

^b One indicates present; 0, absent.

We used paired *t* tests to compare differences in serum levels of the growth factors before and after IVB. Pearson product moment correlation analysis was performed to check the correlation of the serum levels of bevacizumab and VEGF after IVB. We used commercially available statistical software (SAS, version 9.2; SAS Institute Inc) for all data analyses.

Results

We initially included 10 patients in the study; however, 2 patients had severe cardiopulmonary disorders and an unstable clinical course. The pediatricians therefore judged that drawing additional blood samples from these patients would be inappropriate. Their parents also withdrew their consent for enrollment of their child into the study, and these 2 patients were excluded from the analyses. In total, we enrolled 8 patients (5 boys and 3 girls) with type 1 ROP. Among these 8 patients, 4 (50%) had a history of transfusions of packed red blood cells that should have had a minimal or no effect on the components of serum proteins. These transfusions were given at least 3 days before the blood samples were drawn. The demographic characteristics of the patients are summarized in Table 1. Seven patients received IVB in 2 eyes, and 1 patient received IVB in the right eye only. The mean (SD) gestational age of the infants was 27.5 (3.2 [range, 25.3-34.3]) weeks, and the mean birth weight was 911.1 (232.2 [range, 755-1380]) g. All of the patients used IVB as the primary treatment, and none of the infants had received laser photocoagulation of the peripheral avascular retina before IVB. The mean postmenstrual age at IVB was 37.0 (2.9) weeks. All of the eyes had complete resolution of abnormal neovascularization of ROP and continued normal vascularization toward the peripheral retina after IVB. None of the patients experienced recurrence of ROP, and none received another injection during follow-up. Patient 3 died at a postmenstrual age of 38 weeks because of preexisting bowel obstruction, infection with *Candida* species, septic shock, and disseminated intravascular coagulopathy. According to

the pediatrician, the death was caused by these factors and was not related to VEGF suppression. No obvious adverse systemic complications were noted, and the remaining 7 patients were alive after almost 2 years of follow-up.

The volume of each collected blood sample was 2 mL, and the mean time from blood collection to ELISA was 7.6 (3.7 [range, 2-12]) months. The mean serum levels of bevacizumab at the different measurement points were as follows: 0 ng/mL at baseline (n = 8); 1425 (1010 [95% CI, 0-3934]) ng/mL at 1 day after IVB (n = 3) (*P* = .13); 1270 (557 [95% CI, 804-1736]) ng/mL at 1 week after IVB (n = 8) (*P* < .001); 1333 (827 [95% CI, 466-2200]) ng/mL at 2 weeks after IVB (n = 6) (*P* = .01); 803 (87 [95% CI, 21-1586]) ng/mL at 3 weeks after IVB (n = 2) (*P* = .049); 1019 (683 [95% CI, 302-1737]) ng/mL at 4 weeks after IVB (n = 6) (*P* = .02); 448 (67 [95% CI, 0-1048]) ng/mL at 5 weeks after IVB (n = 2) (*P* = .07); 736 (594 [95% CI, 0-1473]) ng/mL at 6 weeks after IVB (n = 5) (*P* = .05); and 285 ng/mL at 8 weeks after IVB (n = 1) (Figure 1).

The mean serum VEGF level was 379 (226 [95% CI, 190-568]) pg/mL at baseline (n = 8), which decreased to the following levels at the subsequent measurements: 71 (27 [95% CI, 4-138]) pg/mL at 1 day after IVB (n = 3) (*P* = .10); 78 (49 [95% CI, 37-119]) pg/mL at 1 week after IVB (n = 8) (*P* = .01); 60 (20 [95% CI, 39-81]) pg/mL at 2 weeks after IVB (n = 6) (*P* = .03); 70 (5 [95% CI, 29-110]) pg/mL at 3 weeks after IVB (n = 2) (*P* = .07); 71 (27 [95% CI, 43-99]) pg/mL at 4 weeks after IVB (n = 6) (*P* = .01); 94 (59 [95% CI, 0-623]) pg/mL at 5 weeks after IVB (n = 2) (*P* = .08); 72 (43 [95% CI, 20-125]) pg/mL at 6 weeks after IVB (n = 5) (*P* = .02); and 216 pg/mL at 8 weeks after IVB (n = 1) (Figure 2). We investigated the correlation between serum levels of bevacizumab and VEGF in the patients after IVB and found a negative correlation (*r* = -0.43 [95% CI, -0.67 to -0.10]; *P* = .01) (Figure 3).

After IVB, no changes were identified in the serum levels of all the other factors, including VEGFR1, VEGFR2, Tie2, erythropoietin, transforming growth factor β 1, insulinlike growth factor 1, angiopoietin 1, angiopoietin 2, and angiopoietinlike 3 (Table 2). Angiopoietin 4 was not detected in any of the measurements.

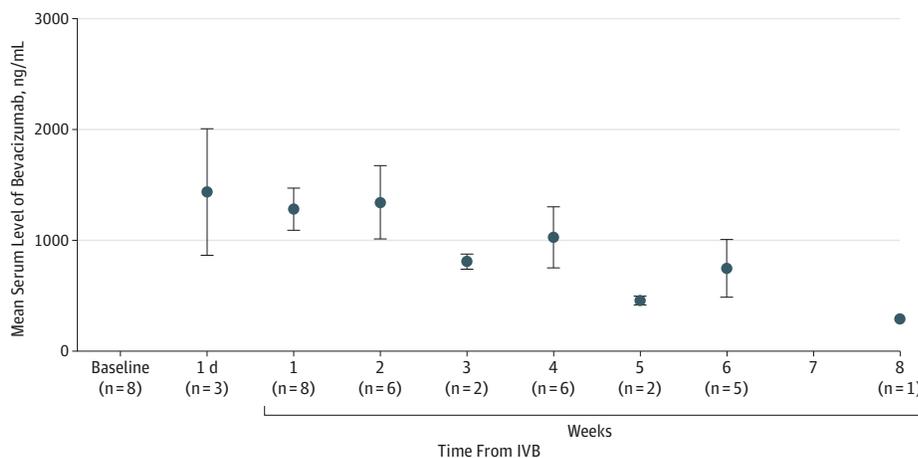
Discussion

Our results showed that the serum bevacizumab level was higher in patients with type 1 ROP after 0.625-mg IVB. At 1 day after IVB, the serum level of bevacizumab increased from 0 ng/mL to a mean of 1425 (1010) ng/mL, and the level of bevacizumab was still detectable as long as 8 weeks after IVB. The mean serum VEGF level was suppressed for the same period. We found a negative correlation between the serum levels of bevacizumab and VEGF in the patients with ROP who received 0.625 mg of bevacizumab. After IVB, no changes were identified in the serum levels of all of the other ROP-associated factors.⁹⁻¹⁵ A previous *in vitro* study¹⁶ showed that a bevacizumab concentration of approximately 500 ng/mL inhibited VEGF activity completely, which could explain the inhibition of systemic VEGF in the present study. The safe range of VEGF serum concentration in premature infants remains unknown, and our data suggest that further studies with long-

term follow-up are needed to determine the effect of VEGF suppression after IVB in patients with ROP.

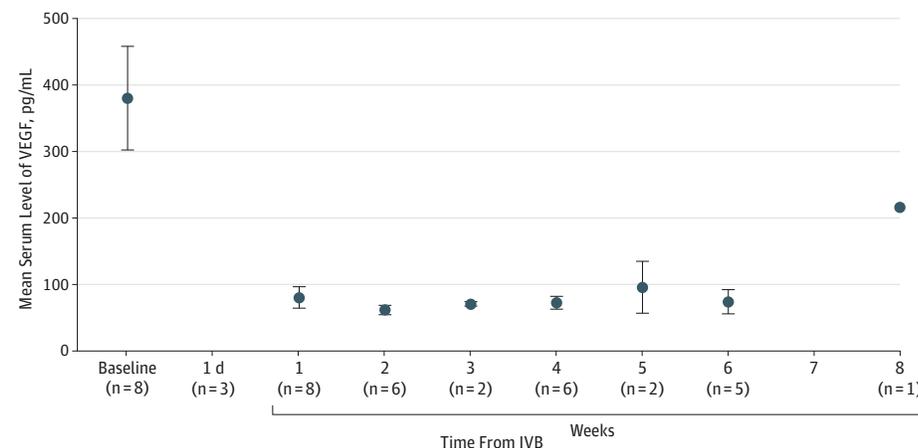
Sato et al⁵ found that serum VEGF levels were suppressed for 2 weeks after IVB in patients with ROP. In the present study, VEGF suppression persisted for as long as 8 weeks after IVB; however, we found several differences between their study and ours. Sato et al⁵ included patients with stages 3, 4, and 5 ROP, whereas our study only included patients with type 1 ROP. This difference could explain in part why their patients had higher serum VEGF levels than our patients before IVB (1628 [929] vs 379 [226] pg/mL). In addition, the injected dose of bevacizumab in their study was 0.25 mg or 0.50 mg compared with 0.625 mg in our study. Furthermore, Sato et al⁵ included patients with prior laser treatment before IVB, whereas we enrolled only patients who had not received laser treatment. In addition, Sato et al⁵ included patients who underwent vitrectomy after IVB, whereas none of our patients did so. In addition to serum VEGF, we also studied growth factors related to ROP development. Finally, our

Figure 1. Serum Levels of Bevacizumab After Intravitreal Injection of Bevacizumab (IVB)



At baseline (before IVB), we found no detectable level of bevacizumab. The serum level of bevacizumab increased 1 day after IVB and remained detectable for 8 weeks. Whiskers indicate SD.

Figure 2. Serum Levels of Vascular Endothelial Growth Factor (VEGF) After Intravitreal Injection of Bevacizumab (IVB)



At baseline (before IVB), the mean serum VEGF level in the patients was close to 400 pg/mL. After IVB, the mean serum level of VEGF decreased and the reduction lasted for 8 weeks. Whiskers indicate SD.

study period was 8 weeks so that the profile of bevacizumab and its interaction with VEGF could be perceived better.

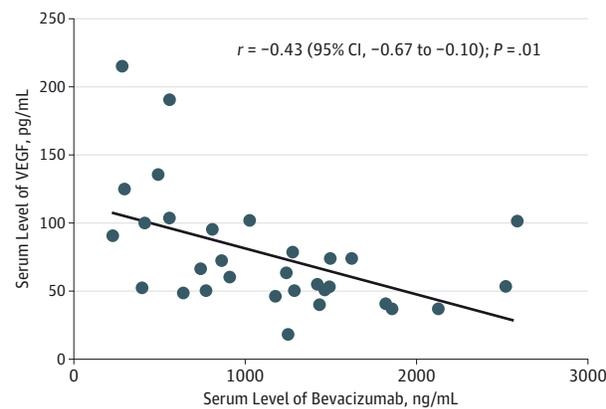
The pharmacokinetics of IVB may be different between newborns and adults.^{5,6} In a study of the treatment of age-related macular degeneration in adults, IVB was shown to suppress VEGF for only 1 week.¹⁷ We found that bevacizumab entered the systemic circulation of the newborns 1 day after IVB and that VEGF was suppressed for as long as 8 weeks. Although no adverse systemic events have been reported in patients with ROP after the administration of IVB,^{2,3} concerns about the safety of using IVB in newborns have been raised owing to a lack of supportive data.⁶ The present study provides more biochemical data concerning the safety of anti-VEGF medications in newborns than previous studies. However, several issues remain to be elucidated, including the safe dose of VEGF in newborns,

whether the suppression of VEGF levels causes organ dysfunction over time, which organs are affected most by VEGF suppression, and whether any compensatory mechanisms are involved in VEGF suppression.

Vascular endothelial growth factor plays an important role in angiogenesis, maintaining organ health, wound repair after injury, and the development of various vital organs in the body.¹⁸⁻²⁹ Because VEGF concentrations are highly elevated in advanced ROP and because VEGF has been found to be a driving force for neovascularization,³⁰⁻³² blocking VEGF with anti-VEGF agents, such as bevacizumab, appears to be a reasonable approach for the treatment of ROP. However, the inhibition of VEGF raises concerns that these important physiological effects associated with VEGF will be inhibited, leading to abnormal organogenesis or neurodevelopment. Additional studies are therefore needed to determine the long-term systemic effects after IVB for patients with ROP.

In this study, a patient with a gestational age of 34.3 weeks developed type 1 ROP (patient 8). Several possible explanations exist for this development. The oxygen saturation targets at our institution are set from 88% to 93%, which constitutes a reasonable range and has been endorsed by other studies.³³ However, suboptimal oxygen management may still be relevant to our findings because the neonatology team may not adhere strictly to this range. Other possible explanations for this finding exist. First, because our center is the major referral center for newborns in Taiwan, our patients may have had more severe disease. Second, the vulnerability to prematurity and genetic predispositions among different ethnicities may be different. Animal models of ROP have shown that pigmented strains of rats are more susceptible to ischemia-induced retinal neovascularization than their albino counterparts.³⁴ In addition, previous studies have shown that, despite a similar overall incidence, a significantly higher rate of Asian children develop more severe ROP.³⁵⁻³⁷ In summary, ROP in older newborns may be related to suboptimal oxygen use, a poor systemic condition of the patients, and different susceptibilities of various ethnicities.

Figure 3. Correlation of Serum Levels of Bevacizumab and Vascular Endothelial Growth Factor (VEGF)



Data points represent correlations; diagonal line, the linear regression fit across all data points. The serum level of VEGF was negatively associated with that of bevacizumab. The Pearson product moment correlation coefficient and P value are shown.

Table 2. Serum Growth Factor Levels Before and After IVB in Patients With Type 1 ROP

Factor	Level, Mean (SD)								
	Baseline (n = 8)	1 d (n = 3)	1 wk (n = 8)	2 wk (n = 6)	3 wk (n = 2)	4 wk (n = 6)	5 wk (n = 2)	6 wk (n = 5)	8 wk (n = 1)
VEGFR1 level, ng/mL	0.17 (0.09)	0.16 (0.06)	0.14 (0.04)	0.15 (0.06)	0.11 (0.01)	0.14 (0.07)	0.11 (0.03)	0.18 (0.07)	0.08
VEGFR2 level, ng/mL	12 (2)	13 (2)	12 (1)	12 (1)	13 (2)	12 (2)	14 (2)	12 (1)	12
Tie2 level, ng/mL	37 (11)	36 (15)	37 (9)	33 (5)	39 (19)	36 (10)	28 (1)	36 (8)	25
Erythropoietin level, IU/L	9 (3)	11 (4)	12 (3)	16 (9)	26 (17)	14 (7)	17 (15)	13 (7)	23
TGF-β1 level, ng/mL	19 (8)	19 (3)	19 (8)	18 (6)	22 (5)	18 (9)	14 (1)	20 (8)	17
IGF-1 level, ng/mL	46 (22)	41 (11)	47 (19)	46 (24)	79 (3)	58 (16)	75 (44)	58 (18)	109
Angiopoietin 1 level, ng/mL	52 (35)	45 (13)	38 (15)	37 (17)	41 (12)	48 (38)	25 (0)	43 (26)	33
Angiopoietin 2 level, ng/mL	11 (6)	8 (3)	10 (5)	11 (6)	8 (3)	6 (1)	7 (1)	6 (2)	4
Angiopoietinlike 3 level, ng/mL	293 (112)	261 (12)	304 (95)	290 (116)	344 (71)	265 (31)	273 (74)	265 (60)	238

Abbreviations: IGF-1, insulinlike growth factor type 1; IVB, intravitreal injection of bevacizumab; ROP, retinopathy of prematurity; TGF-β1, transforming growth factor β1; VEGFR1, vascular endothelial growth factor receptor 1;

VEGFR2, vascular endothelial growth factor receptor 2.

SI conversion factor: To convert IGF-1 to nanomoles per liter, multiply by 0.131.

Serum and plasma samples are used in clinical studies. However, the determination of which sample type is more representative of and better related to the clinical disorders remains controversial. The serum VEGF level is usually higher than the plasma VEGF level because VEGF is stored in the alpha granules³⁸ and is released on platelet activation during clotting.^{39,40} The wide CIs in the measurement of serum VEGF levels could be related to this factor. Plasma concentrations have been suggested to represent a more accurate assessment of circulating VEGF levels.⁴¹ However, because citrated plasma VEGF levels are low and lie at the lower limit of detection of currently available ELISA assays, serum assessments may provide greater sensitivity.⁴² Therefore, serum and plasma levels are important for measurement of VEGF levels in peripheral blood. A recent study of age-related macular degeneration⁴³ also shows that the trend of change in serum and plasma VEGF levels was similar after treatment with aflibercept or ranibizumab.

This study has several limitations, including the small numbers of patients and blood samples available at each time point. Enrolling patients with severe acute ROP into a prospective trial is difficult, and blood samples may not be available because the systemic condition of a newborn may not always be stable enough to allow for blood samples to be drawn safely. The small number of patients may have contributed to the wide CIs in the measurement of serum growth factor levels in this study. Blood samples in the patient who died were not available beyond 3 weeks. In addition, the actual amount of systemic bevacizumab

in the present study was not known, and the presence of bevacizumab in the serum could affect the measurement of VEGF levels by ELISA assay. Vascular endothelial growth factor bound with bevacizumab could not be detected; only the free form of VEGF could be detected by the assay used. However, information about free VEGF is more important because only the free form is biologically active.⁴⁴ Despite these limitations, our results showed that VEGF expression was inhibited for as long as 8 weeks after IVB and that bevacizumab remained in the systemic circulation for that time.

Conclusions

Bevacizumab was found to enter the systemic circulation 1 day after IVB and to remain detectable for 8 weeks in the patients with ROP who received 0.625-mg IVB. This dose was associated with a decrease in VEGF levels as measured by ELISA. We found a negative correlation between the serum levels of bevacizumab and VEGF in the patients with ROP after IVB. No changes were identified in the serum levels of other growth factors. These findings suggest that IVB should be used with caution in the treatment of ROP. Future studies comparing the efficacy of a lower dose of bevacizumab than that used in this study, measuring the serum bevacizumab level for up to 12 weeks or until it is no longer detectable in the circulation, and evaluating the long-term developmental outcomes of these patients are warranted.

ARTICLE INFORMATION

Submitted for Publication: March 30, 2014; final revision received October 16, 2014; accepted October 29, 2014.

Published Online: January 8, 2015.

doi:10.1001/jamaophthalmol.2014.5373.

Author Contributions: Drs Wu and Liao had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition, analysis, or interpretation of data: Wu, Lien, Liao, Y.-P. Chen, K.-J. Chen, T.-L. Chen.

Drafting of the manuscript: Wu.

Critical revision of the manuscript for important intellectual content: All authors.

Statistical analysis: Liao, Wang.

Obtained funding: Wu.

Administrative, technical, or material support: Lien, Y.-P. Chen, Chao, K.-J. Chen, T.-L. Chen, Hwang, Lai. **Study supervision:** Lien.

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none were reported.

Funding/Support: This study was supported in part by research grant CMRPG3AO392 from Chang Gung Memorial Hospital and by research grant SC101-2314-B-182A-055-MY3 from the National Science Council.

Role of the Funder/Sponsor: The funding sources had no role in the design and conduct of the study; collection, management, analysis, and

interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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