CONCENTRATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN AQUEOUS HUMOR OF EYES WITH ADVANCED RETINOPATHY OF PREMATURITY BEFORE AND AFTER INTRAVITREAL INJECTION OF BEVACIZUMAB

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Purpose: To determine whether an intravitreal injection of bevacizumab alters the concentration of vascular endothelial growth factor (VEGF) in the aqueous humor of eyes with retinopathy of prematurity.

Methods: Seven Stage 4 and three Stage 5 eyes of eight patients with retinopathy of prematurity were studied. Bevacizumab (0.75 mg/0.03 mL/eye) was injected intravitreally in six eyes of six patients after approval was obtained from the Institutional Review Board of Nagoya University Hospital and an informed consent was signed by the parents. Aqueous humor was collected just before the surgery or before the intravitreal injection of bevacizumab. Aqueous humor was also collected immediately before vitrectomy 4 to 48 days after the injection of bevacizumab. Aqueous humor was also collected from four patients undergoing congenital cataract surgery as controls. The concentration of VEGF was measured by enzyme-linked immunosorbent assay.

Results: In the 4 control eyes, the concentration of VEGF in 2 eyes was 156 and 158 pg/mL and was not detectable in the other 2 eyes. The average concentration of VEGF was 1,109 pg/mL in the active Stage 4 eyes and 3,520 pg/mL in the active Stage 5 eyes. After bevacizumab injection, the unbound VEGF concentration was 60, 230, and 290 pg/mL in 3 eyes and not detectable in 1 eye.

Conclusion: Intravitreal bevacizumab resulted in a marked decrease in the unbound VEGF concentration in eyes with retinopathy of prematurity.

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Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein that plays an important role in angiogenesis and neovascularization.[1] The retina is known to be ischemic in certain ocular diseases, such as diabetic retinopathy and retinopathy of prematurity (ROP), and the expression of VEGF is up-regulated, which leads to retinal neovascularization. This is important because the neovascularization can progress to vitreous hemorrhage, proliferative membranes, and retinal detachments (RDs).[2–4] Thus, one of the strategies to prevent these vision-threatening changes is to block the upregulation of VEGF.

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Bevacizumab (Avastin; Genentech Inc., San Francisco, CA) is a humanized anti-VEGF monoclonal antibody that has been used systemically to treat patients with cancer.[5] For the eye, an intravitreal injection of bevacizumab was found to be effective in reducing the severity of ocular diseases such as neovascular age-related macular degeneration,[6] retinal vein occlusion,[7] and diabetic retinopathy.[8] Bevacizumab has also been used as a preoperative adjunctive therapy for proliferative diabetic retinopathy (PDR).[9] Sawada et al[10] have reported a marked decrease of ocular unbound VEGF level after an intravitreal injection of bevacizumab in eyes with PDR suggesting that the effectiveness of bevacizumab was due to a reduction of the unbound VEGF level.

ROP is a major cause of serious visual impairment in infants born prematurely, and the number of cases of severe ROP is increasing with the increase in the survival rate of the smallest pronatis.[11] In most cases, retinal photocoagulation is very effective in treating eyes with ROP, and the photocoagulation leads to a quiescent stage. However, despite this treatment, some eyes progress to the advanced stages of ROP with proliferative membranes and RDs. For severe cases, vitrectomy must be performed to reattach the retina, although surgeons must wait for the neovascular membranes to become quiescent, which greatly hinders the prognosis of good vision.

VEGF plays a key role in progression of ROPs, and Chung et al[12] have reported that bevacizumab was effective in treating eyes with ROP. This finding suggested that preoperative bevacizumab can be an effective adjunctive therapy for ROP. To the best of our knowledge, there have been only two studies on the ocular VEGF level in eyes with ROP,[13,14] and neither of these reported the level of VEGF after an intravitreal injection of bevacizumab.

Thus, the purpose of this study was to measure the concentration of VEGF in the aqueous humor in eyes with Stage 4 and Stage 5 ROP before and after an intravitreal injection of bevacizumab.

Methods

Subjects

Approval for this study was obtained from the Institutional Review Board of Nagoya University Hospital, and an informed consent was obtained from the parents. The procedures used in this study conformed to the tenets of the Declaration of Helsinki.

Seven eyes at Stage 4 and three at Stage 5 of eight patients with ROP were studied (Table 1). The mean postmenstrual age of the patients was 41.1 weeks (35–64 weeks). Bevacizumab at a dosage of 0.75 mg/0.03 mL was injected intravitreally in 6 eyes of 6 patients. An encircling or buckling procedure was performed on four eyes at Stage 4 ROP. Vitrectomy with lensectomy was performed on two Stage 4B and on two Stage 5 ROP eyes after bevacizumab injection, and in one Stage 5 ROP eye without an injection.

Aqueous humor was collected just before the surgery from seven eyes at Stage 4 and three at Stage 5 eyes. Aqueous humor was also collected just before the intravitreal injection of bevacizumab from two eyes at Stage 4 and two at Stage 5. For control, aqueous humor was also collected from three eyes with congenital cataract and one eye with persistent pupillary membrane that underwent surgery (1 male and 3 female infants). The mean age of these patients was 4.0 ± 2.1 months with a range of 2 to 7 months. Ophthalmoscopy showed that the fundus was normal in these four eyes. Although the eyes used as control were younger than that used in a previous report,[14] they were still older than the ROP eyes. This difference in the ages might have altered the VEGF level.

Ophthalmologic Examinations and Staging of Retinopathy of Prematurity

Fundus and 15-MHz ultrasound biomicroscopy (RION Inc., Kokubunji, Tokyo) examinations were performed on all eyes in the outpatient clinic. Color fundus photographs were taken with RetCam (Massie Research Laboratories Inc., Dublin, CA). Fluorescein angiography was performed under general anesthesia using the fluorescein angiography unit of the RetCam just before the sample collection.

The stage of the ROP was classified according to international classification,[15] and the vascular activity was classified as active if the eye had 1) plus disease, 2) new vessels growing into the vitreous at the ridge of a tractional RD area, or 3) combined effusive and tractional RD.[14]

Sample Collection and Measurement of Vascular Endothelial Growth Factor

Aqueous humor was collected under general anesthesia with a 27-gauge needle just before the surgery or intravitreal injection of bevacizumab. The amount of undiluted aqueous humor collected ranged from 0.02 mL to 0.1 mL. The samples were not analyzed at the time of collection, but were stored in a deep freezer at −80°C until use. The concentration of VEGF was measured by enzyme-linked immunosorbent assay using a commercially available kit (Quantikine: R&D Systems Inc., Minneapolis, MN), which measures both human $VEGF_{121}$ and $VEGF_{165}$. There

Table 1. Patient Characteristics and the Concentration of VEGF in Aqueous Humor

Patient No.	Gestational Age (Weeks)	Postmenstrual Age (Weeks)	Age (Months)	VEGF Before or Without Bevacizumab (pg/mL)	VEGF After Bevacizumab (pg/mL)
Control					
1			2	156	
2			2	ND	
3			5	ND	
4			7	158	
Stage 4 ROP					
1	22	39		564	
2	26	37		944	
3R	27	37		1,750	
3L	27	37		1,890	
4*	24	64		184	
5	25	41†			60 (4 days‡)
6	22	35, 36†		395	ND (4 days‡)
Stage 5 ROP					
7	28	40, 41†		1,990	230 (7 days‡)
8L	23	36, 43†		5,050	290 (48 days‡)
8R*	23	45		370	

*Inactive.
†Postmenstrual age when aqueous humor was collected after bevacizumab injection.
‡Days after bevacizumab injection.
ND, not detectable.

were three samples in which the VEGF was not detectable. However, as the amount of the samples collected from each eye was different and less than 0.2 mL, the minimum amount necessary for the test, all of the samples had to be diluted with Calibrator Diluent RD6U before use. The sample from Control 2 was diluted 10×, Control 3 was diluted 4×, and ROP 6 after bevacizumab was diluted 5× before the measurement. Thus, the concentration of VEGF in these eyes, in which VEGF was not detectable, might have been higher than 31 pg/mL, the minimum detectable limit of this kit.

Results

The concentration of VEGF in the aqueous humor of 1 of the eyes with congenital cataract was 158 pg/mL, and it was less than the detection level in the other 2 eyes. The concentration in an eye with a persistent pupillary membrane was 156 pg/mL.

The concentration of VEGF in 10 eyes with ROP ranged from 184 to 5,050 pg/mL, which is approximately 1.2× to 32× higher than that in the control eyes (Figure 1). Aqueous humor was collected from seven eyes at Stage 4 and three eyes at Stage 5. One eye at Stage 4 and one at Stage 5 were vascularly inactive. The mean concentration of VEGF in the vascularly active ROP was 1,109 pg/mL in the 5 Stage 4 eyes (395, 564, 944, 1,750, and 1,890 pg/mL in the Stage 4 eyes), and 3,520 pg/mL in the 2 Stage 5 eyes (1,990 and 5,050 pg/mL). In the vascularly inactive eyes, the VEGF level was 184 pg/mL in the 1 Stage 4 eye and 370 pg/mL in 1 Stage 5 eye. Thus, the concentration of VEGF in the vascularly active eyes tended to be higher than in inactive eyes, although statistical analysis could not be performed due to the small number of eyes (Figure 1).

Bevacizumab was injected into six active ROP eyes, four at Stage 4 and two at Stage 5, and aqueous humor was collected from two Stage 4 and two Stage 5 eyes 4 to 48 days after the injection just before vitrectomy. In the Stage 4 eyes, the concentration of

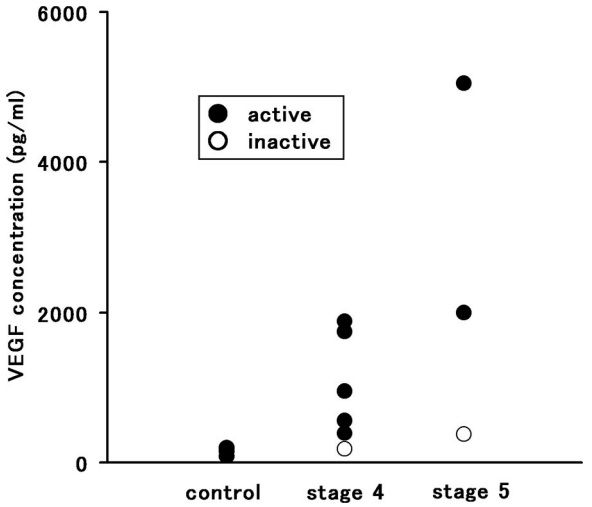

Fig. 1. Concentration of vascular endothelial growth factor (VEGF) in the aqueous humor without or before bevacizumab injection in Stages 4 and 5 ROP and control eyes are shown.

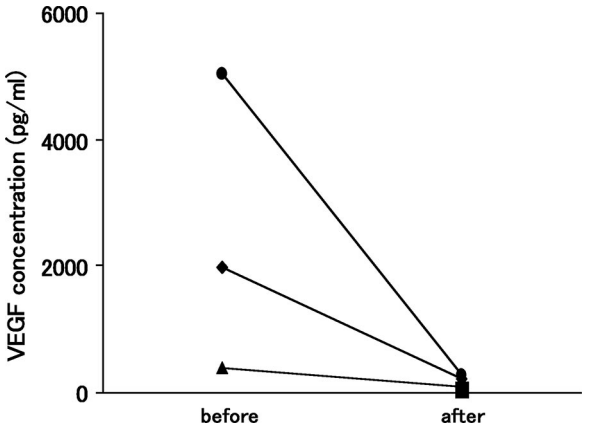

Fig. 2. Vascular endothelial growth factor (VEGF) in the aqueous humor before and/or after bevacizumab injection. The concentration of VEGF in four ROP eyes that had a bevacizumab injection is shown. The concentration before (right) and after (left) injection was measured in three eyes, and after injection in one eye.

unbound VEGF was not detected in 1 eye, and was 60 pg/mL in the other eye 4 days after the injection. In 1 stage 5 eye, the concentration decreased to 230 pg/mL, 7 days after the injection. For 1 Stage 5 eye, vitrectomy was postponed because the patient developed chronic lung disease, and vitrectomy could be safely performed 48 days after the bevacizumab injection. The concentration of VEGF at that time was 290 pg/mL (Figure 2). Fourteen days later, vitrectomy was also performed on the fellow eye without bevacizumab injection, and the concentration of VEGF was 370 pg/mL. The concentration of the VEGF after bevacizumab injection was significantly lower than that of active ROP eyes (145 versus 1,460; $P = 0.04$ with Mann–Whitney U test. The level of VEGF in nondetectable samples was set to 31 pg/mL for statistical analysis). The concentration of VEGF after bevacizumab injection was not measured in two eyes, because the retina was reattached by scleral buckling that was performed together with the injection of bevacizumab, and vitrectomy was not necessary.

Fluorescein angiography was performed before and after the intravitreal injection of bevacizumab in two eyes. In these eyes, there was a considerable decrease of fluorescein leakage from the new vessels after the injection of bevacizumab (Figure 3A and B).

All of the Stage 4 eyes injected with bevacizumab were Stage 4B, and the retina of the two eyes was reattached with one or two vitrectomies without silicon oil tamponade (Figure 3C–F), and two eyes with single encircling surgery (Figure 3G and H). One Stage 5 eye underwent vitrectomy 48 days after the injection, and the retina was reattached under silicon oil (Figure 3I and J). However, one Stage 5 eye with multiple retinal breaks required three surgeries for the retina to be reattached under silicon oil. The silicon oil removal is still being considered for these two eyes.

No ocular complications, such as endophthalmitis, new retinal breaks, or any obvious systemic side effects that were related to bevacizumab were observed.

Comments

The concentration of VEGF in the aqueous humor of eyes at advanced stages of ROP was very high and was higher than that of eyes of adults with PDR (1,460 versus 524; $P = 0.01$ with Mann–Whitney U test) and eyes of infants <1 year without retinal disorders (1,460 versus 94; $P = 0.01$ with Mann–Whitney U test). In these statistical analyses, samples with nondetectable levels of VEGF were set to 31 pg/mL.

Because the concentration of VEGF in the aqueous humor was correlated with the grade of diabetic retinopathy,[16] it would be interesting to know whether there was also a correlation between the VEGF concentration and stage of ROP. Our results showed that the mean VEGF concentration in the Stage 5 eyes was 3× higher than that in the Stage 4 eyes, and one eye at Stage 5 had a level of 5,050 pg/mL. Only three eyes at Stage 4 had <1,000 pg/mL of VEGF. However, Lashkari et al[13] reported that the concentration of VEGF in the subretinal fluid in Stage 4 eyes did not differ significantly from that of Stage 5 ROP eyes. This difference from our results was probably because of the difference in the site where the sample was collected, viz, the aqueous humor or the subretinal fluid. Another reason that might account for this difference might be whether the vessels were active or inactive. Lashkari et al did not report the vascular status of their eye, and it may have been at the vascularly inactive ROP stage. Sonmez et al[14] classified Stage 4 ROP into vascularly active or vascularly inactive state, and reported that the level of VEGF was higher in the active group than in the inactive group as we have found. However, the number of eyes tested in our study was limited, and statistical analysis was not performed. Thus, we cannot make a conclusion on this question.

The intravitreal injection of bevacizumab decreased the concentration of VEGF in the aqueous humor of Stage 4 and Stage 5 ROP eyes markedly, and the concentration decreased to 60, 230, and 290 pg/mL and the level was not detectable in one eye (direct statistical comparison, before and after injection, was not performed because of the small number of the eyes), which is comparable with that of the control eyes of infants. However, it was still higher than that in eyes with PDR after bevacizumab injections reported previously.[10] The concentration in all of these

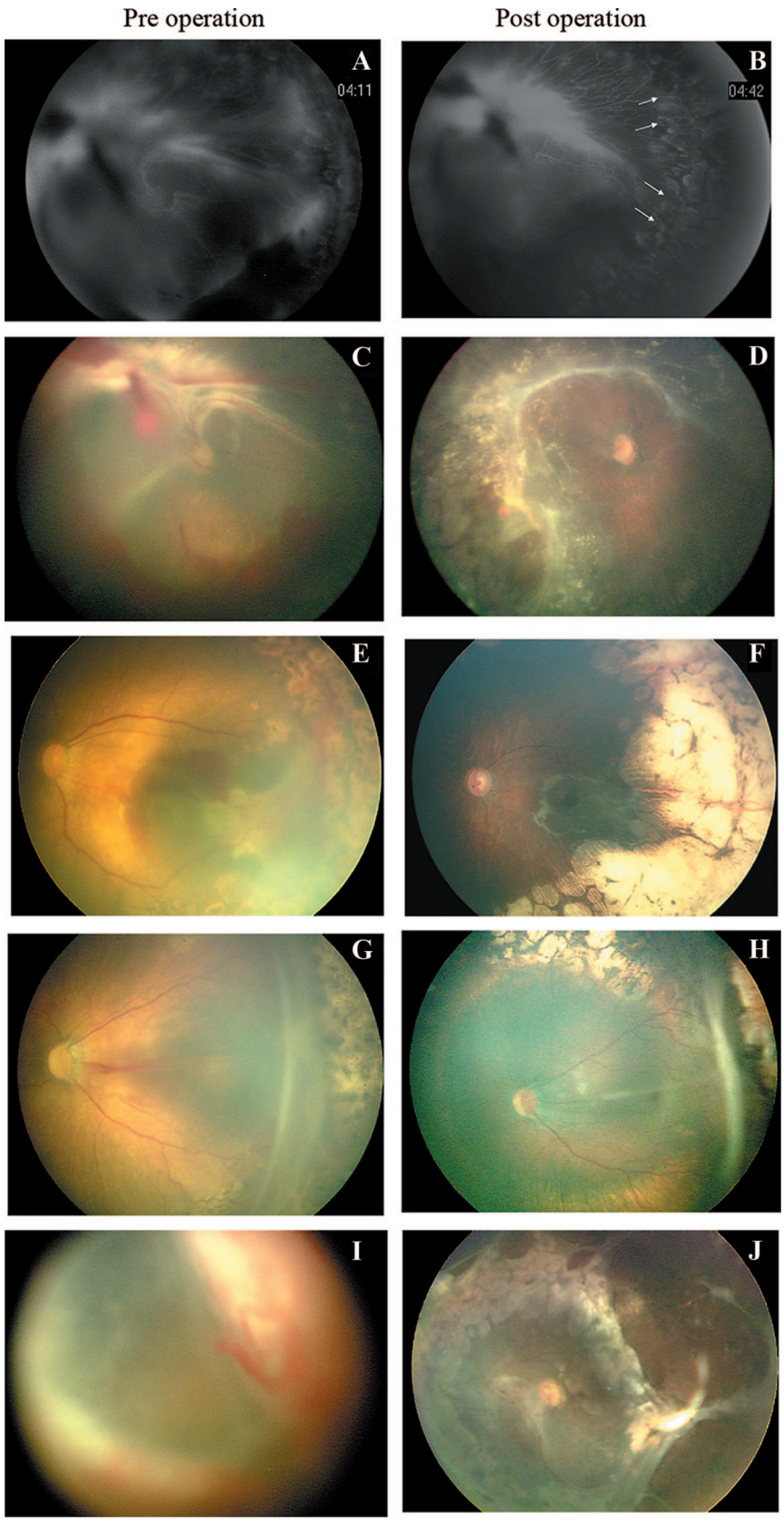

Fig. 3. Clinical outcome of the eyes treated with bevacizumab. (**A**–**D**) Patient 5 (Stage 4, Table 1). Retina was totally detached except the peripheral retina (**A** and **C**) and bevacizumab was injected as a preoperative adjunctive therapy at postmenstrual age of 40 weeks. Four days after injection, decrease of fluorescence leakage (arrow) was observed (**B**) and vitrectomy with lensectomy was performed. One month later, second membrane removal operation was performed, and retina was totally reattached (**D**). The visual acuity of this eye is LS (+) 1 year after the second surgery may be due to the good vision of the fellow eye, and the vision training is performed. (**E** and **F**) Patient 6 (Stage 4, Table 1). Because of the strong proliferation of active new vessels, bevacizumab was injected as preoperative adjunctive therapy at the postmenstrual age of 35 week (**E**). Four days after injection, vitrectomy with lensectomy was performed, and the retina was totally reattached. Four months after operation, macular is formed (**F**) and vision tracking is obtained. (**G** and **H**) Patient 2 (Stage 4, Table 1). Encircling surgery was performed at postmenstrual age of 35 weeks, but as the vascular activity was high and the retinal detachment remained (**G**), vitrectomy was planned. However, after the bevacizumab injection at 14 days after the encircling procedure, the progression stopped and the retina was reattached. Six months after the injection, the macular configuration was present (**H**) and visual acuity of 0.75 cycles/degree (Teller Acuity Cards) was obtained. (**I** and **J**): Patient 8L (Stage 5, Table 1). At postmenstrual age of 36 weeks, the retina was totally detached to closed funnel shape with multiple retinal breaks (**I**). Bevacizumab was injected, but vitrectomy was postponed because of the chronic lung disease. Vitrectomy with lensectomy was performed 48 days after the bevacizumab injection at the postmenstrual age of 43 weeks, and silicon oil was injected because of the retinal break. One year after the operation, the retina is reattached (**J**), and silicone oil removal is considered.

eyes was below the detection level of 31 pg/mL (no statistical analysis was performed). This difference might be because the VEGF concentration before the injection was much higher in the eyes with ROP, or the amount of bevacizumab injected in PDR eyes was greater. However, because the assays were run by different methods, at different institutes, and at different times, it might not be proper to compare these results. Although there was detectable VEGF remaining in some eyes, it was decided not to increase the amount of bevacizumab for these infants because we did observe a decrease of fluorescein leakage after the injection and less bleeding during vitrectomy with this dosage, and because of the possible systemic side effects. It also is possible that the residual level of VEGF might have prevented the topical side effects, e.g., obstruction of the development of normal vessels and neural retina, as the level is similar to that of control eyes, although the distribution of VEGF might be different from normal.

One Stage 5 eye became vascularly inactive and its VEGF level was 370 pg/mL, 62 days after the bevacizumab was injected into the fellow eye. However, it is not clear whether this was due to circulating bevacizumab or just a natural course of ROP. Because there was no ROP eye in which samples were collected twice in 4 or 7 days without bevacizumab as control, there is a possibility that decrease in ocular VEGF in other bevacizumab injected eyes can be the natural course of this disease. However, as a rapid inactivation of ROP was observed in these eyes and bevacizumab is shown to decrease VEGF in other diseases such as PDR,[10] this rapid decrease of VEGF is likely to be the effect of this drug.

From August 2004 to November 2006, vitrectomy was performed for Stage 4B (3 eyes) and Stage 5 (17 eyes) eyes without bevacizumab injection at our hospital. The average postmenstrual age of these infants was 49.7 weeks (38–67 weeks). Without bevacizumab, vitrectomy was performed 2.1 ± 0.9 times/eye (1–5 times), and the rate of reattachment was 55%. However, vitrectomy could be performed on the eyes treated with bevacizumab without severe bleeding during membrane removal at postmenstrual age of 40.3 weeks (36–43 weeks) which is earlier than that without bevacizumab, without severe bleeding during membrane removal. In two Stage 4B eyes, reattachment of the retina was obtained without silicon oil tamponade with one or two surgeries, and one Stage 5 eye, reattachment of the retina under silicon oil was obtained with one surgery. The removal of the silicon oil is being considered. For one Stage 5 eye with multiple retinal breaks, reattachment of the retina under silicon oil was obtained with three surgeries, and removal of the silicon oil is still being considered. Moreover, two Stage 4B eyes, which received an injection of bevacizumab just before or 2 weeks after encircling surgery, had a reattachment of the retina without additional operations. From August 2004 to November 2006, encircling surgery was performed without bevacizumab at our hospital for two Stage 4B eyes, but both eyes progressed to Stage 5, and vitrectomy was required for reattachment. These results suggest that bevacizumab injection might be useful adjunctive therapy for both vitrectomy and encircling surgery for severe ROP, although the number of the eyes studied was very limited.

In conclusion, an intravitreal injection of bevacizumab decreased the VEGF level markedly in the aqueous humor of ROP eyes although a direct comparison, before and after injection, was not performed statistically. However, the concentration of VEGF was not below the detection level in most of the eyes after injection. Our findings indicate that bevacizumab might be useful for preoperative adjunctive therapy for ROP, however the number of eyes studied was very limited and further studies are needed.

Key words: retinopathy of prematurity, vascular endothelial growth factor, bevacizumab, intravitreal injection, aqueous humor.

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