Vitreous Levels of Angiopoietin-1 and Angiopoietin-2 in Eyes With Retinopathy of Prematurity

TATSUHIKO SATO, CHIHARU SHIMA, AND SHUNJI KUSAKA

• PURPOSE: To determine the vitreous levels of angiopoietin (Ang)-1 and Ang-2 in eyes with retinopathy of prematurity (ROP), and to determine the correlation between the 2 levels.
• DESIGN: Retrospective case-control study.
• METHODS: Forty-eight eyes with stage 4 ROP were studied. Six eyes with congenital cataract were used as controls. The ROP eyes were classified by the vascular activity into highly (n = 22), moderately (n = 15), and mildly (n = 11) vascular-active ROP. Eyes with highly vascular-active ROP initially received 0.5 mg of intravitreal bevacizumab (IVB) and underwent vitrectomy within 1 week. The others underwent vitrectomy without IVB. Vitreous samples were collected at the beginning of vitrectomy, and the vitreous levels of Angs were measured by enzyme-linked immunosorbent assay.
• RESULTS: The mean concentrations of Ang-1 and Ang-2 were 201.9 and 7832.1 pg/mL in highly vascular-active ROP eyes, 216.1 and 7731.2 pg/mL in moderately vascular-active ROP eyes, 533.8 and 1685.9 pg/mL in mildly vascular-active ROP eyes, and 0 and 41.5 pg/mL in control eyes. The vitreous Ang-1 level was significantly higher ($P < .05$) in highly, moderately, and mildly vascular-active ROP eyes than in control eyes. The vitreous Ang-2 level was significantly higher ($P < .05$) in highly and moderately vascular-active ROP eyes than in control eyes. There was a significant negative correlation ($r = -0.406$; $P = .040$) between the Ang-1 and Ang-2 levels in moderately and mildly vascular-active ROP eyes.
• CONCLUSIONS: The balance of Ang-1 and Ang-2 in the vitreous may be important in the pathogenesis of ROP. (Am J Ophthalmol 2011;151:353–357. © 2011 by Elsevier Inc. All rights reserved.)

RETINOPATHY OF PREMATURITY (ROP), FIRST REported as retrolental fibroplasia by Terry in 1942,[1] is a retinal vascular disorder that develops in eyes with incomplete blood vessel development at birth. The sprouting of new blood vessels, pathologic neovascularization, induced by hypoxia is the crucial event in the pathology of ROP. In severe cases, the pathologic neovascularization leads to fibrovascular proliferation, vitreoretinal traction, and tractional retinal detachment, which then leads to severe loss of vision. Various factors may be involved in regulating the pathologic neovascularization, and among them vascular endothelial growth factor (VEGF) has been reported to be the dominant factor.[2–7]

The vitreous level of VEGF has been shown to be higher in stage 4 ROP eyes than in control (congenital cataract) eyes, and the VEGF level was correlated with the vascular activity.[3–5] In addition, anti-VEGF therapy, for example, intravitreal injection of bevacizumab, a humanized monoclonal antibody against VEGF, can reduce the angiogenic activity in ROP eyes.[6,7]

The angiopoietins (Angs) are growth factors that modulate the processes of not only physiological angiogenesis but also pathologic neovascularization, particularly associated with VEGF.[8] Ang-1, Ang-2, Ang-3, and Ang-4 are members of the Ang family.[8] Among them, Ang-1 and Ang-2 are ligands of tyrosine kinase receptor Tie2 and have similar binding affinities for Tie2.[8,9] Ang-1 is expressed by pericytes in vitro and in vivo.[10] It induces autophosphorylation of Tie2 and promotes remodeling, maturation, and stabilization of blood vessels by recruiting surrounding support cells and extracellular matrix.[11–14] Ang-2, on the other hand, is expressed by endothelial cells in vivo and is an antagonist for the Tie2 receptor by inhibiting its autophosphorylation.[9,15,16] Ang-2 induces endothelial destabilization and promotes angiogenesis in the presence of VEGF, and destabilization by Ang-2 in the absence of VEGF leads to the regression of fragile vessels.[14,17] The production of Ang-2 is upregulated by hypoxia[18] and VEGF.[18,19]

The purpose of this study was to measure the vitreous levels of Ang-1 and Ang-2 in ROP eyes and to compare the levels to that in eyes with congenital cataracts. We also determined the correlation between the Ang-1 and Ang-2 levels in the vitreous.

METHODS

FORTY-EIGHT EYES OF 36 INFANTS (17 FEMALE AND 19 MALE infants) with stage 4 ROP (4A, 36 eyes; 4B, 12 eyes) were studied. The mean gestational age of the infants was 24.2 weeks (range, 22–26 weeks), and the mean birth weight was 640 grams (range, 332–977 grams). All of the infants

Accepted for publication Aug 25, 2010.
From the Departments of Applied Visual Science (T.S., S.K.) and Ophthalmology (C.S.), Osaka University, Graduate School of Medicine, Suita, Japan.
Inquiries to Shunji Kusaka, Department of Ophthalmology, Sakai Hospital Kinki University Faculty of Medicine, 2-7-1 Harayamadai, Minami-ku, Sakai, 590-0132 Osaka, Japan; e-mail: kusaka-ns@umin.net

0002-9394/$36.00
doi:10.1016/j.ajo.2010.08.037

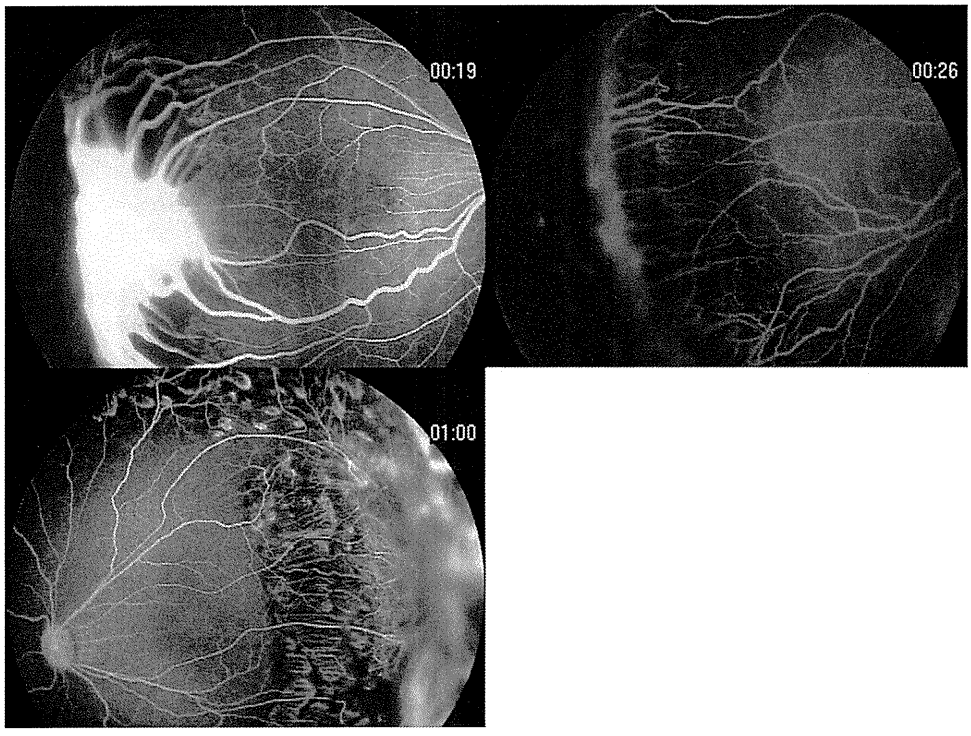

FIGURE 1. Fluorescein angiography in eyes with stage 4 retinopathy of prematurity (ROP). (Top left) Representative fluorescein angiogram in an eye with highly vascular-active ROP. The angiogram shows dilation and tortuosity of the posterior retinal vessels and marked fluorescein leakage from the neovascularization. (Top right) Representative fluorescein angiogram in an eye with moderately vascular-active ROP. The angiogram shows dilation and tortuosity of the posterior retinal vessels and weak fluorescein leakage from neovascularization. (Bottom left) Representative fluorescein angiogram in an eye with mildly vascular-active ROP. The angiogram shows that the posterior retinal vessels are not dilated and tortuous and shows the mild fluorescein leakage from the neovascularization.

underwent primary vitreous surgery at the Osaka University Hospital, Osaka, Japan from July 5, 2007 through December 24, 2009. All of the eyes underwent indirect photocoagulation of the avascular peripheral retina before the vitrectomy.

Fundus examinations with a slit lamp and contact lens (Volk Quad Pediatric Lens; Volk Optical Inc, Mentor, Ohio, USA) were performed under general anesthesia. During the examinations, fundus photographs and fluorescein angiograms were taken with a RetCam 120 digital fundus camera (Massie Research Laboratories, Inc, Pleasanton, California, USA). The stage of the ROP was based on the International Classification of Retinopathy of Prematurity.[20] In addition, the stage 4 ROP eyes were classified into 3 groups: highly vascular-active ROP, moderately vascular-active ROP, and mildly vascular-active ROP, as described (Figure 1).[4,5] Eyes with highly vascular-active ROP initially received 0.5 mg of intravitreal bevacizumab (IVB) and underwent vitrectomy 1 to 7 days after the injection.[7] Vitrectomy was performed on moderately and mildly vascular-active ROP eyes without IVB.

Six eyes of 5 infants with congenital cataract (3 female and 2 male infants), whose ages ranged from 1 month to 4 years, were studied as controls. All of these infants were full-term babies and did not have any other ocular or systemic complications.

Undiluted vitreous samples were collected from eyes with ROP during 3-port closed vitrectomy with a 23-gauge system before the infusion valve was opened. In the eyes with congenital cataract, the 2-port limbal approach was used. After lens aspiration, the aqueous humor in anterior chamber was replaced by viscoelastic material. A posterior continuous curvilinear capsulorrhexis was performed,[21] and undiluted vitreous samples were collected during the anterior vitrectomy.

The vitreous samples were collected in sterile tubes, which were then placed on dry ice and stored at −80°C until the assay. For the protein assay, the vitreous samples were thawed and centrifuged at 15 000 rpm for 10 minutes at 4°C. The supernatants were used to determine the vitreous levels of Ang-1 and Ang-2 by enzyme-linked immunosorbent assay with kits for human anti-Ang-1 and anti-Ang-2 (R & D Systems, Minneapolis, Minnesota, USA). The minimum detectable levels of the tests were 3.45 pg/mL for Ang-1 and 8.29 pg/mL for Ang-2. If the raw data were less than the minimum detectable levels, they were set to 0 for the statistical analyses. Each assay used 30 μL for Ang-1 and 25 μL for Ang-2 of the vitreous

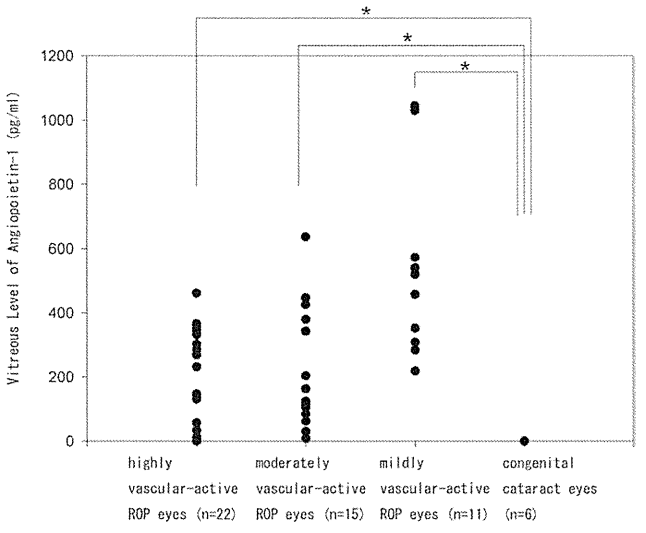

FIGURE 2. Vitreous level of angiopoietin-1 in eyes with retinopathy of prematurity and congenital cataract. The horizontal axis represents the sample type and the vertical axis represents the vitreous level of angiopoietin-1. Statistical analyses were performed by Kruskal-Wallis 1-way analysis of variance, followed by Dunn's method (*$P < .05$). ROP = retinopathy of prematurity.

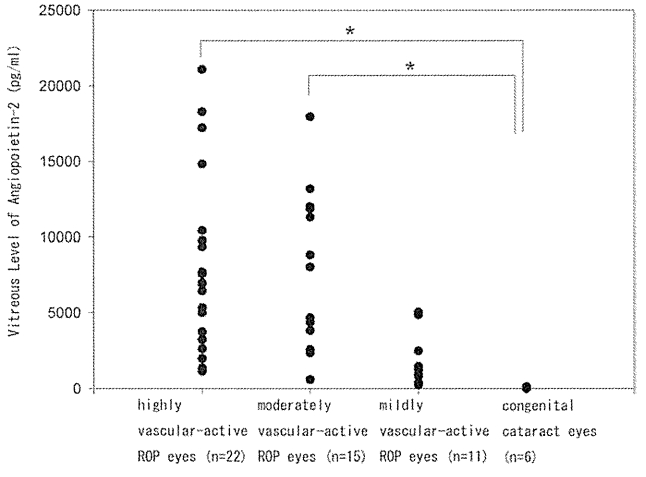

FIGURE 3. Vitreous level of angiopoietin-2 in eyes with retinopathy of prematurity and congenital cataract. The horizontal axis represents the sample type and the vertical axis represents the vitreous level of angiopoietin-2. Statistical analyses were performed by Kruskal-Wallis 1-way analysis of variance, followed by Dunn's method (*$P < .05$). ROP = retinopathy of prematurity.

sample/well, and the assay was performed twice. The optical density was determined at 450 nm with an absorption spectrophotometer ($ARVO_{MX}$; PerkinElmer Japan, Kanagawa, Japan) with the correction wavelength set at 540 nm.

Statistical analyses were performed using SPSS software (Sigma Stat; Systat Software, Inc, San Jose, California, USA). Data are presented as the means and ranges.

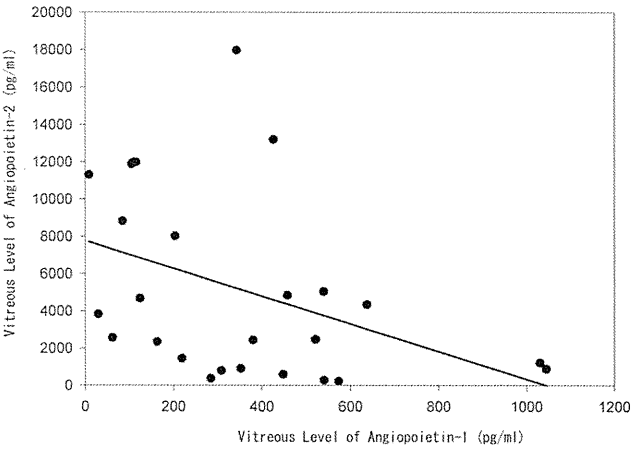

FIGURE 4. Correlation between angiopoietin-1 and -2 levels in the vitreous of eyes with retinopathy of prematurity (ROP). The horizontal and vertical axes represent the vitreous levels of angiopoietin-1 and -2 in moderately and mildly vascular-active ROP eyes, respectively. Statistical analyses were performed by Pearson product moment correlation ($r = -0.406$, $P = .040$).

Kruskal-Wallis 1-way analysis of variance (ANOVA) on ranks was used to compare the vitreous concentrations of Ang-1and Ang-2 among the groups, followed by Dunn's method to detect significant difference between 2 groups. The correlation between the Ang-1 and Ang-2 levels was determined by the Pearson product moment correlation. A P value less than .05 was considered to be statistically significant.

RESULTS

AMONG THE EYES WITH STAGE 4 ROP, 22 EYES WERE CLASsified as highly vascular-active ROP, 15 eyes as moderately vascular-active ROP, and 11 eyes as mildly vascular-active ROP. The mean vitreous level of Ang-1 was 201.9 pg/mL (range, 0 to 461.7 pg/mL) in the highly vascular-active ROP eyes, 216.1 pg/mL (range, 8.5 to 637.0 pg/mL) in the moderately vascular-active ROP eyes, 533.8 pg/mL (range, 218.9 to 1044.9 pg/mL) in the mildly vascular-active ROP eyes, and 0 pg/mL (range, 0 to 0 pg/mL) in the control eyes. The vitreous levels of Ang-1 were significantly different ($P < .001$) among the 4 groups. The levels of Ang-1 in the highly, moderately, and mildly vascular-active ROP eyes were significantly ($P < .05$) higher than that in the control eyes (Figure 2).

The vitreous levels of Ang-2 were significantly different ($P < .001$) among the 4 groups. The mean vitreous level of Ang-2 in the highly vascular-active ROP eyes that had received IVB was 7832.1 pg/mL (range, 1136.6 to 21 078.1 pg/mL). The mean vitreous level of Ang-2 was 7731.2 pg/mL (range, 599.5 to 17 956.0 pg/mL) in the moderately vascular-active ROP eyes, 1685.9 pg/mL (range, 231.0 to 5055.1 pg/mL) in the mildly vascular-active ROP eyes, and

41.5 pg/mL (range, 0 to 125.5 pg/mL) in the control eyes. The levels of Ang-2 in the highly and moderately vascular-active ROP eyes were significantly ($P < .05$) higher than that in control eyes (Figure 3).

Because the vitreous level of Ang-2 in highly vascular-active ROP eyes could be affected by the IVB,[18,19] the correlation of the vitreous levels between Ang-1 and Ang-2 was analyzed only in the moderately and mildly vascular-active ROP eyes. The analysis showed a significant negative correlation ($r = -0.406$; $P = .040$) between the Ang-1 and Ang-2 levels in the vitreous of these ROP eyes (Figure 4).

DISCUSSION

THE MAJOR FINDINGS IN THIS STUDY ARE: FIRST, THE VITreous level of Ang-1 was significantly higher in the highly, moderately, and mildly vascular-active ROP eyes than in control eyes. Second, the vitreous Ang-2 level was significantly higher in the highly and moderately vascular-active ROP eyes than in control eyes. And third, there was a significant negative correlation between the Ang-1 and Ang-2 levels in the vitreous fluid of the moderately and mildly vascular-active ROP eyes.

Ang-1 was first described in 1996 to be a ligand for the Tie2 receptor.[12] In vivo experiments on transgenic mice that overexpressed Ang-1[22] or of recombinant adenoviruses expressing Ang-1[23] showed that Ang-1 induced the development of blood vessels that were not leaky and vessels that did not leak when exposed to inflammatory agents. Mice lacking Ang-1 had angiogenic deficits similar to those seen in mice lacking Tie2. These findings indicated that Ang-1 was a primary physiologic ligand for Tie2, and that it played a critical role in in vivo angiogenic activity.[13] Ang-1 has also been demonstrated in diabetic rats where it prevents and reverses diabetic retinal vascular changes, such as blood-retinal barrier breakdown, in both new and established diabetes.[24] We found that the vitreous level of Ang-1 was significantly higher in the highly, moderately, and mildly vascular-active ROP eyes than that in the control eyes. Taking these results together, Ang-1 may contribute to the antipermeability and stabilization of new blood vessels in ROP eyes with any increased degree of vascular activity.

Ang-2 was first described in 1997 as a naturally occurring antagonist against Ang-1 and Tie2.[9] Transgenic mice overexpressing Ang-2 had disruptions in the formation of embryonic blood vessels.[9] Ang-2 also caused pericyte dropout in normal retinas.[25] The inhibition of Ang-2 by an intravitreal injection of sTie2-Fc reduced retinal neovascularization in murine oxygen-induced retinopathy (OIR), a commonly used mouse model of ROP.[16] The vitreous level of Ang-2 has been shown to be higher in eyes with proliferative diabetic retinopathy than that in eyes without proliferative diabetic retinopathy, and the level increased as the vascular activity increased.[26] We found that the level of Ang-2 was significantly higher in the highly and moderately vascular-active ROP eyes than that of control eyes. Taken together, Ang-2 may contribute to vascular plasticity and angiogenesis in ROP eyes.

We also found that the vitreous levels of Ang-1 and Ang-2 were significantly negatively correlated in the moderately and mildly vascular-active ROP eyes. These results indicated that the balance of positive and negative regulation by both Ang-1 and Ang-2 may be crucial to angiogenic events in ROP.

We did not determine the vitreous level of VEGF. However, we have measured the vitreous level of VEGF in stage 4 ROP eyes[4,5] and found that the level in highly vascular-active ROP eyes that had received 0.5 mg of IVB was not significantly different from that of control (congenital cataract) eyes.[4] This suggested that a dose of 0.5 mg IVB may be enough to inhibit the activity of VEGF in ROP eyes. On the other hand, although the IVB led to reduced neovascular activity in most cases of ROP, we have never had a complete resolution of leakage after IVB.[7] The reason for this discrepancy between the VEGF and neovascular activity has not been determined. One possibility is that a molecule (eg, Ang-2) other than VEGF may play a significant role in the vascular activity in ROP eyes.

The origins of vitreous Ang-1 and Ang-2 were also not determined. Because the serum concentrations of the Angs were not measured, we cannot eliminate the possibility of leakage of the Angs from blood into the vitreous cavity. However, the vitreous Angs are more likely derived from the eye. One of the reasons for this is that the production of Ang-2 has been demonstrated to be upregulated by hypoxia and VEGF,[18,19] which is similar to the condition in ROP eyes. In fact, we have investigated the comprehensive gene expression changes in murine OIR, and demonstrated that the mRNA level of Ang-2 is upregulated at the time when the extraretinal neovascularization is the most prominent.[27] These results suggested that the Angs may be produced in ROP eyes in response to the clinical conditions. Further studies are needed to determine the origin of Angs in the vitreous.

In summary, the results of this study showed that the vitreous levels of Ang-1 and Ang-2 in stage 4 ROP eyes were significantly higher than those of control eyes. The levels of Ang-1 and Ang-2 showed a significant negative correlation. These results indicate that the balance of positive and negative regulation by both Ang-1 and Ang-2 may be important to the pathology of ROP.

PUBLICATION OF THIS ARTICLE WAS SUPPORTED BY GRANT-IN-AID 17591832 AND 20592041 FROM THE MINISTRY OF Education, Culture, Sports, Science and Technology, Tokyo, Japan. The authors indicate no financial conflict of interest. Involved in conception and design (T.S., S.K.); analysis and interpretation (T.S., S.K.); writing the article (T.S., S.K.); critical revision (T.S., S.K.); final approval (T.S., C.S., S.K.);

data collection (T.S., C.S., S.K.); provision of patients and resources (T.S., C.S., S.K.); statistical expertise (T.S., S.K.); and literature search (T.S., S.K.). The procedures used in this study conformed to the tenets of the Declaration of Helsinki and were approved by the Institutional Review Board of Osaka University Hospital. The parents of all of the patients provided written informed consent after an explanation of the nature and possible consequences of this study.

REFERENCES

1. Terry TL. Fibroblastic overgrowth of persistent tunica vasculosa lentis in infants born prematurely: II. Report of cases-clinical aspects. Trans Am Ophthalmol Soc 1942;40: 262–284.
2. Mechoulam H, Pierce EA. Retinopathy of prematurity: molecular pathology and therapeutic strategies. Am J Pharmacogenomics 2003;3(4):261–277.
3. Sonmez K, Drenser KA, Capone A Jr, Trese MT. vitreous levels of stromal cell-derived factor 1 and vascular endothelial growth factor in patients with retinopathy of prematurity. Ophthalmology 2008;115(6):1065–1070.
4. Sato T, Kusaka S, Shimojo H, Fujikado T. Vitreous levels of erythropoietin and vascular endothelial growth factor in eyes with retinopathy of prematurity. Ophthalmology 2009;116(9): 1599–1603.
5. Sato T, Kusaka S, Shimojo H, Fujikado T. Simultaneous analyses of vitreous levels of 27 cytokines in eyes with retinopathy of prematurity. Ophthalmology 2009;116(11):2165–2169.
6. Mintz-Hittner HA, Kuffel RR Jr. Intravitreal injection of bevacizumab (avastin) for treatment of stage 3 retinopathy of prematurity in zone I or posterior zone II. Retina 2008;28(6):831–838.
7. Kusaka S, Shima C, Wada K, et al. Efficacy of intravitreal injection of bevacizumab for severe retinopathy of prematurity: a pilot study. Br J Ophthalmol 2008;92(11):1450–1455.
8. Koh GY, Kim I, Kwak HJ, Yun MJ, Leem JC. Biomedical significance of endothelial cell specific growth factor, angiopoietin. Exp Mol Med 2002;34(1):1–11.
9. Maisonpierre PC, Suri C, Jones PF, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997;277(5322):55–60.
10. Sundberg C, Kowanetz M, Brown LF, Detmar M, Dvorak HF. Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis in vivo. Lab Invest 2002;82(4):387–401.
11. Sato TN, Tozawa Y, Deutsch U, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 1995;376(6535):70–74.
12. Davis S, Aldrich TH, Jones PF, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996;87(7):1161–1169.
13. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996;87(7):1171–1180.
14. Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene 1999;18(38):5356–5362.
15. Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci U S A 2003;100(19):10623–10628.
16. Takagi H, Koyama S, Seike H, et al. Potential role of the angiopoietin/tie2 system in ischemia-induced retinal neovascularization. Invest Ophthalmol Vis Sci 2003;44(1):393–402.
17. Asahara T, Chen D, Takahashi T, et al. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulated VEGF-induced postnatal neovascularization. Circ Res 1998; 83(3):233–240.
18. Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. Hypoxia and vascular endothelial growth factor selectively up-regulated angiopoietin-2 in bovine microvascular endothelial cells. J Biol Chem 1999;274(22):15732–15739.
19. Mandriota SJ, Pepper MS. Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. Circ Res 1998;83(8):852–859.
20. International committee for the classification of retinopathy of prematurity. The international classification of retinopathy of prematurity revisited. Arch Ophthalmol 2005;123(7):991–999.
21. Gimbel HV, DeBroff BM. Posterior capsulorhexis with optic capture: maintaining a clear visual axis after pediatric cataract surgery. J Cataract Refract Surg 1994;20(6):658–664.
22. Thurston G, Suri C, Smith K, et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 1999;286(5449):2511–2514.
23. Thurston G, Rudge JS, Ioffe E, et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 2000;6(4):460–463.
24. Joussen AM, Poulaki V, Tsujikawa A, et al. Suppression of diabetic retinopathy with angiopoietin-1. Am J Pathol 2002; 160(5):1683–1693.
25. Hammes HP, Lin J, Wagner P, et al. Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. Diabetes 2004;53(4):1104–1110.
26. Watanabe D, Suzuma K, Suzuma I, et al. Vitreous levels of angiopoietin 2 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. Am J Ophthalmol 2005;139(3):476–481.
27. Sato T, Kusaka S, Hashida N, Saishin Y, Fujikado T, Tano Y. Comprehensive gene expression profile in murine oxygen-induced retinopathy. Br J Ophthalmol 2009;93(1):96–103.

Biosketch

Tatsuhiko Sato, MD, received his medical degree from Osaka University Graduate School of Medicine, Suita, Japan, in 2001. He completed residency at Osaka University Hospital. Now he is an attending staff in Ophthalmology at Osaka Rosai Hospital. His field of interest includes surgical treatment of vitreoretinal disease such as diabetic retinopathy, retinal detachment, and retinopathy of prematurity.

Serum Concentrations of Bevacizumab (Avastin) and Vascular Endothelial Growth Factor in Infants With Retinopathy of Prematurity

TATSUHIKO SATO, KAZUKO WADA, HITOMI ARAHORI, NORIYUKI KUNO, KENJI IMOTO, CHIHARU IWAHASHI-SHIMA, AND SHUNJI KUSAKA

• PURPOSE: To determine the serum concentrations of bevacizumab and vascular endothelial growth factor (VEGF) in infants with retinopathy of prematurity (ROP) who received intravitreal bevacizumab; and to determine whether the changes in the serum concentration of bevacizumab were significantly correlated with the serum concentration of VEGF after intravitreal bevacizumab.

• DESIGN: Case series.

• METHODS: Eleven infants (4 girls and 7 boys) with ROP were studied. They received 0.25 mg or 0.5 mg of intravitreal bevacizumab to either 1 eye (unilateral cases) or both eyes (bilateral cases) with vascularly active ROP. Serum samples were collected before and 1 day, 1 week, and 2 weeks after the intravitreal bevacizumab. The serum concentrations of bevacizumab and VEGF were measured by enzyme-linked immunosorbent assay, and the correlation in the serum levels between the 2 was determined.

• RESULTS: The serum concentration of bevacizumab before and 1 day, 1week, and 2 weeks after a total of 0.5 mg of intravitreal bevacizumab was 0 ng/mL, 195 ± 324 ng/mL, 946 ± 680 ng/mL, and 1214 ± 351 ng/mL, respectively. The serum bevacizumab level before and 1 day and 1 week after a total 1.0 mg of intravitreal bevacizumab was 0 ng/mL, 248 ± 174 ng/mL, and 548 ± 89 ng/mL, respectively. The serum concentration of VEGF before and 1 day, 1 week, and 2 weeks after a total of 0.5 mg intravitreal bevacizumab was 1628 ± 929 pg/mL, 427 ± 140 pg/mL, 246 ± 110 pg/mL, and 269 ± 157 pg/mL, respectively. There was a significant negative correlation ($r = -0.575$, $P = .0125$) between the serum concentration of bevacizumab and VEGF when a total of 0.25 mg or 0.5 mg of bevacizumab was injected.

• CONCLUSIONS: These results indicate that bevacizumab can escape from the eye into the systemic circulation and reduce the serum level of VEGF in infants with ROP. Continued extensive evaluations of infants are warranted for possible effects after intravitreal bevacizumab in ROP patients. (Am J Ophthalmol 2011; xx:xxx. © 2011 by Elsevier Inc. All rights reserved.)

Accepted for publication Jul 22, 2011.

From the Departments of Applied Visual Science (T.S., S.K.), Pediatrics (K.W., H.A.), and Ophthalmology (C.I.S.), Osaka University Graduate School of Medicine, Suita, Japan; Research and Development Center, Santen Pharmaceutical Co, Ltd (N.K., K.I.), Nara, Japan; and Department of Ophthalmology (S.K.), Sakai Hospital, Kinki University Faculty of Medicine, Osaka, Japan.

Inquiries to Shunji Kusaka, Department of Ophthalmology, Sakai Hospital, Kinki University Faculty of Medicine, 2-7-1 Harayamadai, Minami-ku, Sakai, 590-0132 Osaka, Japan; e-mail: kusaka-ns@umin.net

RETINOPATHY OF PREMATURITY (ROP) IS THE LEADing cause of infant blindness, especially in developed countries. Retinal photocoagulation of the peripheral avascular retina is commonly used to treat eyes with ROP without retinal detachment, and scleral buckling or vitrectomy is used in ROP eyes with retinal detachment. Recently, early vitrectomy has been used to treat eyes with ROP to obtain favorable functional and structural outcomes.[1] However, some of the ROP eyes have high vascular activity, and vitrectomy in these eyes usually results in poor surgical outcomes.[2]

For such cases with high vascular activity, we have performed vitrectomy combined with a preoperative intravitreal injection of an antibody against vascular endothelial growth factor (VEGF).[3] VEGF is the main growth factor responsible for angiogenesis and is considered to be the primary angiogenic factor that mediates retinal neovascularization in eyes with ROP.[4] Studies of patients with stage 4 ROP showed that the vitreous concentration of VEGF in eyes with vascularly active ROP was significantly higher than in eyes with vascularly inactive ROP,[5–7] and anti-VEGF therapy has been shown to be effective in reducing the angiogenic activity in eyes with ROP.[3,8,9]

Bevacizumab (Avastin; Genentech Inc, South San Francisco, California, USA) is a recombinant humanized monoclonal antibody that is directed against all isoforms of VEGF. Many studies have reported on the effectiveness of intravitreal bevacizumab on neovascular disorders, for example, age-related macular degeneration,[10] proliferative diabetic retinopathy,[11] neovascular glaucoma,[12] and ROP.[3,8,9] In addition, the results of a randomized clinical trial that compared intravitreal bevacizumab as monotherapy with laser therapy in the treatment of ROP have been published.[13] As intravitreal bevacizumab was shown to be of significant benefit compared to laser therapy in zone I stage 3+ ROP, the use of intravitreal bevacizumab in the treatment of ROP is likely to be more common in the near future. However, there are also studies that have reported that intravitreal bevacizumab had adverse sys-

0002-9394/$36.00
doi:10.1016/j.ajo.2011.07.005

TABLE 1. Demographics of Infants with Retinopathy of Prematurity

Patient	Sex	Eye/Stage	Intravitreal Bevacizumab (mg)	Gestational Age (Weeks)	Body Weight at Birth (g)	Postmenstrual Age at Intravitreal Bevacizumab (Weeks)	Body Weight at Intravitreal Bevacizumab (g)	Time of Intravitreal Bevacizumab to Vitrectomy (Days)
1	Male	Right/5	—	24	753	—	1820	Not applicable
		Left/4A	0.5			36		2
2	Male	Right/4B	—	23	611	—	2490	1
		Left/4A	0.5			39		1
3	Female	Right/4A	0.5	23	492	37	1354	3
		Left/4A	0.5			37		Not applicable
4	Male	Right/5	0.5	24	332	38	1384	2
		Left/4A	0.5			38		2
5	Male	Right/4A	0.5	23	686	41	2098	2
		Left/3	0.5			41		Not applicable
6	Female	Right/3	0.5	26	826	33	1214	Not applicable
		Left/3	0.5			33		Not applicable
7	Female	Right/3	0.25	25	768	41	2600	Not applicable
		Left/4B	—			—		0
8	Male	Right/3	0.25	27	454	51	2151	Not applicable
		Left/3	0.25			51		Not applicable
9	Male	Right/3	0.25	26	828	35	1476	Not applicable
		Left/3	0.25			35		Not applicable
10	Female	Right/3	0.25	23	472	38	940	6
		Left/5	0.25			38		6
11	Male	Right/3	0.25	27	1042	32	1398	Not applicable
		Left/3	0.25			32		Not applicable

temic effects.[14,15] These adverse effects (for example, systemic thrombotic events and hypertension) are similar to the ones reported after intravenous administration of bevacizumab for cancer treatments. Although no systemic adverse event has been reported after intravitreal bevacizumab in eyes with ROP,[3,8,9,13] the serum concentration of bevacizumab after intravitreal bevacizumab has not been determined.

Thus, the purpose of this study was to determine the serum concentrations of bevacizumab and VEGF in ROP infants who received intravitreal bevacizumab.

METHODS

THE FUNDUS OF INFANTS WITH ROP WAS EXAMINED WITH a slit lamp and contact lens (Volk Quad Pediatric Lens; Volk Optical Inc, Mentor, Ohio, USA) under general anesthesia. During the examinations, fundus photographs and fluorescein angiograms were taken with a RetCam 120 digital fundus camera (Clarity Medical Systems, Inc, Pleasanton, California, USA). The stage of the ROP was based on the International Classification of Retinopathy of Prematurity.[16] The ROP eyes were also classified into 3 groups according to the vascular activity: highly vascular-active ROP, moderately vascular-active ROP, and mildly vascular-active ROP.[17] The eyes with highly vascular-active ROP initially received 0.25 mg or 0.5 mg of intravitreal bevacizumab and underwent 23-gauge pars plicata vitrectomy without cannula system. The surgery was performed within 1 week after the injection when considered to be necessary.[3]

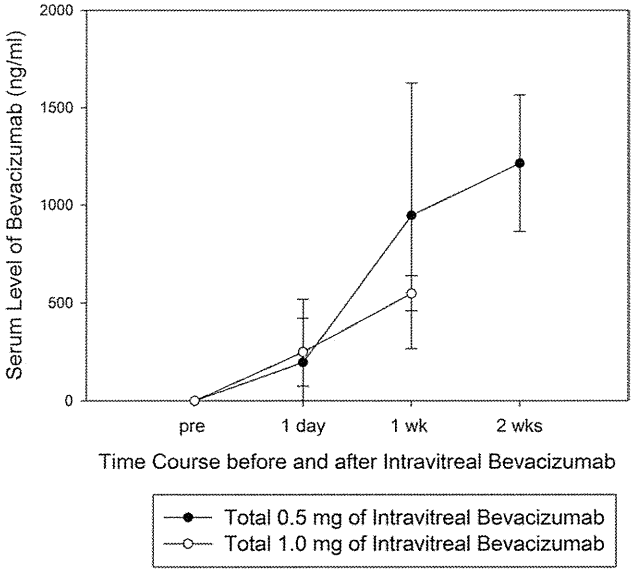

FIGURE 1. Time course of serum level of bevacizumab in infants with retinopathy of prematurity who received intravitreal bevacizumab. The abscissa represents the time before and after intravitreal bevacizumab and the ordinate represents the serum level of bevacizumab.

TABLE 2. Serum Levels of Bevacizumab (Avastin) and Vascular Endothelial Growth Factor in Infants With Retinopathy of Prematurity

		Serum Level of Bevacizumab (ng/mL)				Serum Level of Vascular Endothelial Growth Factor (pg/mL)			
Eye/Stage	Total Dosage of Intravitreal Bevacizumab (mg)	Before Intravitreal Bevacizumab	1 Day After Intravitreal Bevacizumab	1 Week After Intravitreal Bevacizumab	2 Weeks After Intravitreal Bevacizumab	Before Intravitreal Bevacizumab	1 Day After Intravitreal Bevacizumab	1 Week After Intravitreal Bevacizumab	2 Week Intrav Bevaci
Right/5 Left/4A	0.5	0	23	NA	NA	NA	NA	NA	N
Right/4B Left/4A	0.5	0	31	81	NA	NA	NA	NA	N
Right/4A Left/4A	1.0	0	206	665	NA	NA	NA	NA	N
Right/5 Left/4A	1.0	0	396	513	NA	NA	NA	NA	N
Right/4A Left/3	1.0	0	19	560	NA	NA	NA	NA	N
Right/3 Left/3	1.0	0	372	453	NA	NA	NA	NA	N
Right/3 Left/4B	0.25	0	11	113	NA	418	303	301	N
Right/3 Left/3	0.5	0	33	1204	NA	603	227	106	N
Right/3 Left/3	0.5	0	36	610	844	1140	515	208	1
Right/3 Left/5	0.5	0	841	1905	1255	2110	433	331	4
Right/3 Left/3	0.5	0	209	928	1542	2660	533	337	2

e serum levels of bevacizumab and vascular endothelial growth factor in the blank cells could be not measured because of the limited sample volumes.

Blood samples were collected before and 1 day, 1 week, and 2 weeks after the intravitreal bevacizumab. This schedule was based on the data from animal experiments demonstrating that the maximum blood level of bevacizumab is achieved about 1 to 2 weeks after the intravitreal bevacizumab.[18–20] The blood samples were collected in sterile tubes by an anesthesiologist or a neonatologist and centrifuged at 5000 rpm for 10 minutes until a clear separation between serum and the cell components was seen. The serum was transferred to sterile tubes and stored at −80 C until the assay.

The serum concentration of bevacizumab was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Protein Detector ELISA Kit; Kirkegaard & Perry Laboratories, Inc, Gaithersburg, Maryland, USA), according to the manufacturer's protocol and also according to an earlier report with slight modifications.[21] Briefly, microwell plates (Immuno 96 MicroCell solid plates; Nunc, Roskilde, Denmark) were coated with recombinant human $VEGF_{165}$ (PeproTech, Rocky Hill, New Jersey, USA) at a concentration of 1.0 μg/mL for 1 hour at room temperature (100 μL/well). After blocking the wells to reduce nonspecific binding, 100 μL of each sample and different concentrations of the standard were added to the plates. A standard curve was prepared with bevacizumab ranging from 1 ng/mL to 5000 ng/mL. The bound bevacizumab was made visible with 0.1 μg/mL of horseradish peroxidase–goat anti-human IgG (H+L) conjugate prepared by the ELISA kit. The optical density was determined at 405 nm with an absorption spectrophotometer ($ARVO_{MX}$; PerkinElmer Japan, Kanagawa, Japan). The background absorbance was subtracted from all values. This assay measures the free bevacizumab, and all measurements were performed twice according to the manufacturer's recommendation.

The serum concentration of VEGF was measured with an ELISA kit for human anti-VEGF (R & D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol. The anti-VEGF kit can detect the 121 and 165 isoforms of VEGF. The minimum detectable level of the test was 9.0 pg/mL for VEGF. The optical density was determined at 450 nm with the absorption spectrophotometer with the correction wavelength set at 540 nm. The assay was also performed in duplicate.

Statistical analyses were performed using the SPSS software (Sigma Stat; Systat Software, Inc, San Jose, California, USA). Data are presented as the means and standard deviations. If the data were normally and equally distributed, 1-way repeated-measures analysis of variance was used to compare 3 or more matched groups, followed by the Holm-Sidak method to detect significant differences between each set of data. If the data were not normally or equally distributed, Friedman repeated-measures analysis of variance on ranks was performed to compare 3 or more matched groups, followed by Dunn's method to detect significant differences between each set of data. The significance of differences between 2 groups was determined by *t* tests if the data were normally and equally distributed and by the Mann-Whitney rank sum test if not normally distributed. The correlation between 2 parameters was determined by the Spearman rank order correlation because the residuals were not normally distributed with constant variance. A *P* value less than .05 was considered to be statistically significant.

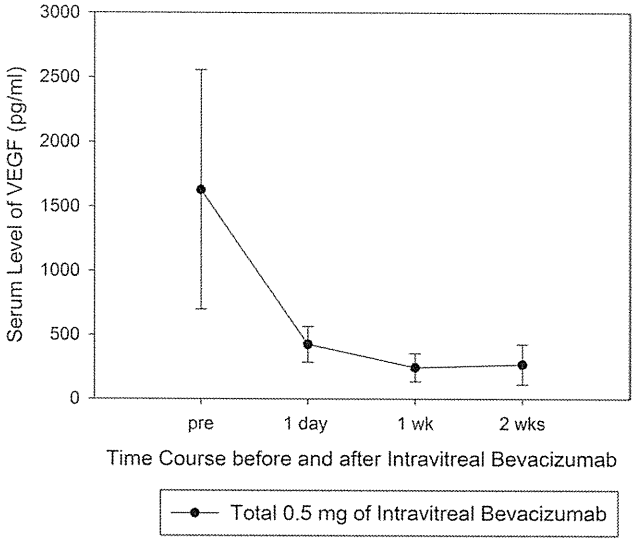

FIGURE 2. Time course of serum level of vascular endothelial growth factor (VEGF) in infants with retinopathy of prematurity who received a total of 0.5 mg of intravitreal bevacizumab. The abscissa represents the time before and after intravitreal bevacizumab and the ordinate represents the serum level of VEGF.

RESULTS

ELEVEN INFANTS (4 GIRLS AND 7 BOYS) WITH HIGHLY VASCULAR-active ROP were studied. The demographics of the patients are summarized in Table 1. Three patients received intravitreal bevacizumab in 1 eye and the other 8 received intravitreal bevacizumab in both eyes. The mean gestational age of the infants was 25 weeks (range, 23–27 weeks), and the mean body weight at birth was 660 grams (range, 332–1042 grams). All of the infants had received laser photocoagulation of the peripheral avascular retina before the intravitreal bevacizumab. The mean postmenstrual age of the infants at the time of intravitreal bevacizumab was 38 weeks (range, 32–51 weeks), and the mean body weight at the time of the intravitreal bevacizumab was 1720 grams (range, 940–2600 grams). In Patients 6, 8, 9, and 11 with stage 3 ROP, vitrectomy was not performed after the intravitreal bevacizumab because of the reduction of vascular activities. In the remaining patients, vitrectomy was performed 0 to 6 days after the intravitreal bevacizumab.

The average serum levels of bevacizumab before and 1 day, 1 week, and 2 weeks after a total of 0.5 mg of

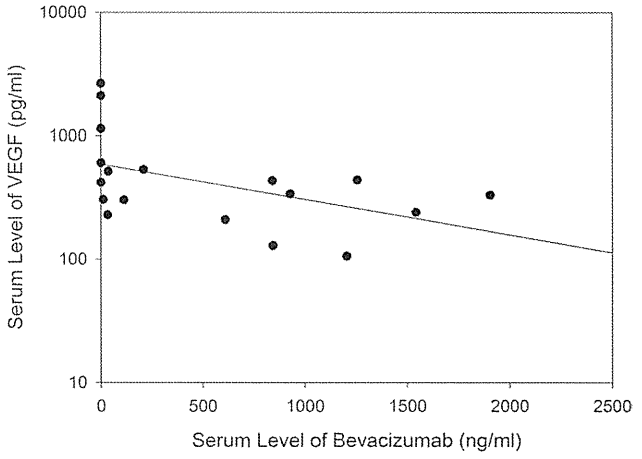

FIGURE 3. Correlation between bevacizumab and vascular endothelial growth factor (VEGF) levels in the serum of patients with retinopathy of prematurity (ROP). The abscissa represents the serum levels of bevacizumab and the ordinate represents the VEGF level in ROP infants. Statistical analyses were performed by Spearman rank order correlation ($r = -0.575$, $P = .0125$).

intravitreal bevacizumab were 0 ng/mL, 195 ± 324 ng/mL, 946 ± 680 ng/mL, and 1214 ± 351 ng/mL, respectively (Figure 1, Table 2). In Patients 2, 8, 9, 10, and 11, the serum bevacizumab levels were significantly different ($P = .008$) before and 1 day and 1week after the intravitreal bevacizumab, and the serum bevacizumab level at 1 week after the intravitreal bevacizumab was significantly higher ($P < .05$) than that before the intravitreal bevacizumab.

The average serum bevacizumab levels before and 1 day and 1 week after a total of 1.0 mg of intravitreal bevacizumab were 0 ng/mL, 248 ± 174 ng/mL, and 548 ± 89 ng/mL, respectively (Figure 1, Table 2). In Patients 3 through 6, the serum bevacizumab levels were significantly different ($P = .005$) before and 1 day and 1 week after the intravitreal bevacizumab. The serum bevacizumab level at 1 week after the intravitreal bevacizumab was significantly higher ($P < .05$) than that before the intravitreal bevacizumab. The differences in the serum bevacizumab levels after a total of 0.5 mg and 1.0 mg of intravitreal bevacizumab were not significant at any time points.

The average serum concentrations of VEGF before and 1 day, 1 week, and 2 weeks after a total of 0.5 mg of intravitreal bevacizumab were 1628 ± 929 pg/mL, 427 ± 140 pg/mL, 246 ± 110 pg/mL, and 269 ± 157 pg/mL, respectively (Figure 2, Table 2). The serum VEGF level in Patients 1 through 6 could not be measured because the volumes of the samples collected were too small. In Patients 8 through 11, who received a total of 0.5 mg of intravitreal bevacizumab, the serum VEGF levels were significantly different ($P = .005$) before and 1 day and 1 week after the intravitreal bevacizumab. The serum VEGF level at 1 week after the intravitreal bevacizumab was significantly lower ($P < .05$) than that before the intravitreal bevacizumab.

The correlation of serum levels of bevacizumab and VEGF was investigated in Patients 7 through 11, who received a total of 0.25 mg or 0.5 mg of intravitreal bevacizumab. The results showed that there was a significant negative correlation between the 2 levels ($r = -0.575$, $P = .0125$; Figure 3).

DISCUSSION

OUR RESULTS SHOWED THAT THE SERUM BEVACIZUMAB level was significantly higher at 1 week after than before the intravitreal bevacizumab in the ROP infants who received a total of 0.5 mg or 1.0 mg of intravitreal bevacizumab. In addition, the serum VEGF level was significantly lower at 1 week after than before the intravitreal bevacizumab in the ROP infants who underwent a total of 0.5 mg of intravitreal bevacizumab. Our results showed that there was a significant negative correlation between the serum levels of bevacizumab and VEGF in the ROP infants who received a total of 0.25 mg or 0.5 mg of intravitreal bevacizumab.

With regard to the serum bevacizumab level, animal experiments showed that the blood level of bevacizumab peaked at about 1 to 2 weeks after intravitreal bevacizumab.[18–20] In our patients, the serum level of bevacizumab was significantly increased 1 week after the intravitreal bevacizumab after a total of 0.5 mg or 1.0 mg of intravitreal bevacizumab, and the highest serum level of bevacizumab was achieved 2 weeks after the intravitreal bevacizumab after a total of 0.5 mg of intravitreal bevacizumab.

An in vitro experiment using human umbilical vein endothelial cells demonstrated that about 500 ng/mL of bevacizumab was able to completely block the VEGF activities.[22] Our results showed that the average serum level of bevacizumab exceeded 500 ng/mL at 1 week after the intravitreal bevacizumab in ROP infants who received a total of 0.5 mg or 1.0 mg of intravitreal bevacizumab. These results account for the decreased serum levels of VEGF after intravitreal bevacizumab in ROP infants. The results of an in vivo experiment (interview form for bevacizumab, Chugai Oncology, Tokyo, Japan; available only in Japanese) showed that a once-weekly intravenous injection of 2 mg/kg of bevacizumab in young macaque monkeys did not induce any obvious side effects 26 weeks after the beginning of the injections. The in vivo experiment demonstrated that the bevacizumab concentration in the serum 1 week after 1 intravenous injection of 2 mg/kg bevacizumab was over 10 μg/mL, which is much higher than the maximum serum level of bevacizumab in our study. Fortunately, the patients in our study did not show any systemic adverse events as far as our neonatologists

(K.W. and H.A.) could determine. However, a careful long-term study is necessary.

Our results also showed that the VEGF level significantly decreased 1 week after intravitreal bevacizumab, and the VEGF level was significantly correlated negatively with the serum bevacizumab level. These results suggest that bevacizumab escapes from the vitreous into the systemic circulation and reduces the VEGF concentrations in ROP infants after the intravitreal bevacizumab.

Data regarding the safe range of VEGF serum concentrations in premature infants with ROP have not been reported, although the systemic levels of VEGF in infants with or without ROP have been investigated.[23,24] Villegas-Becerril and associates[23] reported that at 4 to 6 weeks after birth, the mean serum VEGF concentration in premature babies with ROP was 708 pg/mL, which was significantly higher than the 511 pg/mL in premature babies without ROP. Pieh and associates[24] reported that the median plasma level of VEGF in ROP infants was 904 pg/mL at 32 weeks and 344 pg/mL at 36 weeks of postmenstrual age, and that in infants without ROP was 658 pg/mL at 32 weeks and 437 pg/mL at 36 weeks of postmenstrual age. The differences in the VEGF levels between ROP and non-ROP infants at both 32 and 36 weeks were not significant.[24]

The average serum VEGF level before the intravitreal bevacizumab in our ROP infants (Patients 7 through 11), whose average gestational age was 26.0 weeks, was 1386 pg/mL at an average postmenstrual age of 39.8 weeks. After the intravitreal bevacizumab, the average serum VEGF level was comparable to those of the 2 reports[23,24] for both ROP and non-ROP infants. Thus, the intravitreal bevacizumab did not induce an extreme inhibition of VEGF activities in ROP infants, although the serum VEGF level was significantly reduced 1 week after the 0.5 mg of intravitreal bevacizumab in ROP infants. Further studies are needed to determine the safe range of VEGF in ROP infants in order to establish the appropriate dose of intravitreal bevacizumab in ROP infants.

There are some limitations in this study. The number of patients was limited mainly because of the small number of infants with severe ROP. The number of blood samples at various time points was also limited because of the technical difficulties in obtaining blood samples from low-birth weight infants. These limitations made the statistical analyses difficult, and there is a possibility that the serum bevacizumab level may reach its maximal point more than 2 weeks after the intravitreal bevacizumab. Another limitation is that all of the infants had received laser photocoagulation to the peripheral avascular retina before the intravitreal bevacizumab. The laser photocoagulation may break down the retinal barrier.[25] Thus, although bevacizumab is a large molecule so that it has difficulty in escaping from the eye,[13] there is a possibility that the retinal photocoagulation led to the higher systemic levels of bevacizumab. In addition, the eyes with preoperative intravitreal bevacizumab received vitrectomy in Patients 1, 2, 3, 4, 5, and 10. Thus, the possible role of vitrectomy should be determined by either allowing the systemic diffusion of bevacizumab by opening the eye or decreasing it by washing out the intravitreal bevacizumab.

In conclusion, the serum levels of bevacizumab and VEGF were determined in vascularly active ROP infants who received intravitreal bevacizumab. The results suggest that bevacizumab escapes from the vitreous into systemic circulation and could suppress the VEGF concentration in infants with ROP after intravitreal bevacizumab. Although no systemic adverse events were observed in our patients, continued extensive evaluation of infants is warranted for possible effects after intravitreal bevacizumab in ROP patients.

PUBLICATION OF THIS ARTICLE WAS SUPPORTED BY GRANT-IN-AID 20592041 FROM THE MINISTRY OF EDUCATION, CULTURE, Sports, Science and Technology, Tokyo, Japan. All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. N. Kuno and K. Imoto are employees of Santen Pharmaceutical Co, Ltd. Involved in conception and design (S.K.); analysis and interpretation (T.S., S.K.); writing the article (T.S., S.K.); critical revision (S.K.); final approval (S.K.); data collection (K.W., H.A., C.I.-S.); provision of patients and resources (S.K.); statistical expertise (T.S., S.K.); obtaining funding (S.K.); literature search (T.S., S.K.); and technical support (N.K., K.I.). The sponsor or funding organization had no role in the design or conduct of this research. The procedures used in this study conformed to the tenets of the Declaration of Helsinki. The Institutional Review Board of Osaka University Hospital approved this retrospective study. The parents of all of the patients provided written informed consent after an explanation of the nature and possible consequences of this study.

REFERENCES

1. Azuma N, Ishikawa K, Hama Y, Hiraoka M, Suzuki Y, Nishina S. Early vitreous surgery for aggressive posterior retinopathy of prematurity. Am J Ophthalmol 2006;142(4):636–643.
2. Hartnett ME. Features associated with surgical outcome in patients with stages 4 and 5 retinopathy of prematurity. Retina 2003;23(3):322–329.
3. Kusaka S, Shima C, Wada K, et al. Efficacy of intravitreal injection of bevacizumab for severe retinopathy of prematurity: a pilot study. Br J Ophthalmol 2008;92(11):1450–1455.
4. Mechoulam H, Pierce EA. Retinopathy of prematurity: molecular pathology and therapeutic strategies. Am J Pharmacogenomics 2003;3(4):261–277.
5. Sonmez K, Drenser KA, Capone A Jr, Trese MT. Vitreous levels of stromal cell-derived factor 1 and vascular endothelial growth factor in patients with retinopathy of prematurity. Ophthalmology 2008;115(6):1065–1070.
6. Sato T, Kusaka S, Shimojo H, Fujikado T. Vitreous levels of erythropoietin and vascular endothelial growth factor in eyes with retinopathy of prematurity. Ophthalmology 2009;116(9):1599–1603.

7. Sato T, Kusaka S, Shimojo H, Fujikado T. Simultaneous analyses of vitreous levels of 27 cytokines in eyes with retinopathy of prematurity. Ophthalmology 2009;116(11): 2165–2169.
8. Mintz-Hittner HA, Kuffel RR Jr. Intravitreal injection of bevacizumab (Avastin) for treatment of stage 3 retinopathy of prematurity in zone I or posterior zone II. Retina 2008; 28(6):831–838.
9. Wu WC, Yeh PT, Chen SN, Yang CM, Lai CC, Kuo HK. Effects and complications of bevacizumab use in patients with retinopathy of prematurity: a multicenter study in Taiwan. Ophthalmology 2011;118(1):176–183.
10. Avery RL, Pieramici DJ, Rabena MD, Castellarin AA, Nasir MA, Giust MJ. Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. Ophthalmology 2006;113(3):363–372.
11. Avery RL, Pearlman J, Pieramici DJ, et al. Intravitreal bevacizumab (Avastin) in the treatment of proliferative diabetic retinopathy. Ophthalmology 2006;113(10):1695–1705.
12. Oshima Y, Sakaguchi H, Gomi F, Tano Y. Regression of iris neovascularization after intravitreal injection of bevacizumab in patients with proliferative diabetic retinopathy. Am J Ophthalmol 2006;142(1):155–158.
13. Mintz-Hittner HA, Kennedy KA, Chuang AZ; BEAT-ROP Cooperative Group. Efficacy of intravitreal bevacizumab for stage 3+ retinopathy of prematurity. N Engl J Med 2011; 364(7):603–615.
14. Fung AE, Rosenfeld PJ, Reichel E. The international intravitreal bevacizumab safety survey: using the internet to assess drug safety worldwide. Br J Ophthalmol 2006;90(11):1344–1349.
15. Shima C, Sakaguchi H, Gomi F, et al. Complications in patients after intravitreal injection of bevacizumab. Acta Ophthalmol 2008;86(4):372–376.
16. International Committee for the Classification of Retinopathy of Prematurity. The international classification of retinopathy of prematurity revisited. Arch Ophthalmol 2005; 123(7):991–999.
17. Sato T, Shima C, Kusaka S. Vitreous levels of angiopoietin-1 and angiopoietin-2 in eyes with retinopathy of prematurity. Am J Ophthalmol 2011;151(2):353–357.
18. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Singh RJ. Pharmacokinetics of intravitreal bevacizumab (Avastin). Ophthalmology 2007;114(5):855–859.
19. Miyake T, Sawada O, Kakinoki M, et al. Pharmacokinetics of bevacizumab and its effect on vascular endothelial growth factor after intravitreal injection of bevacizumab in macaque eyes. Invest Ophthalmol Vis Sci 2010;51(3): 1606–1608.
20. Nomoto H, Shiraga F, Kuno N, et al. Pharmacokinetics of bevacizumab after topical, subconjunctival, and intravitreal administration in rabbits. Invest Ophthalmol Vis Sci 2009; 50(10):4807–4813.
21. Zhu Q, Ziemssen F, Henke-Fahle S, et al. Vitreous levels of bevacizumab and vascular endothelial growth factor-A in patients with choroidal neovascularization. Ophthalmology 2008;115(10):1750–1755.
22. Wang Y, Fei D, Vanderlaan M, Song A. Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro. Angiogenesis 2004;7(4):335–345.
23. Villegas-Becerril E, Gonzalez-Fernandez R, Perula-Torres L, Gallardo-Galera JM. IGF-1, VEGF, and bFGF as predictive factors for the onset of retinopathy of prematurity (ROP). Arch Soc Esp Oftalmol 2006;81(11):641–646.
24. Pieh C, Agostini H, Buschbeck C, et al. VEGF-A, VEGFR-1, VEGFR-2 and Tie2 levels in plasma of premature infants: relationship to retinopathy of prematurity. Br J Ophthalmol 2008;92(5):689–693.
25. Sato Y, Berkowitz BA, Wilson CA, deJuan E Jr. Blood-retinal barrier breakdown caused by diode vs argon laser endophotocoagulation. Arch Ophthalmol 1992;110(2):277–281.

Biosketch

Tatsuhiko Sato, MD, received his medical degree from Osaka University Medical School, Osaka, Japan, in 2001. He completed residency at Osaka University Hospital. Now he is an attending staff in Ophthalmology at Osaka Rosai Hospital. His field of interest includes surgical treatment of vitreoretinal disease such as diabetic retinopathy, retinal detachment, and retinopathy of prematurity.

Acta Ophthalmologica

ACTA OPHTHALMOLOGICA 2011

Long-term efficacy and safety of ranibizumab administered *pro re nata* in Japanese patients with neovascular age-related macular degeneration in the EXTEND-I study

Yasuo Tano[1] and Masahito Ohji[2] on behalf of the EXTEND-I Study Group*

[1]Department of Ophthalmology, Osaka University Medical School, Osaka, Japan
[2]Department of Ophthalmology, Shiga University of Medical Science, Shiga, Japan

ABSTRACT.

Purpose: **To evaluate the long-term efficacy and safety of ranibizumab administered *pro re nata* (PRN) in Japanese patients with choroidal neovascularization secondary to age-related macular degeneration during the extension phase of the EXTEND-I study.**

Methods: **EXTEND-I, an open-label, multicenter, Phase I/II study comprised: a single-injection (Group A); a multiple-injection (Groups A and B; the latter consisted of patients who did not participate in the single-injection phase); and an extension phase. In the extension phase, a PRN regimen of ranibizumab (0.3 or 0.5 mg) guided by monthly best-corrected visual acuity (BCVA) score and other ophthalmic examinations was employed. The efficacy variables included the mean BCVA change from Month 12 to the last visit in Group B. Safety was assessed in all patients.**

Results: **In the extension phase, efficacy was assessed only in Group B patients. The number of ranibizumab injections per year in the 0.3 and 0.5 mg Group B patients was 4.19 and 4.27, respectively. The mean BCVA change (SD) from Month 12 to the last visit was −3.6 (14.82) letters for 0.3 mg (n = 28) and −2.2 (7.92) letters for 0.5 mg groups (n = 33) in Group B. Conjunctival haemorrhage and nasopharyngitis were the most commonly reported adverse events. Of the 13 serious adverse events reported, cerebral infarction (two incidences) was suspected to be study-drug related.**

Conclusions: ***Pro re nata*** **regimen of ranibizumab guided by monthly BCVA and other ophthalmic examinations appears effective in sustaining the BCVA gained with 12 monthly injections while reducing the number of injections during the extension phase. Ranibizumab was well tolerated during the extension phase.**

Key words: age-related macular degeneration – best-corrected visual acuity score – efficacy – individualized flexible interval regimen – Japanese patients – PRN – ranibizumab – safety – subfoveal choroidal neovascularization

Acta Ophthalmol. 2011: 89: 208–217

doi: 10.1111/j.1755-3768.2010.02065.x

*The EXTEND-I study group: Tomohiro Iida (Fukushima Medical University Hospital), Tatsuro Ishibashi (Kyushu University Hospital), Susumu Ishida (Hokkaido University Hospital), Shoji Kishi (Gunma University Hospital), Masahito Ohji (Shiga University of Medical Science Hospital), Annabelle A Okada (Kyorin University Hospital), Yukihiro Sato (Jichi Medical University Hospital), Fumio Shiraga (Kagawa University Hospital), Kunihiko Shiraki (Osaka City University Medical School Hospital), Yasuo Tano (Osaka University Hospital), Hiroko Terasaki (Nagoya University Hospital), and Mitsuko Yuzawa (Surugadai Nihon University Hospital).

Introduction

Age-related macular degeneration (AMD) is a leading cause of vision loss in the elderly population. Of the two types of AMD, the wet form caused by choroidal neovascularization (CNV) is mainly responsible for AMD-related vision loss (Bressler 2004). According to the Hisayama study (prospective cohort study in Japan), the prevalence of neovascular AMD in residents aged 50 years or older was 0.67% in 1998, which was lower than that observed in the Caucasians (Oshima et al. 2001). However, another recent study (The Funagata study) in Japanese residents aged 35 years or older suggested that the prevalence of neovascular AMD in Japanese men was similar to that seen in the Caucasian men (Kawasaki et al. 2008).

Current evidence points to the role of vascular endothelial growth factor (VEGF) in CNV proliferation, and hence agents that block its activity are considered as a suitable therapeutic intervention in the management of this form of AMD (Ferrara et al. 2006; Waisbourd et al. 2007). Ranibizumab (Lucentis®; Novartis Pharma AG, Basel, Switzerland and Genentech Inc, South San Francisco, CA, USA)

is a humanized monoclonal antibody fragment that inhibits active forms of VEGF-A, the main factor responsible for CNV proliferation and vascular permeability (Ferrara et al. 2006, 2007). Benefits of ranibizumab treatment in improving best-corrected visual acuity (BCVA) have been shown in the Caucasian population (Brown et al. 2006, 2009; Rosenfeld et al. 2006; Mitchell et al. 2010). Ranibizumab is currently approved in the United States, European Union, Japan and several other countries. EXTEND-I was the first study in Japanese patients that showed the safety and efficacy of monthly ranibizumab treatment (12-month results) during multiple-injection phase in terms of BCVA gain, reduction in total area of leakage from CNV plus retinal pigment epithelium staining and foveal retinal thickness, which were consistent with the pivotal studies performed in the Caucasian population (Tano & Ohji 2010). After the patients had completed the 12-month multiple-injection phase, all patients who provided written consent and were eligible based on the inclusion and exclusion criteria of the extension phase had the opportunity to continue to receive the 'individualized flexible interval regimen' [namely, *pro re nata* (PRN), as needed] until the approval of ranibizumab in Japan. This also provided a means to assess its long-term safety and efficacy. The PRN regimen was expected to maintain the improved visual acuity (VA) with less frequent injections in the extension phase. Current treatment guidelines in Europe recommend three initial monthly dosing followed by a maintenance phase, wherein the ranibizumab administration is decided based on monthly BCVA observation (Holz et al. 2010; Mitchell et al. 2010). This recommendation is based mainly on the results of the ranibizumab pivotal randomized phase III studies, namely MARINA (Rosenfeld et al. 2006) and ANCHOR (Brown et al. 2006) with monthly ranibizumab treatment. In these studies, the improvement of the BCVA score had stabilized (almost reached a plateau) by Month 3, and further increase in BCVA was minimal during the subsequent monthly treatments. On the other hand, in another pivotal randomized Phase IIIb study, PIER, quarterly treatment regimen could not maintain the improvement in BCVA score that was obtained by the three initial monthly injections (Regillo et al. 2008). However, there were also patients who maintained their gain in BCVA score during the quarterly regimen.

The extension phase of this study was initiated, therefore, to investigate whether ranibizumab administered PRN based on monthly BCVA scores and other ophthalmic examinations at two consecutive visits could maintain the improvement in BCVA scores. The reduction in dosing frequency was expected to reduce the risk of adverse events (AEs) associated with the intravitreal injection procedure in the elderly population as well as to address the difficulties in treating AMD through monthly injection of ranibizumab in a clinical setting.

Based on the 6-month interim results of the extension phase with PRN regimen as well as the 6- and 12-month interim analyses of monthly multiple-injection phase of this study, and the results of pivotal studies in the Caucasian population, ranibizumab was approved in Japan in January 2009. This paper presents the final data on long-term efficacy (in terms of BCVA) and safety of ranibizumab with PRN regimen from whole period of the extension phase of EXTEND-I.

Methodology

Study design

EXTEND-I was an open-label, multicentre, Phase I/II study comprising three phases: a single-injection phase, a multiple-injection phase and an extension phase (Fig. 1). The single-injection phase (Group A) was designed to sequentially evaluate the safety of intravitreal injections of 0.3 and 0.5 mg ranibizumab (six patients treated with each dose). The patients who successfully completed the single-dose phase (i.e., did not experience a Grade-3 targeted AE) could enter a multiple-injection phase wherein they received the same dose for an additional 11 months. The 12-month multiple-injection phase (Groups A and B; the latter consisted of patients who did not partic-

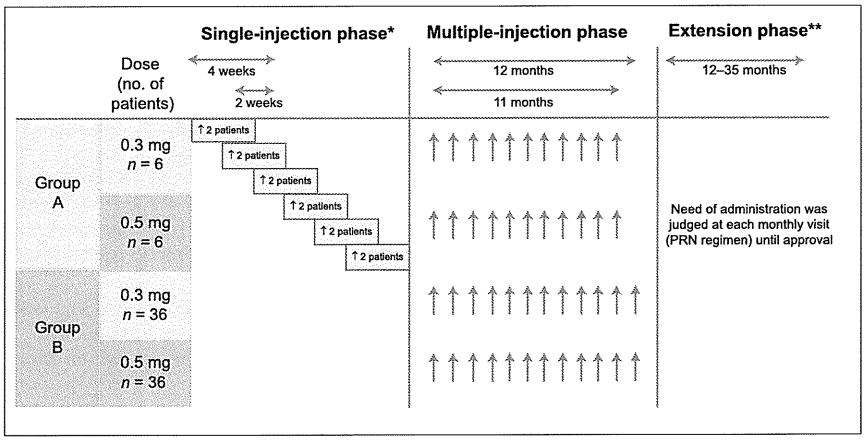

Fig. 1. EXTEND-I treatment schedule. *Upon completion of the single-dose phase, patients in Group A were eligible to enter the multiple-injection phase, which began ≥4 weeks after the final visit of the single-injection phase. Multiple injections did not begin until both doses were shown to be well tolerated in all cohorts. **Upon completion of the multiple dose phase, based on prespecified inclusion/exclusion criteria, patients could enter the extension phase. For the extension phase, treatment as per *pro re nata* regimen; dose same as core phase; retreatment at the monthly visit if loss of >5 letters in best-corrected visual acuity (BCVA) on two consecutive visits (except unscheduled visits), considering other ophthalmic examinations, such as slit-lamp examination, ophthalmoscopy, fundus photography, fluorescein angiography and optical coherence tomography, the investigator decided whether ranibizumab treatment would be performed. Similarly, if the BCVA score decreased on two consecutive visits (except unscheduled visits) by ≤5 letters using ETDRS-like visual acuity chart, a decision was taken whether treatment could be withheld. In any case, other ophthalmic examinations were taken into consideration. For the extension phase, the number of patients for Group A was 3 in the 0.3 mg group and 6 in the 0.5 mg group; the number of patients for Group B was 28 in the 0.3 mg group and 33 in the 0.5 mg group.

ipate in the single-injection phase) evaluated the safety and efficacy of both doses administered as monthly intravitreal injections in two parallel groups of 0.3 mg dose and 0.5 mg dose (Tano & Ohji 2010). The multiple-injection phase was followed by an extension phase in which the ranibizumab (0.3 or 0.5 mg) administration was on a PRN basis, but assessments were carried out on a monthly basis. If the BCVA score decreased at two consecutive visits (except unscheduled visits) by >5 letters, considering other ophthalmic examinations, such as slit-lamp examination and ophthalmoscopy for safety, fundus photography, fluorescein angiography and optical coherence tomography for efficacy, the investigator decided whether ranibizumab treatment would be administered although no specific retreatment criteria were provided for fundus photography, fluorescein angiography and optical coherence tomography and were at the discretion of the investigators. Similarly, if the BCVA score decreased at two consecutive visits (except unscheduled visits) by ≤ 5 letters, in conjunction with other ophthalmic examinations, a decision was taken whether the treatment could be withheld.

This study was conducted in accordance with the Declaration of Helsinki, International Conference on Harmonization Good Clinical Practice (GCP) guidelines and Japanese GCP. The study was approved by Institutional Review Boards at each study centre. All patients provided written informed consent before participating in the study and the extension. The trial is registered with clinicaltrials.gov (NCT00275821).

Inclusion and exclusion criteria

All patients with subfoveal CNV secondary to AMD who completed the multiple-injection phase in either of the ranibizumab groups (Groups A or B), provided written consent and met all of the inclusion criteria set at the beginning of the study (Tano & Ohji 2010) were eligible to enrol in the extension phase. Patients were allowed to participate in the extension phase regardless of the time elapsed between the exit visit of the multiple-injection phase and the participation in the extension phase.

Patients were excluded from the extension phase if they had received any anti-angiogenic drugs (bevacizumab, pegaptanib, ranibizumab, anecortave acetate, corticosteroids or protein kinase C inhibitors) or participated in any other clinical study of an investigational drug during the period from the exit visit of the multiple-injection phase to participation in the extension phase. However, as the extension phase was not started on the day of the exit visit from the multiple-injection phase, photodynamic therapy with verteporfin was allowed for the study eye during the transition period.

Efficacy assessments

The efficacy variables of the extension phase included mean change from Month 12 in BCVA score of the study eye using ETRDS chart (at a starting distance of 2 m) at the last visit of the extension phase for Group B patients only. Group A patients were not included as they were not assessed for efficacy, but only for safety throughout the study. The other efficacy variables included the proportion of patients at the last visit with a BCVA score loss <15 letters, and ≥ 30 letters, or a BCVA score gain of ≥ 15 letters in the study eye. Proportion of patients with BCVA <34 letters, approximate Snellen equivalent of 20/200 or worse, were also evaluated (ETDRS charts at a starting distance of 2 m). In the extension phase, colour fundus photography, fluorescein angiography and optical coherence tomography were performed in accordance with the routine procedures specified at each study site.

Safety assessments

All safety evaluations were based on the enrolled population (Groups A and B) of the extension phase. Safety assessments consisted of recording the frequency of the treatment collecting all AEs, serious adverse events (SAEs), with their severity, and relationship to study drug. It also included monitoring of haematology, serum chemistry, urinalysis and regular assessments of vital signs. Grade 3 targeted AEs (Tano & Ohji 2010), intraocular inflammation, myocardial infarction and stroke and AEs potentially related to systemic VEGF inhibition were analysed separately. Serum samples for the evaluation of immunoreactivity to ranibizumab (antiranibizumab antibodies) were obtained from patients prior to study administration at Month 23 and the last visit for Group A patients, and Month 24 and the last visit for Group B patients. At the last visit as well as at early termination, the assessments were performed if at least 6 months had passed since the previous measurement, on or after Month 11 for Group A patients and Month 12 for Group B patients. The last measurement in the multiple-injection phase of the study was performed at Month 11 for Group A and Month 12 for Group B.

Statistical analysis

The patient population included all enrolled patients in the extension phase. This population was used for all analyses in Groups A and B. All efficacy data presented were for observed cases without the last observation carried forward method.

Descriptive statistics of the number of injections, duration of exposure and reason of injection were presented for the enrolled population. The duration of treatment varied for each patient in the extension phase. To reduce a possible bias because of the patients who discontinued early without injection, the number of injections per year was calculated as $365.25 \times$ sum of total number of injections in the group/duration of the PRN regimen for the respective group. The number of injections per year was calculated for the respective group and not per patient. Duration of the PRN regimen was the date of the last potential treatment visit minus the date of Month 11 visit (the last treatment visit of multiple-injection phase) plus 1.

All efficacy analyses were based on the study eye. Descriptive statistics (mean, median, standard deviation, standard error, minimum and maximum) of the change from baseline (the single-injection phase of Group A and the multiple-injection phase of Group B), Month 11 and Month 12 in Group B were performed by treat-

ment and visit. The 95% confidence intervals based on *t*-distributions and p-values based on paired *t*-tests were determined for the change from baseline. Exact 95% confidence intervals were calculated for the proportion of patients with the specified response rates.

Results

Patients

Overall, 70 patients at 11 sites participated in the extension phase from 20 March 2007 to 20 January 2009: 9 in Group A (3 and 6 in the 0.3 and 0.5 mg dose groups, respectively) and 61 in Group B (28 and 33 in the 0.3 and 0.5 mg dose groups, respectively) as shown in Table 1. In Group A, a total of seven patients were not discontinued in the extension phase. Two patients in the 0.3 mg dose group withdrew from the study, as their condition did not further require the study drug. In Group B, 22 patients in the 0.3 mg dose group and 21 patients in the 0.5 mg dose groups were not discontinued in the extension phase. Six patients in the 0.3 mg dose group and 12 patients in the 0.5 mg dose group withdrew from the extension study. The maximum number of patients discontinued as they did not require the study drug because of improvement in VA (n = 9, two in the 0.3 mg dose group and seven in the 0.5 mg dose group); other reasons being AEs (n = 4, two in each dose group), withdrawal of consent (n = 4, one in the 0.3 mg dose group and three in the 0.5 mg dose group) and protocol violation (n = 1, one in the 0.3 mg dose group). None of the AEs leading to study discontinuation was thought to be related to the study drug.

The mean duration of treatment (standard deviation, SD) during the extension phase was 1.70 (0.35) years in the 0.3 mg group and 1.93 (0.09) years in the 0.5 mg dose group in Group A (Table 1). In Group B patients, the mean duration of treatment was 1.45 (0.33) years and 1.36 (0.39) years in the 0.3 and 0.5 mg dose groups, respectively.

The baseline demographic and ocular characteristics of enrolled patients at the start of the extension phase are given in Table 2. The mean (SD) BCVA score of the study eye at the start of the extension phase was 59.1 (11.69) letters and 59.8 (15.07) letters in the 0.3 and 0.5 mg dose groups of Group B, respectively. Overall, approximate Snellen equivalent VA of almost all patients was better than 20/200 except for two patients in the 0.5 mg dose group.

Of the 61 patients in Group B, approximately 90% (25/28 and 27/33 in the 0.3 mg and the 0.5 mg dose groups, respectively, Table 3) completed Month 24 from the baseline of the multiple-injection phase of the study, i.e., these patients received treatment of ranibizumab with PRN for 12 months in the extension phase. The duration of treatment of each patient in the extension phase varied with respect to the study entry and the longest was 35 months from baseline for the 0.3 mg dose group (n = 1). For the 0.5 mg dose group, the longest was 34 months (n = 1), as shown in Fig. 2.

Table 1. Patient disposition in the extension phase.

Disposition/patients studied	Group A Ranibizumab 0.3 mg	Group A Ranibizumab 0.5 mg	Group B Ranibizumab 0.3 mg	Group B Ranibizumab 0.5 mg
Patients (n %)				
Enrolled	3 (100.0)	6 (100.0)	28 (100.0)	33 (100.0)
Not discontinued	1 (33.3)	6 (100.0)	22 (78.6)	21 (63.6)
Discontinued	2 (66.7)	0 (0.0)	6 (21.4)	12 (36.4)
Main cause of discontinuation				
Adverse event (s)	0 (0.0)	0 (0.0)	2 (7.1)	2 (6.1)
Patient's condition does not requires study drug	2 (66.7)	0 (0.0)	2 (7.1)	7 (21.2)
Protocol violation	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Patient withdrew consent	0 (0.0)	0 (0.0)	1 (3.6)	3 (9.1)
Mean duration, years, of the extension phase (SD)	1.70 (0.35)	1.93 (0.09)	1.45 (0.33)	1.36 (0.39)

Table 2. Baseline demographics of enrolled patients and ocular characteristics (study eye) at the start of the extension phase.

Characteristic	Category/statistic	Group A Ranibizumab 0.3 mg N = 3	Group A Ranibizumab 0.5 mg N = 6	Group B Ranibizumab 0.3 mg N = 28	Group B Ranibizumab 0.5 mg N = 33
Gender – n (%)	Male	3 (100.0)	5 (83.3)	19 (67.9)	28 (84.8)
	Female	0 (0.0)	1 (16.7)	9 (32.1)	5 (15.2)
Age, years	Mean (SD)	68.0 (10.15)	72.0 (4.82)	69.8 (8.72)	70.2 (7.83)
Race (%)	Asian	3 (100.0)	6 (100.0)	28 (100.0)	33 (100.0)
Best-corrected visual acuity score	Mean (SD)	72.0 (4.58)	58.5 (15.66)	59.1 (11.69)	59.8 (15.07)
	Range	68–77	42–77	39–80	36–85
Approximate Snellen equivalent n (%)	Median	40.0	70.0	71.5	63.0
	20/200 or worse	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.1)
	Better than 20/200 but worse than 20/40	1 (33.3)	3 (50.0)	20 (71.4)	20 (60.6)
	20/40 or better	2 (66.7)	3 (50.0)	8 (28.6)	11 (33.3)
Intraocular pressure (mmHg)	Mean (SD)	13.3 (1.53)	14.2 (3.06)	13.5 (2.92)	13.7 (3.09)
	Range	12–15	9–18	8–20	9–23

Data of ocular characteristics are based on Month 11 visit in Group A and Month 12 visit in Group B.
N = number of enrolled patients, n = number of patients.

Table 3. Summary of patient exposure to ranibizumab for 12 months (from Month 12 to Month 24) in the extension phase (Group B, enrolled patients).

Cumulative number of injections	Ranibizumab 0.3 mg (N = 28)	Ranibizumab 0.5 mg (N = 33)
Month 24		
n	25	27
Mean (SD)	4.1 (4.12)	3.9 (4.63)
Range	0–13	0–13
0	7	9
1–2	3	8
3–6	9	2
7–9	2	3
10–12	3	4
13	1	1
Number of injections per Year	4.19	4.27

The number of injections per year is calculated as: 365.25 × total number of injections/duration of the *pro re nata* (PRN) regimen.
Number of injections per year is calculated for total group, not per patient.
Duration of the PRN regimen: date of last potential treatment visit – date of Month 11 visit + 1.
N = number of enrolled patients, n = number of patients.

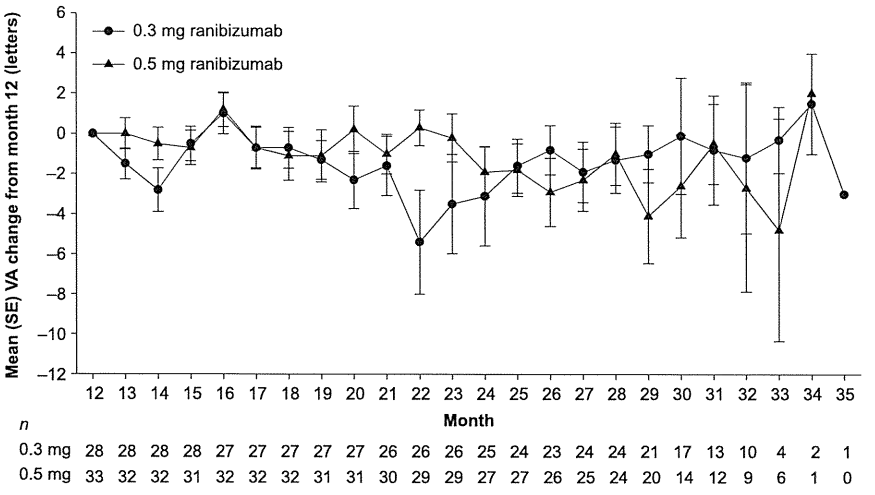

Fig. 2. Mean change from Month 12 (the start of Extension phase) in best-corrected visual acuity score (±SE) of study eye by visit during extension phase (Group B patients).

The exposure to ranibizumab in the extension phase of Group B is shown in Table 3. At Month 24, the patients had been treated with the PRN regimen for 12 months in the extension phase, and hence the maximum achievable number of injections by this visit was 13. The injection frequency of ranibizumab for individual patient varied from 0 to 13 times for this 12 months in the extension phase. The estimated number of injections per year in the extension phase was 4.19 and 4.27 in the 0.3 and 0.5 mg dose groups in Group B, respectively.

Efficacy

The mean change (SD) from Month 12 in BCVA score of the study eye to the last visit in the extension phase was −3.6 (14.82) letters in the 0.3 mg group and −2.2 (7.92) letters in the 0.5 mg group of Group B using the PRN regimen (Table 4). Furthermore, the mean change (SD) from baseline in BCVA score of the study eye to the last visit in the extension phase was 7.5 (19.12) letters in the 0.3 mg group and 7.7 (13.02) letters in the 0.5 mg group (p = 0.0475 for the 0.3 mg dose group and p = 0.0019 for the 0.5 mg dose group) (Table 4). Overall, the improvement in BCVA score at Month 12 by monthly ranibizumab injection was sustained throughout the extension phase with the PRN regimen (Fig. 2).

Table 5 shows the proportion of patients with respect to VA outcome at the last visit in the extension phase. The proportion of patients who lost < 15 letters from baseline in BCVA in the study eye was 85.7% (24/28) and 97.0% (32/33) in the 0.3 and 0.5 mg dose groups, respectively. Nine patients each in the 0.3 mg (32.1%) and 0.5 mg (27.3%) dose groups gained ≥15 letters from the baseline. One patient each in the 0.3 mg (3.6%) and 0.5 mg (3.0%) dose groups lost ≥30 letters from the baseline. The proportion of patients with approximate Snellen equivalent of 20/200 or worse was 14.3% (4/28) and 6.1% (2/33) in the 0.3 and 0.5 mg groups, respectively.

The mean time to first retreatment in the extension phase since Month 11 of the multiple-injection phase (when the last monthly injection was done) in Group B was 218.5 days (range: 29–512 days) for the 0.3 mg group and 255.6 days (range: 29–571 days) for the 0.5 mg group.

Safety

In Group A patient population (n = 9), two of three (66.7%) patients in the 0.3 mg dose group and three of six (50.0%) patients in the 0.5 mg dose group experienced at least one ocular AE in the study eye during the extension phase. In Group B, 20 of 28 (71.4%) patients in the 0.3 mg dose group and 18 of 33 (54.5%) patients in the 0.5 mg dose group experienced at least one ocular AE in the study eye during the extension phase. The most common ocular AE in the study eye in Group B was conjunctival haemorrhage. Other frequent ocular AEs included retinal haemorrhage, retinal detachment and increased intraocular pressure (Table 6). Two patients in the 0.3 mg dose group of Group B experienced Grade 3 targeted AEs (intraocular inflammation, reduced VA, increased intraocular pressure, vitreous haemorrhage, retinal tear or detachment, and retinal haemorrhage). One patient experienced retinal detachment, retinal haemorrhage and vitreous haemorrhage in the study eye, and the other patient experienced retinal haemorrhage in the fellow eye.

One patient in the 0.3 mg dose group of Group B experienced iritis in the study eye among the ocular AEs defined under the group of intraocular inflammation (iritis, iridocyclitis, vitritis, uveitis, hypopyon and anterior chamber inflammation). Two kinds of ocular AEs in six patients of Group B were suspected to be study-drug related: increased intraocular pressure (two patients in the 0.3 mg dose group and three patients in the 0.5 mg dose group) and retinal haemorrhage (one patient in the 0.5 mg dose group).

Table 4. Mean change from baseline in best-corrected visual acuity score of the study eye at the last visit in the extension phase (Group B, enrolled patients).

Visual acuity (letters)	Ranibizumab 0.3 mg $N = 28$	Ranibizumab 0.5 mg $N = 33$
Baseline		
Mean (SD)	47.9 (12.59)	50.0 (10.38)
Month 12 (start of extension phase)		
Mean (SD)	59.1 (11.69)	59.8 (15.07)
Last visit		
Mean (SD)	55.4 (17.14)	57.6 (15.36)
Change from baseline		
Mean (SD)	7.5 (19.12)	7.7 (13.02)
95% CI of the mean*	0.1, 14.9	3.0, 12.3
p-value†	0.0475	0.0019
Change from Month 12		
Mean (SD)	−3.6 (14.82)	−2.2 (7.92)
95% CI of the mean*	−9.4, 2.1	−5.0, 0.6
p-value†	0.2042	0.1186

Observed values are presented. Patients must have values at both Month 12 and last visit to be included. Baseline value is defined as the last available measurement prior to the first injection in the multiple-injection phase of the study. End of study differed between the patients and this was more evident from Month 30. Month 35 was the longest analysis point.
N = number of enrolled patients.
* Derived from t-distribution.
† Derived from paired t-test.

Nonocular AEs were observed in four patients (44.4%) in Group A (two each in the 0.3 and 0.5 mg dose groups), 19 patients (67.9%) in the 0.3 mg group and 24 patients (72.7%) in the 0.5 mg group in Group B. Nasopharyngitis was the most common AE in Group B patients (Table 6).

Adverse events potentially related to systemic VEGF inhibition were observed in four patients (14.3%) and two patients (6.1%) in the 0.3 and 0.5 mg dose groups of Group B, respectively. One patient in each dose group experienced cerebral infarction; three patients (0.3 mg dose group) and one patient (0.5 mg dose group) experienced hypertension. In Group A, AEs potentially related to systemic VEGF inhibition were observed in two patients in the 0.3 mg dose group (blood pressure increased and haematuria in one patient and hypertension in another patient).

Nonocular AEs suspected to be related to study drug were cerebral infarction, dementia and hypertension (one patient each) in 0.3 mg group, cerebral infarction and malaise (one patient each) in 0.5 mg dose group.

There were no deaths during the extension phase. Serious adverse events were reported for one of three (33.3%) patients in the 0.3 mg dose group and one of six (16.7%) patients in the 0.5 mg dose group in Group A, four patients (14.3%) in the 0.3 mg dose group and seven patients (21.2%) in the 0.5 mg dose group of Group B. Summary of ocular and nonocular SAEs is shown in Table 7. Of the SAEs, cerebral infarction (one patient each in the 0.3 and 0.5 mg dose groups of Group B) was suspected to be related to study drug and resolved with medical treatment in both patients. Four patients (two patients each from both dose groups) in Group B discontinued from the study because of SAEs. These SAEs that led to discontinuation were, however, not suspected to be study-drug related.

During the extension phase, immunoreactivity to ranibizumab (anti-ranibizumab antibodies) was not detected in patients of Group A; however, it was detected in two patients in the 0.3 mg dose group and one patient in the 0.5 mg dose group of Group B in the extension phase. In one patient in the 0.3 mg dose group, immunoreactivity to ranibizumab was detected at Month 12 (for the first time) and at study completion visit, but not at Month 24. In another patient in the 0.3 mg dose group, immunoreactivity to ranibizumab was detected at Month 24 (for the first time) and at study completion visit. In the 0.5 mg dose group, immunoreactivity to ranibizumab was detected in one patient at Month 12 (for the first time), Month 24 and at study completion visit. Of the three patients, AEs were reported in two patients. One patient in the 0.3 mg dose group experienced mild iritis as ocular AE and moderate glaucomatocyclitic crises as ocular SAE in the study eye as well as mild back injury and fall as nonocular AE. Iritis, back injury and fall were resolved without treatment and glaucomatocyclitic crises were resolved with medical treatment. One patient in the 0.5 mg dose group experienced both of conjunctival hyperaemia and intraocular pressure increased in the study eye, and both events were mild and resolved without treatment. All these events, except for intraocular pressure increased, were not suspected to be study-drug related.

Discussion

EXTEND-I was the first study with ranibizumab in Japanese patients with primary or recurrent subfoveal CNV secondary to AMD. The 6-month results indicated that monthly ranibizumab treatment significantly improved BCVA scores at Month 6 compared with baseline; the mean change (SD) observed was of +8.1 (12.65) letters and +9.0 (9.62) letters in BCVA score in the 0.3 and 0.5 mg dose groups, respectively. The improved BCVA scores at Month 6 were maintained until Month 12 by monthly treatment; the mean change (SD) observed was of +9.5 (12.79) letters and +10.5 (11.14) letters in BCVA score in the 0.3 and 0.5 mg dose groups, respectively. Monthly intravitreal injections of ranibizumab were shown to be safe and well tolerated over 12 months in Japanese patient population (Tano & Ohji 2010).

In the extension phase, the efficacy and safety of individualized flexible interval regimen (PRN regimen) of ranibizumab was assessed. In other words, the study consecutively investigated 12 monthly injections in the multiple-injection phase followed by the extension phase with PRN regimen guided by monthly BCVA score and by other ophthalmic examina-