Figure I Findings in a representative case of age-related macular degeneration associated with an occult choroidal neovascularization. The eye was treated with three-monthly intravitreal injections of ranibizumab. The serous retinal detachment promptly disappeared after three injections. There was no change in waveforms and amplitudes of the full-field cone electroretinograms after three intravitreal injections of ranibizumab. The amplitudes of the focal macular electroretinograms are slightly larger at 6 months and thereafter.

Abbreviations: IVRs, intravitreal injections of ranibizumab; BCVA, best corrected visual acuity; ERG, electroretinogram; PhNR, photopic negative response; OP, oscillatory potential.

averaged amplitude of the focal macular ERGs increased, and significant recovery was observed at 3, 6, and 12 months after starting the IVR (Figure 4, $P < 0.05$–0.0005). The degree of recovery of the b wave and PhNR after the IVR was not significantly different. As a result, the PhNR/b wave

amplitude ratio was unchanged throughout the observation period (Figure 5B).

Before IVR, the averaged implicit times of the a waves and b waves of the focal macular ERGs were significantly longer in affected eyes than in unaffected eyes ($P < 0.005$
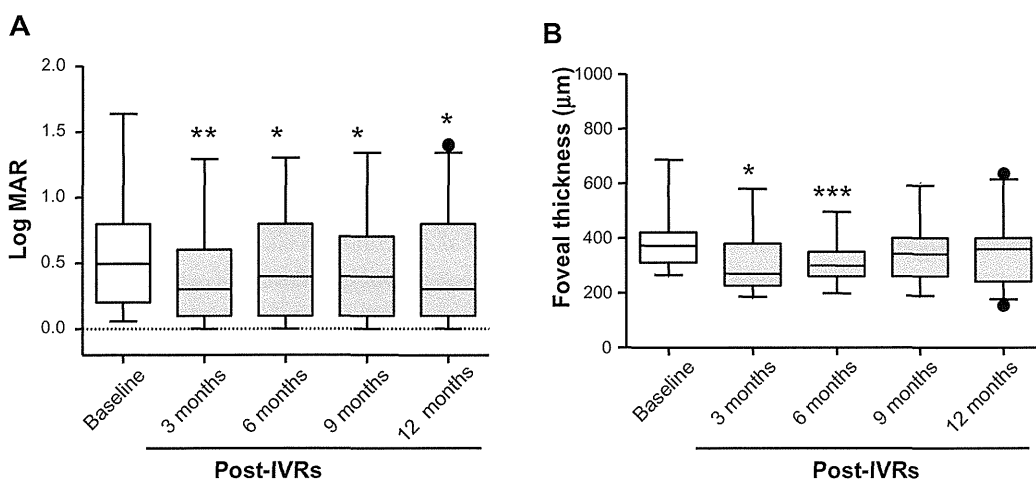

Figure 2 Averaged best-corrected visual acuity in logarithm of the minimum angle resolution (logMAR) units before and after intravitreal injections of ranibizumab (IVRs). (A) Averaged foveal thickness measured by optic coherence tomography before and after IVRs. (B) The white boxes represent the values before (baseline) and the gray boxes the values after IVRs.

Notes: The boxes represent the 25%–75% interquartile ranges. The horizontal line represents median values, and the bars represent the 5% and 95% confidence intervals. Filled circles represent data beyond the confidence intervals. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$.
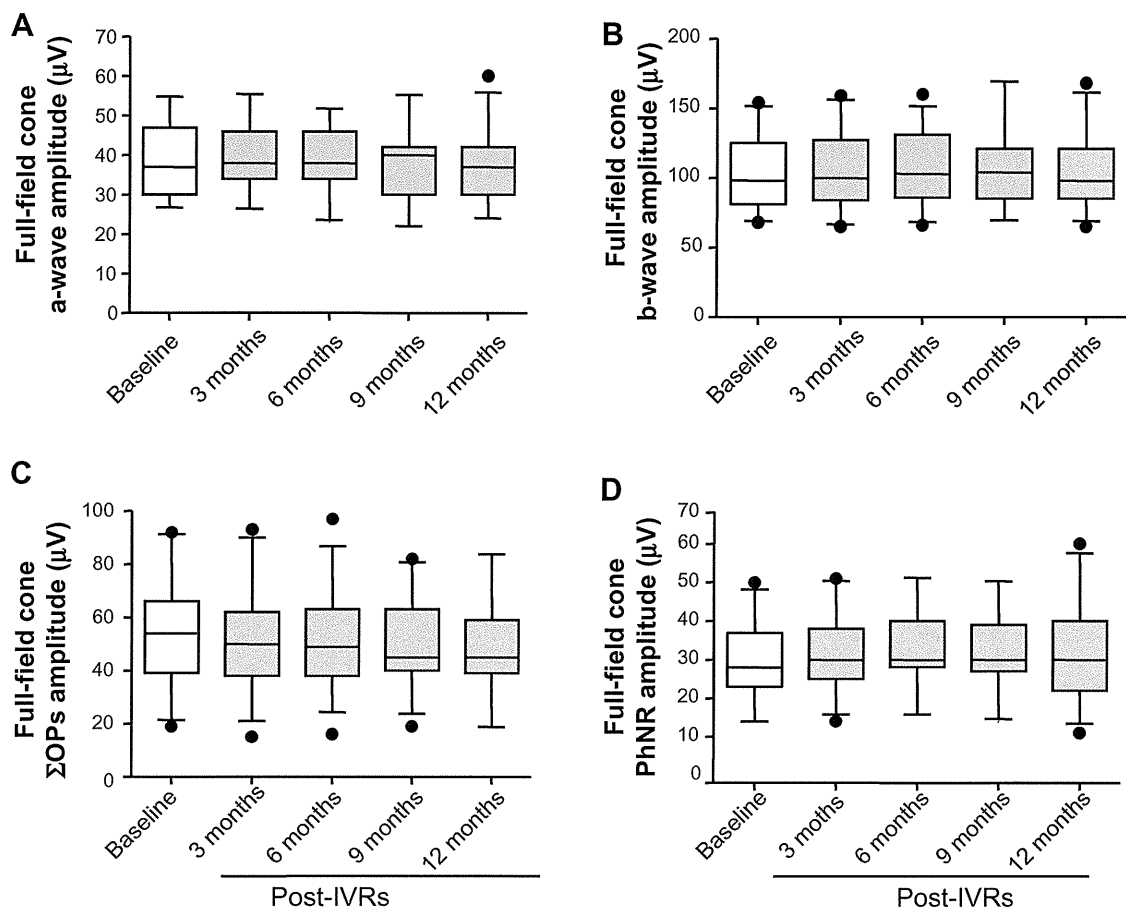
Figure 3 Averaged amplitudes of a waves (**A**) and b waves (**B**), oscillatory potentials (OPs), (**C**) and photopic negative response (PhNR), (**D**) for full-field cone electroretinograms. **Notes:** The white boxes represent values before (baseline) and the gray boxes after intravitreal injections of ranibizumab (IVRs). The boxes represent 25%–75% interquartile ranges. The horizontal line represents median values, and the bars represent the 5% and 95% confidence intervals. Filled circles represent data beyond the confidence intervals.

for a wave; $P < 0.05$ for b wave). After IVR, the implicit times were significantly shortened compared with baseline values ($P < 0.05$–0.005, Figures 6C and D). There was no significant difference in amplitudes and implicit times of all ERG components in eyes with typical AMD and polypoidal choroidal vasculopathy.

Correlation of number of treatments with ERG changes

The number of IVR ranged from three to seven, with a mean of 4.09 for the 12-month observation period. The differences in PhNR amplitude and PhNR/b wave amplitude ratio between baseline and 12 months after the IVR were plotted against numbers of IVR for the full-field (Figure 7A and B) and focal macular ERGs (Figure 7C and D). There was no significant correlation between the number of IVR and changes in PhNR amplitude and PhNR/b wave amplitude ratio for the full-field ERG. Focal PhNR amplitude was significantly reduced with numbers of IVR ($r = 0.21$; $P < 0.01$). However,

the focal PhNR/b wave amplitude ratio was not significantly correlated with number of IVR.

Discussion

We examined the photopic full-field and focal macular ERGs in AMD patients before and after three consecutive monthly IVR. Our results demonstrate that full-field cone ERGs did not change significantly after IVR at any time. However, focal macular ERGs were significantly improved, and accompanied by recovery of central vision and the anatomical structure of the macula. These findings indicate that IVR did not have adverse effects on any retinal layers, including the retinal ganglion cells.

Full-field and focal macular ERG changes

The full-field cone ERGs represent the sum of the electrical activities of all of the retinal neurons in the cone pathway. Therefore, the absence of any significant changes in the amplitudes and implicit times of the a waves and b waves, OPs, and
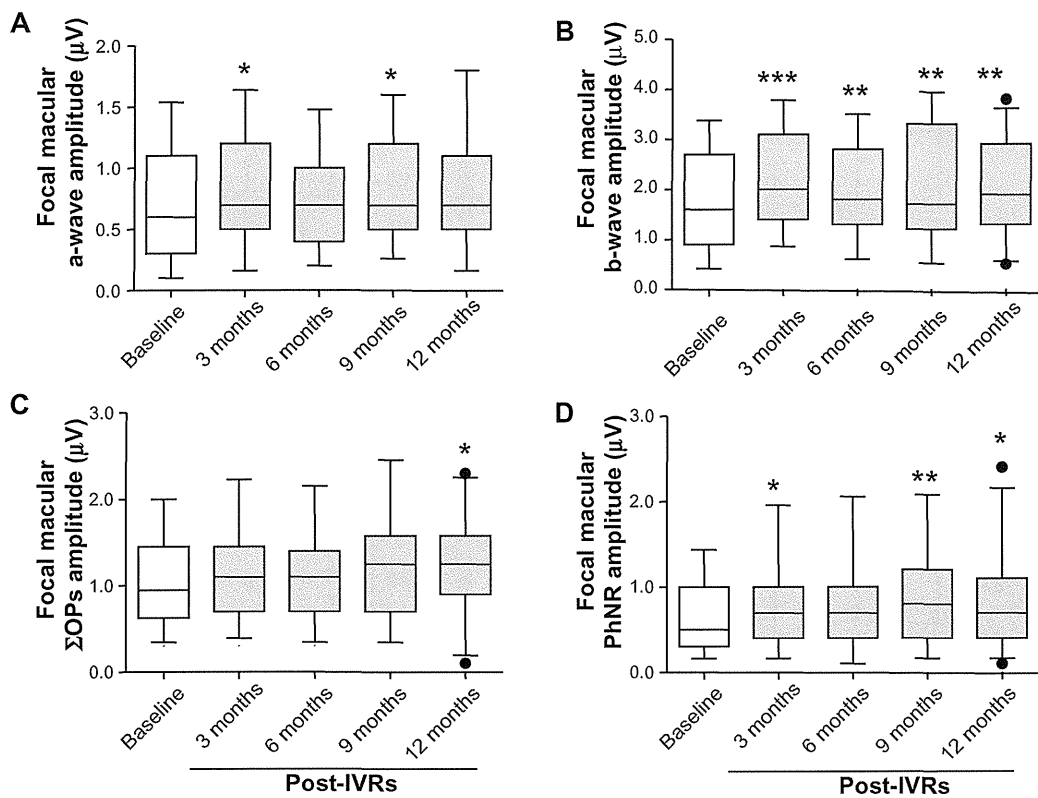
Figure 4 Averaged amplitudes of a waves (**A**) and b waves (**B**), oscillatory potentials (OPs), (**C**) and photopic negative response (PhNR), (**D**) for focal macular electoretinograms.
Notes: The white boxes represent values before (baseline) and the gray boxes after intravitreal injections of ranibizumab (IVRs). The boxes represent 25%–75% interquartile ranges. The horizontal line represents the median values, and the bars represent 5% to 95% confidence intervals. Filled circles represent data beyond the confidence intervals.
$*P < 0.05$; $**P < 0.005$; $***P < 0.0005$.

PhNR indicate that cells in each retinal layer, including the cone photoreceptors, cone bipolar cells, amacrine cells, and retinal ganglion cells of the entire retina, were not affected by IVR.

On the other hand, the amplitudes and implicit times of the focal macular ERGs did improve in association with recovery

of central vision and disappearance of exudative changes. These findings are compatible with an earlier report that the amplitude of multifocal ERGs increased after anti-VEGF therapy.[22,28] Recovery of outer retinal function probably led to an increase in signals to the inner retina, resulting in an
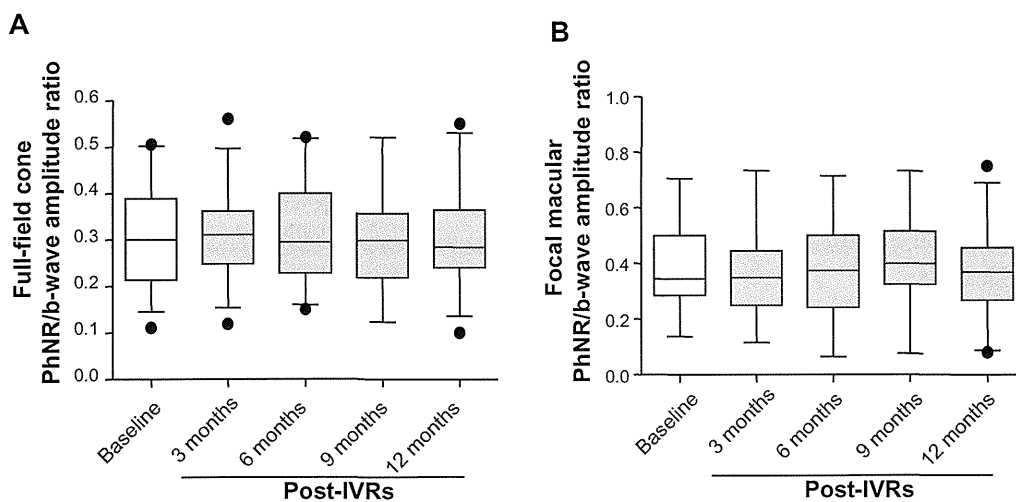

Figure 5 Averaged amplitude ratio of the photopic negative response (PhNR)/b wave for the full-field cone (**A**) and focal macular electoretinograms. (**B**) The white boxes represent values before (baseline) and the gray boxes after the intravitreal injections of ranibizumab (IVRs).
Notes: The boxes represent the 25%–75% interquartile ranges. The horizontal line represents median values, and the bars represent 5% and 95% confidence intervals. Filled circles represent data beyond the confidence intervals.

Figure 6 Averaged implicit times of a waves and b waves for full-field cone (**A** and **B**) and focal macular electroretinograms. (**C** and **D**).
Notes: The white boxes represent the values before (baseline) and the gray boxes after the intravitreal injections of ranibizumab (IVRs). The boxes represent the 25%–75% interquartile ranges. The horizontal line represents median values, and the bars represent 5% and 95% confidence intervals. Filled circles represent data beyond the confidence intervals. $*P < 0.05$; $**P < 0.005$.
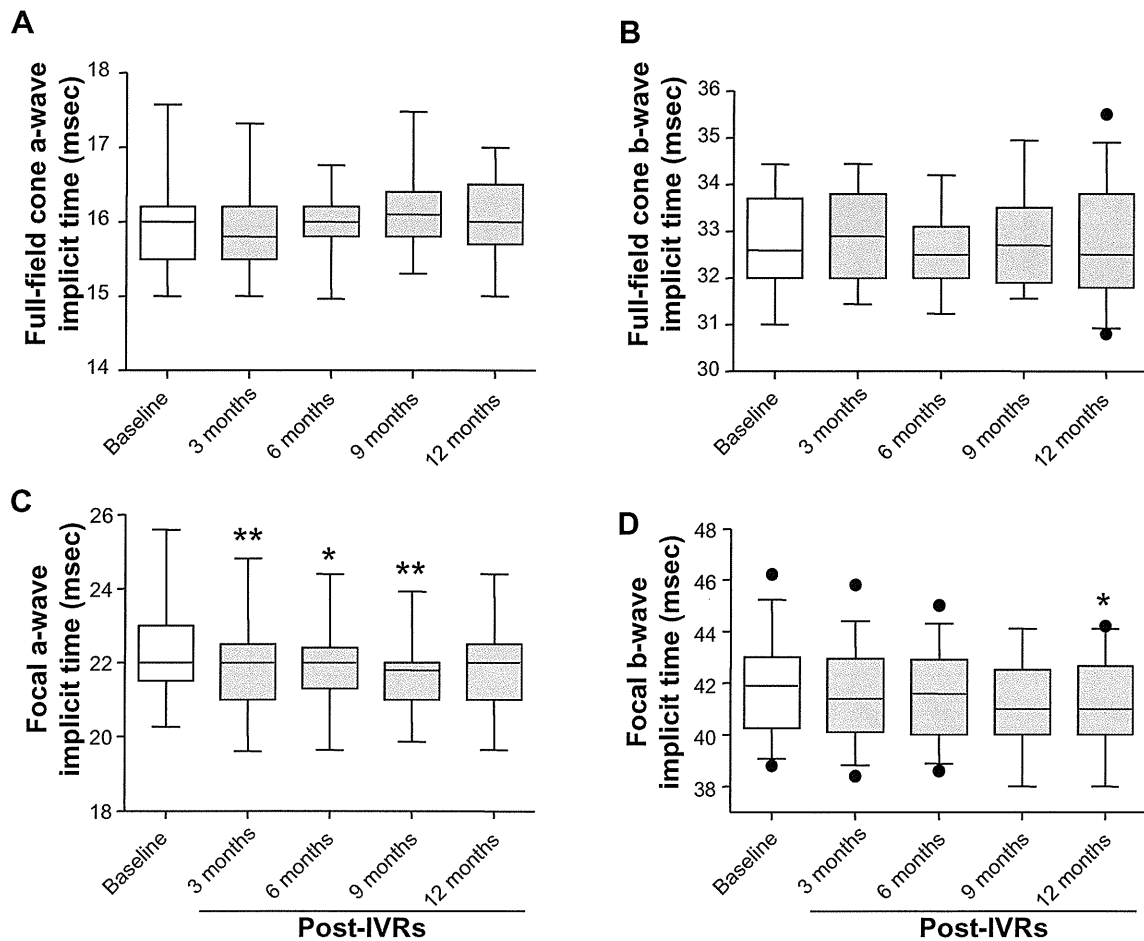
increase of ΣOPs and PhNR amplitudes in the focal macular ERGs. However, the increased PhNR amplitude of the focal macular ERG does not necessarily indicate an increase in the activity of retinal ganglion cells. Because of this, we used the PhNR/b wave amplitude ratio as a functional indicator of retinal ganglion cells in the focal macular ERGs. The amplitude ratio did not change after treatment, suggesting that IVR did not adversely affect the retinal ganglion cell function of the macula.

Comparison with results of earlier studies

It has been reported that repeated intravitreal injections of anti-VEGF-A monoclonal antibody lead to degeneration of retinal ganglion cells in rats.[2] This is inconsistent with our observation that the amplitude of the PhNR of the full-field and focal macular ERGs did not decrease. There are several possible reasons for this difference. First, there

was a difference in the protocol of injections between these studies. Nishijima et al injected anti-VEGF-A monoclonal antibody weekly for 6 weeks, in contrast with our monthly injections for the first 3 months and additional injections when needed.[2,26] Second, ranibizumab is a Fab with a lower molecular weight than the full-length antibody, and its half-life in the vitreous is approximately one-half of that of full-length bevacizumab.[29,30] Therefore, intraocular ranibizumab is quickly leached out after each injection, which may be less toxic to retinal ganglion cells.

The number of injections was not significantly correlated with changes in the full-field cone ERG components, including PhNR. These findings indicate that the dose-dependent toxic effect was not seen in patients after multiple IVR. This is consistent with the results of an earlier study demonstrating that an average of 16 IVR did not adversely affect retinal nerve fiber layer thickness around the optic nerve head, as measured by OCT.[31]
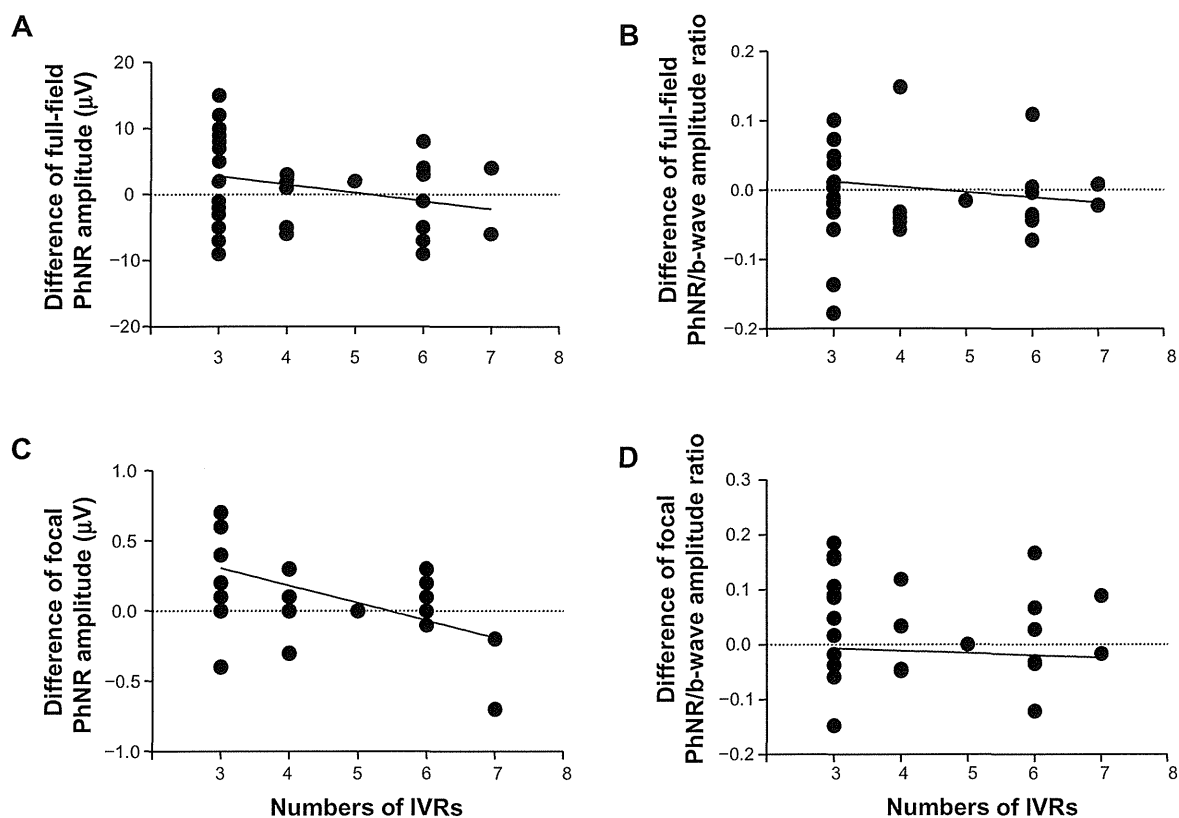
Figure 7 Photopic negative response (PhNR) amplitudes and PhNR/b wave amplitude ratios of the baseline and 12 months after the intravitreal injections of ranibizumab (IVRs) are plotted against the number of IVRs for the full-field (**A** and **B**) and focal macular electroretinograms (**C** and **D**).

Effect of vasoconstriction and retinal ischemia on PhNR and OPs

We have reported that, after a central retinal artery occlusion, PhNR amplitude was predominantly reduced with relatively good preservation of the b wave.[32] It has also been reported that PhNR is reduced in eyes with a central retinal vein occlusion or with diabetic retinopathy.[27,33,34] These observations indicate that the PhNR could be a good indicator of retinal function in ischemic diseases of the retina. The OPs represent the neural activity of the inner retina and has been used as a sensitive indicator of retinal ischemia, such as in eyes with diabetic retinopathy.[35]

It was shown that IVR can induce retinal arteriolar vasoconstriction that worsens with time.[36] In an animal experiment, it was shown that intravitreal injections of anti-VEGF-A antibody resulted in closure of retinal capillaries.[37] Previous clinical reports have suggested that retinal ischemia became worse following intravitreal administration of anti-VEGF-A antibody.[38–40] Taken together, if retinal ischemia develops after IVR, this is important because it can lead to glutamate excitotoxicity, to which retinal ganglion cells are particularly susceptible.[41] The ischemia should then reduce

the amplitudes of the PhNR and OPs, but our results show that they were not significantly changed, indicating that retinal ischemia after the IVR, if it occurred, did not alter the function of the inner retina of our AMD patients.

Correlation between PhNR changes and number of IVR

In cases that required additional IVR, focal macular ERG amplitudes were reduced despite the treatments because of recurrences of exudative changes. We found a significant correlation between changes in PhNR amplitudes of the focal macular ERGs and the number of IVR. However, this does not indicate a decrease in retinal ganglion cell function because the focal PhNR/b wave amplitude ratio was not significantly correlated with the number of treatments. This suggests that exudation affected the outer retina which then reduced the signal to the middle and inner retina in the macular area.

Study limitations

One limitation of this study is the fact that we recorded only cone-mediated ERGs. Therefore, we cannot rule out

possible impairment of retinal ganglion cell function in the rod-mediated pathway. The scotopic threshold response[42,43] is a negative-going wave recorded by very weak stimuli under dark adaptation, representing retinal ganglion cell function in the rod-mediated pathway.[44] Recording the scotopic threshold response is difficult in the clinic because complete dark adaptation is required for the recordings. In addition, the cellular origin of the scotopic threshold response is still controversial in humans.[45] Therefore, we decided to evaluate the retinal ganglion cell function by analyzing the PhNR.

Conclusion

The amplitudes of focal macular ERGs, including PhNR, improved after repeated IVR, accompanied by recovery of visual acuity and macular structure. Results of the full-field cone ERG indicate that retinal ganglion cell function in the cone-mediated pathway is not altered by repeated IVR.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

References

1. Storkebaum E, Lambrechts D, Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. *Bio Essays*. 2004;26:943–954.
2. Nishijima K, Ng YS, Zhong L, et al. Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. *Am J Pathol*. 2007; 171:53–67.
3. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med*. 2006;355:1432–1444.
4. Rosenfeld PJ, Brown DM, Heier JS, et al. MARINA study group: ranibizumab for neovascular age-related macular degeneration. *N Engl J Med*. 2006;355:1419–1431.
5. Brown DM, Michels M, Kaiser PK, et al. ANCHOR study group: ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration: two-year results of the ANCHOR study. *Ophthalmology*. 2009;116:57–65.
6. Mitchell P, Korobelnik JF, Lazetta P, et al. Ranibizumab (Lucentis) in neovascular age-related macular degeneration: evidence from clinical trials. *Br J Ophthalmol*. 2010;94:2–13.
7. Van Wijngaarden P, Coster DJ, Williams KA. Inhibitors of ocular neovascularization: promises and potential problems. *JAMA*. 2005;293: 1509–1513.
8. Viswanathan S, Frishman LJ, Robson JG, et al. The photopic negative response of the macaque electroretinogram: reduction by experimental glaucoma. *Invest Ophthalmol Vis Sci*. 1999;40:1124–1136.
9. Viswanathan S, Frishman LJ, Robson JG, Walter JW. The photopic negative response of the flash electroretinogram in primary open angle glaucoma. *Invest Ophthalmol Vis Sci*. 2001;42:514–522.
10. Gotoh Y, Machida S, Tazawa Y. Selective loss of the photopic negative response in patients with optic nerve atrophy. *Arch Ophthalmol*. 2004; 122:341–346.
11. Rangaswamy NV, Frishman LJ, Dorotheo EU, et al. Photopic ERGs in patients with optic neuropathies: comparison with primate ERGs after pharmacological blockade of inner retina. *Invest Ophthalmol Vis Sci*. 2004;45:3827–3837.
12. Miyata K, Nakamura M, Kondo M, et al. Reduction of oscillatory potentials and photopic negative response in patients with autosomal dominant optic atrophy with OPA1 mutations. *Invest Ophthalmol Vis Sci*. 2007;48:820–824.
13. Machida S, Gotoh Y, Toba Y, et al. Correlation between photopic negative response and retinal nerve fiber layer thickness and optic disc topography in glaucomatous eyes. *Invest Ophthalmol Vis Sci*. 2008;49: 2201–2207.
14. Miyake Y, Yanagida K, Kondo K, Ota I. Subjective scotometry and recording of local electroretinogram and visual evoked response. System with television monitor of the fundus. *Jpn J Ophthalmol*. 1981;25:439–448.
15. Colotto A, Falsini B, Salgarello T, et al. Photopic negative response of the human ERG: losses associated with glaucomatous damage. *Invest Ophthalmol Vis Sci*. 2000;41:2205–2211.
16. Machida S, Toba Y, Ohtaki A, et al. Photopic negative response of focal electroretinogram in glaucomatous eyes. *Invest Ophthalmol Vis Sci*. 2008;49:5636–5644.
17. Machida S, Tamada K, Oikawa T, et al. Sensitivity and specificity of photopic negative response of focal electroretinograms in detecting glaucomatous eyes. *Br J Ophthalmol*. 2010;94:202–208.
18. Machida S, Tamada K, Oikawa T, et al. Comparison of photopic negative response between full-field and focal electroretinograms in detecting glaucomatous eyes. *J Ophthalmol*. September 30, 2010. [Epub ahead of print.]
19. Kondo M, Kurimoto Y, Sakai T, et al. Recording focal macular photopic negative response (PhNR) from monkeys. *Invest Ophthalmol Vis Sci*. 2008;49:3544–3550.
20. Tamada K, Machida S, Oikawa T, et al. Correlation between photopic negative response of focal electroretinograms and local loss of retinal neurons in glaucoma. *Curr Eye Res*. 2010;35:155–164.
21. Bhurayanontachai P, Ratanasukon M, Jirarattanasopa P. Electrophysiologic changes after intravitreal ranibizumab injection for the treatment of choroidal neovascular membrane (CNVM). *Clin Ophthalmol*. 2011;5:567–572.
22. Lüke M, Januschowski K, Lüke J, et al. Effects of ranibizumab (Lucentis) on retinal function in isolated vertebrate retina. *Br J Ophthalmol*. 2009;93:1396–1400.
23. Sheybani A, Brantley MA Jr, Apte RS. Pattern electoretinography in age-related macular degeneration. *Arch Ophthalmol*. 2011;129:580–584.
24. Mafei L, Fiorrentini A, Bisti S, Holländer H. Pattern ERG in the monkey after section of the optic nerve. *Exp Brain Res*. 1985;59:423–425.
25. Mafei L, Fiorrentini A. Electroretinographic responses to alternating gratings before and after section of the optic nerve. *Science*. 1981;211: 953–955.
26. Lalwani GA, Rosenfeld PJ, Fung AE, et al. A variable-dosing regimen with intravitreal ranibizumab for neovascular age-related macular degeneration: year 2 of the PrONTO study. *Am J Ophthalmol*. 2009;148:43–58.
27. Kizawa J, Machida S, Kobayashi T, et al. Changes of oscillatory potentials and photopic negative response in patients with early diabetic retinopathy. *Jpn J Ophthalmol*. 2006;50:367–373.
28. Moschos MM, Brouzas D, Apostolopoulos M, et al. Intravitreal use of bevacizumab (Avastin) for choroidal neovascularization due to ARMD: a preliminary multifocal-ERG and OCT study. Multifocal-ERG after use of bevacizumab in ARMD. *Doc Ophthalmol*. 2007;114: 37–44.
29. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Ezzat MK, Singh RJ. Pharmacokinetics of intravitreal bevacizumab (Avastin). *Ophthalmology*. 2007;114:855–859.

30. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Ezzat MK, Singh RJ. Pharmacokinetics of intravitreal ranibizumab (Lucentis). *Ophthalmology.* 2007;114:2179–2182.

31. Horsley MB, Mandava N, Maycotte MA, Kahook MY. Retinal nerve fiber layer thickness in patients receiving chronic anti-vascular endothelial growth factor therapy. *Am J Ophthalmol.* 2010;150:558–561.

32. Machida S, Gotoh Y, Tanaka M, Tazawa Y. Predominant loss of the photopic negative response in central retinal artery occlusion. *Am J Ophthalmol.* 2004;37:938–940.

33. Chen H, Wu D, Huang S, Yan H. The photopic negative response of the flash electroretinogram in retinal vein occlusion. *Doc Ophthalmol.* 2006;113:53–59.

34. Chen H, Zhang M, Huang S, Wu D. The photopic negative response of flash ERG in nonproliferative diabetic retinopathy. *Doc Ophthalmol.* 2008;117:129–135.

35. Yonemura D, Aoki T, Tsuzuki K. Electroretinogram in diabetic retinopathy. *Arch Ophthalmol.* 1962;68:19–24.

36. Papadopoulou DN, Medrinos E, Mangioris G, Donati G, Pouraras CJ. Intravitreal ranibizumab may induce retinal arteriolar vasoconstriction in patients with neovascular age-related macular degeneration. *Ophthalmology.* 2009;116:1755–1761.

37. Ameri H, Chader GJ, Kim JG, et al. The effects of intravitreous bevacizumab on retinal neovascular membrane and normal capillaries in rabbits. *Invest Ophthalmol Vis Sci.* 2007;48:5708–5715.

38. Kim KS, Chang HR, Song S. Ischemic change after intravitreal bevacizumab (Avastin) injection of macular for macular oedema secondary to non-ischemic central retinal vein occlusion. *Acta Ophthalmol.* 2008;86:925–927.

39. Yokoyama K, Choshi T, Kimoto K, et al. Retinal circulatory disturbances following intracameral injection of bevacizumab for neovascular glaucoma. *Acta Ophthalmol.* 2008;86:927–928.

40. Shimura M, Yasuda K. Macular ischaemia after intravitreal bevacizumab injection in patients with central retinal vein occlusion and a history of diabetes and vascular disease. *Br J Ophthalmol.* 2010; 94:381–383.

41. Siliprandi R, Canella R, Carmignoto G, et al. N-methyl-D-aspartate-induced neurotoxicity in the adult rat retina. *Vis Neurosci.* 1992;8: 567–573.

42. Sieving PA, Frishman LJ, Steinberg RH. Scotopic threshold response of proximal retina in cat. *J Neurophysiol.* 1986;56:1049–1061.

43. Sieving PA, Nino C. Scotopic threshold response (STR) of the human electroretinogram. *Invest Ophthalmol Vis Sci.* 1988;29:1608–1614.

44. Frishman LJ, Shen FF, Du L, et al. The scotopic electroretinogram of macaque after retinal ganglion cell loss from experimental glaucoma. *Invest Ophthalmol Vis Sci.* 1996;37:125–141.

45. Sieving PA. Retinal ganglion cell loss does not abolish the scotopic threshold response (STR) of the cat and the human ERG. *Clin Vision Sci.* 1991;6:149–158.

REDUCTION OF LASER-INDUCED CHOROIDAL NEOVASCULARIZATION BY INTRAVITREAL VASOHIBIN-1 IN MONKEY EYES

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Purpose: To determine whether intravitreal vasohibin-1 will reduce the grade of the choroidal neovascularization in monkey eyes.

Methods: Choroidal neovascularizations were induced in 12 monkey eyes by laser photocoagulation. Three monkeys were evaluated for the safety of the vasohibin-1 injections, 6 monkeys for the effects of a single injection, and 3 monkeys for repeated injections of vasohibin-1. Ophthalmoscopy, fluorescein angiography, focal electroretinograms, and optical coherence tomography were used for the evaluations. The level of vascular endothelial growth factor in the aqueous was determined by enzyme-linked immunosorbent assay. Immunohistochemistry was performed.

Results: An intravitreal injection of 10 μg of vasohibin-1 induced mild intraocular inflammation. Eyes with an intravitreal injection of 0.1 μg and 1.0 μg of vasohibin-1 had significant less fluorescein leakage from the choroidal neovascularizations and larger amplitude focal electroretinograms than that of vehicle-injected eyes. Similar results were obtained by repeated injections of 0.1 μg of vasohibin-1. Immunohistochemistry showed that vasohibin-1 was expressed mainly in the endothelial cells within the choroidal neovascularizations. The vascular endothelial growth factor level was not significantly altered by intravitreal vasohibin-1.

Conclusion: The reduction of the laser-induced choroidal neovascularizations and preservation of macular function in monkey by intravitreal vasohibin-1 suggest that it should be considered for suppressing choroidal neovascularizations in humans.
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A ge-related macular degeneration (AMD) is one of the most common sight-threatening disease in developed countries.[1] A choroidal neovascularization (CNV) is a typical finding in eyes with the wet-type AMD, and the CNV can lead to subretinal hemorrhages, exudative lesions, serous retinal detachment, and disciform scars.[2] Many different types of treatments have been used to treat AMD, for example, laser photocoagulation,[3] surgery,[4,5] transpupillary thermotherapy,[6] photodynamic therapy,[7] and intravitreal injection of anti–vascular endothelial growth factor (VEGF).[8,9] Each of these treatments has advantages and disadvantages, and the best treatment of AMD has still not been determined.

Different pro- and antiangiogenic factors play important roles in the development and progression of CNVs.[10] Among the proangiogenic factors, VEGF has been shown to play a major role.[11] Thus, anti-VEGF

therapy is being used to successfully treat CNVs in patients with AMD.[8,9] However, this method requires repeated injections that can lead to irritation, infection, and other adverse side effects.[12] In addition, not all patients respond to the therapy.[13] Thus, other types of therapy need to be developed to treat AMD eyes with a CNV.

Vasohibin-1 is a VEGF-inducible molecule expressed by human cultured endothelial cells (ECs) and has antiangiogenic properties.[14] Its expression is selectively induced in ECs not only by VEGF but also by several other proangiogenic factors such as basic fibroblast growth factor.[15] Vasohibin-1 inhibits the formation of EC networks in vitro, corneal neovascularization,[14] and retinal neovascularization in a mouse model of oxygen-induced ischemic retinopathy.[16] Vasohibin-1 is found in the vitreous and in

proliferative membranes of patients with diabetic retinopathy. The level of vasohibin-1 is significantly correlated with the VEGF level in the vitreous of patients with proliferative diabetic retinopathy.[17] Vasohibin-1 is also expressed in the CNV membranes of patients with AMD.[18] Eyes with lower vasohibin-1/VEGF expression ratios tend to have larger CNV lesions, whereas those with higher vasohibin-1/VEGF ratios have subretinal fibrosislike lesions.[18]

We have found that the laser-induced CNVs were less active in mice injected intravitreally with vasohibin-1 than those injected with the vehicle.[19] Thus, the purpose of this study was to determine the effect of intravitreal vasohibin-1 on the laser-induced CNVs in monkey eyes. We shall show that the intravitreal vasohibin-1 was safe and reduced the degree of the CNVs in monkey eyes.

Methods

Animals

The procedures used in the animal experiments followed the guidelines of the The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved by the Animal Care Committee of Tohoku University Graduate School of Medicine. Twelve Japanese macaque monkeys (*Macaca fuscata*) between ages 4 and 6 years and weighing between 4.2 kg and 10.1 kg were used (Table 1). For all procedures, the monkeys were anesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg), and the pupils were dilated with topical 2.5% phenylephrine and 1% tropicamide. Oxybuprocaine hydrochloride (0.4%) was also used for local anesthesia. Three monkeys were

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Table 1. Monkey Eyes Used in This Study

	Vasohibin (mg)	Number of Eyes	Inflammation
Nontreated	0	1	0/1
	0.01	1	0/1
	0.1	1	0/1
	1	1	0/1
	10	1	1/1
	100	1	1/1
Laser application	0	3	0/3
	0.01	3	0/3
	0.1	3	0/3
	1	3	1/3
Laser application	0	3	0/3
	0.1	3	0/3
Total		24	3/24

Inflammation shows clinical inflammation signs that were observed during the experiments.

used to evaluate the safety of intravitreal vasohibin-1, 6 monkeys for dose dependency of a single injection of vasohibin-1, and 3 monkeys for repeated injections of vasohibin-1.

Experimental Choroidal Neovascularization

An argon green laser was used to rupture of the choroidal membrane using a slit-lamp delivery system (Ultima 2000SE; Lumenis, Yokneam, Israel) with a contact lens.[20] The laser settings were as follows: 50-μm diameter, 0.1-second duration, and 650-mW to 750-mW intensity. Five laser burns were made around the macula within 15° of the fovea. The foveola was not treated. Each burn was confirmed to have induced subretinal bubbles indicating a rupture of Bruch membrane.

Expression and Purification of Human Vasohibin-1 Polypeptide

Human vasohibin-1 was purified from *Escherichia coli* as described.[21] Human vasohibin-1 was isolated as a thioredoxin fusion protein. The fusion protein was dialyzed and digested with blood coagulation Factor Xa (Novagen, Darmstadf, Germany). The released vasohibin-1 was collected, eluted, and dialyzed against 20 mM glycine–HCl buffer (pH 3.5). Then, the vasohibin-1 was resolubilized with 50 mM Tris–HCl buffer containing 50 mM NaCl, 5 mM tris(2-carboxyethyl)phosphine, 0.5 mM ethylenediaminetetraacetic acid, 5% glycerol, and 4.4% N-lauroylsarcosine (pH 8.0) and was dialyzed against 20 mM sodium phosphate buffer at pH 8.0. This buffer was also used as the vehicle.

The protein concentration was determined by the Bradford method with a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as a standard protein.

Intravitreal Injection of Recombinant Vasohibin-1 Polypeptide

Vasohibin-1 was injected intravitreally in 3 groups of monkeys (Table 1). The first group of 6 eyes did not have a laser burn and received a single injection of vehicle, or 0.01, 0.1, 1, 10, or 100 μg of vasohibin-1/50 μL of vehicle. The second group of 12 eyes of 6 monkeys (3 eyes for each concentration) received a single injection of vehicle or 0.01, 0.1, and 1 μg of vasohibin-1/50 μL of vehicle 4 days after the laser burn. The third group of 3 eyes had 3 injections of 0.1 μg of vasohibin-1/50 μL of vehicle in the right eyes and 50 μL of vehicle in 3 fellow eyes on 0, 4, and 7 days after the laser burn. We examined the natural course of laser-induced CNVs in mice, and the CNVs were most active around Day 14 after the laser burn, and then gradually regressed, especially 28 days after laser burn. When we injected vasohibin-1 into the vitreous of mice after laser burns, we found that the injection of vasohibin-1 on Day 4 after the laser burn was most effective, followed by Days 7 and 1. Other days were less effective. In addition, immuno-histochemical studies for vasohibin-1 in the mouse CNV membranes showed that the later the laser burn, the more vasohibin-1 staining was observed.[19] So we decided to do the repeated vasohibin-1 injections on 0, 4, and 7 days after the laser burn (relatively early days after laser burn).

For the intravitreal injections, the monkeys were anesthetized and pupils were dilated. The intravitreal injections were made with a 30-gauge needle attached to a 1-mL syringe. The needle was inserted through the sclera into the vitreous cavity ~1.5 mm posterior to the limbus while observing the eye with an operating microscope. The fundus was examined after the injection to confirm that the retina and lens were not damaged.

Ophthalmic Examinations

In addition to the routine ophthalmologic examinations, fluorescein angiography (FA) with an imaging system (GENESIS-Df; Kowa, Tokyo, Japan), optical coherence tomography (OCT, RS3000; NIDEK, Tokyo, Japan), and focal and full-field electroretinography (ERG) were performed on the selected days. Fluorescein angiography was performed 1, 2, and 4 weeks after the laser photocoagulation. Two retinal specialists (R.W. and T.A.) graded the angiograms in a masked way using a grading system[22]: Grade 1, no hyperfluorescence; Grade 2, hyperfluorescence without leakage; Grade 3, hyperfluorescence in the early or middle phase and leakage in the late phase; and Grade 4, bright

hyperfluorescence in the transit and leakage in late phase beyond the treated areas.

The central macular thickness was determined from the macular thickness maps (3.45 mm in diameter) of the scans by OCT 4 weeks after the laser photocoagulation. The volume of the lesion was also calculated using the same program.

The pupils were maximally dilated for the ERG recordings 4 weeks after intravitreal vasohibin-1 injections. The ERGs were amplified and digitally band-pass filtered from 0.5 Hz to 500 Hz for the full-field ERGs and from 5 Hz to 500 Hz for the focal ERGs (PuREC; Mayo, Aichi, Japan). The animals were dark adapted for at least 30 minutes before the full-field ERG recordings. The light for the stimulus was obtained from light-emitting diodes (EW-102; Mayo Co., Nagoya, Japan) embedded in a contact lens electrode. The intensity and duration of the stimuli were controlled by an electronic stimulator (WLS-20; Mayo Co.). Chlorided silver plate electrodes were placed on the forehead and right ear lobe as reference and ground electrodes, respectively. The intensity of the stimulus was 1,000 cd/m^2 and the duration was 3 milliseconds.

Focal ERGs were recorded 4 weeks after the laser photocoagulation with a focal ERG system (PuREC; Mayo; ER-80; Kowa) that was integrated into an infrared fundus camera. This system was developed and described in detail by Miyake et al.[23,24] The stimulus spot was 15° in diameter and was placed on the macula by viewing the ocular fundus on a monitor. The intensity of the stimulus was 1,000 cd/m^2, and the background light was 1.5 cd/m^2. The stimulus duration was 100 milliseconds. A Burian–Allen bipolar contact lens electrode (Hansen Ophthalmic Laboratories, Iowa City, IA) was inserted into the anesthesized conjunctival sac to record the focal ERGs. A chlorided silver electrode was placed on the left ear lobe as the ground electrode. Two hundred to 300 responses were averaged at a stimulation rate of 5 Hz.

The a-waves were measured from the baseline to the trough of the first negative response, and the b-wave from the first trough to the peak of the following positive wave. The amplitudes of a-waves and b-waves from the three untreated monkeys were used as control. The number of monkeys used in this experiment was not added to the total number of monkeys.

Immunohistochemistry

Immunostaining for vasohibin-1 was done on eyes with laser-induced CNVs 28 days after the laser application. From the results of CNV experiments on

mice,[19] the laser-induced CNV lesions were self-resolved >28 days after the laser burn. Thus, we decided to enucleate the eyes 28 days after the laser burn, although there may be differences between mice and monkeys. The eyes were enucleated and fixed in 4% paraformaldehyde overnight, and the anterior segment and lens were removed. The posterior segment was embedded in paraffin, and 3-μm serial sections were cut, and adjacent sections were stained with hematoxylin and eosin.

The immunohistochemical staining for vasohibin-1 was performed with the peroxidase method and for cytokeratin by the alkaline phosphatase method. Mouse monoclonal antibodies against vasohibin-1 (1:400) and mouse monoclonal anti-pan cytokeratin (1:200; Sigma-Aldrich, St. Louis, MO) were applied to the sections overnight at 4°C. Then the sections were incubated in biotin-conjugated anti-mouse immunoglobulin (Histfine SAB-PO(M) kit; Nichirei, Tokyo, Japan). The slides for vasohibin were incubated with peroxidase-conjugated streptavidin (Histfine SAB-PO(M) kit; Nichirei), and the slides for cytokeratin were incubated with alkaline phosphatase–conjugated streptavidin (Histfine; Nichirei). HistoGreen (HISTOPRIME HistoGreen substrate kit for peroxidase; Ab Cys SA) was used for the chromogen of vasohibin, and VECTOR RED (alkaline phosphatase substrate kit 1; Vector, Burlingame, CA) was used for the chromogen of cytokeratin. The slides were counterstained with hematoxylin. For control, pre-immune mouse immunoglobulin G was used instead of the primary antibody.

Enzyme-Linked Immunosorbent Assay for Vascular Endothelial Growth Factor

Aqueous was collected by a 30-gauge needle from the anterior chamber of each monkey 4 weeks after the laser photocoagulation. The level of the VEGF peptide was quantified by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R & D Systems, Mckinley, MN; Quantikine Human VEGF immunoassay) using 50 μL of aqueous. The intensity of the color of the reaction products was measured with a MAXline microplate reader (Molecular Devices Corporation, Palo Alto, CA). The measurements were made in duplicate, and the mean was used. The concentration of VEGF was expressed as the amount of protein in picograms per milliliter (pg/mL).

Statistical Analyses

Analysis of variance with Scheffe test for post hoc analysis was used to examine the differences in the leakage and intensity of the CNVs in the fluorescein angiograms, amplitudes of the ERGs, mean central thickness, and volume of the CNV. The differences were also compared using the Student two-sample t-tests.

Results

Safety Evaluations and Outcomes

Before any of the procedures, the retina and choroid were normal in all the monkeys. Then 6 nontreated eyes were injected intravitreally with vehicle or 0.01, 0.1, 1, 10, or 100 μg of vasohibin-1/50 μL. After 0.01, 0.1, and 1 μg of vasohibin-1, the appearance of the retina and choroid did not differ from that of the vehicle-injected eyes. When 10 μg or 100 μg/50 μL of vasohibin-1 polypeptide was injected, a mild inflammation (Grade 1)[25] was detected in the vitreous on the day after the injection. The inflammation was less with 10 μg than with 100 μg of vasohibin, and the inflammation was resolved in 2 days after 10 μg and in 1 week after 100 μg (Table 1). When we injected 1 μg/50 μL of vasohibin-1 once in the laser-treated eyes, 1 of the 3 eyes developed inflammation in the aqueous. An inflammation was not observed when 0.1 μg of vasohibin-1 was injected even after 3 injections. When we injected 50 μL of vehicle with almost the same amount of endotoxin (400 U/mL) as that of 100 μg of vasohibin-1, no inflammation was detected. These results indicated that mild inflammation can develop with $\geq$10 μg of vasohibin-1 injection into the vitreous in nontreated monkey eyes.

The amplitudes of the a- and b-waves of the full-field ERGs of eyes injected with 0.01 μg to 100 μg of vasohibin-1 did not differ significantly from the vehicle-injected eyes. The a-wave amplitudes ranged from 87.3 μV to 180.3 μV (average, 119.3 ± 36.6 μV) before and from 100.7 μV to 195.8 μV (average, 131.3 ± 53.7 μV; P = 0.444) after the vasohibin-1 injection. The b-wave amplitudes ranged from 219.6 μV to 340.6 μV (average 250.6 ± 54.7 μV) before and from 240.8 μV to 345.2 μV (average 274.4 ± 82.0 μV, P = 0.801) after the vasohibin-1 injection.

Effect of Different Concentrations of Vasohibin-1

After the laser photocoagulation, we injected vehicle or 0.01, 0.1, or 1 μg of vasohibin-1/50 μL of vehicle in 3 eyes of each dosage for a total of 12 eyes (Table 1). From the results of safety evaluations, we selected the maximum amount of vasohibin-1 as 1 μg of vasohibin-1/50 μL of vehicle. Representative results of FA at 1, 2, and 4 weeks after the laser application for each dose of vasohibin-1 are shown in Figure 1. Color fundus photographs and focal ERGs recorded at 4 weeks are also shown.
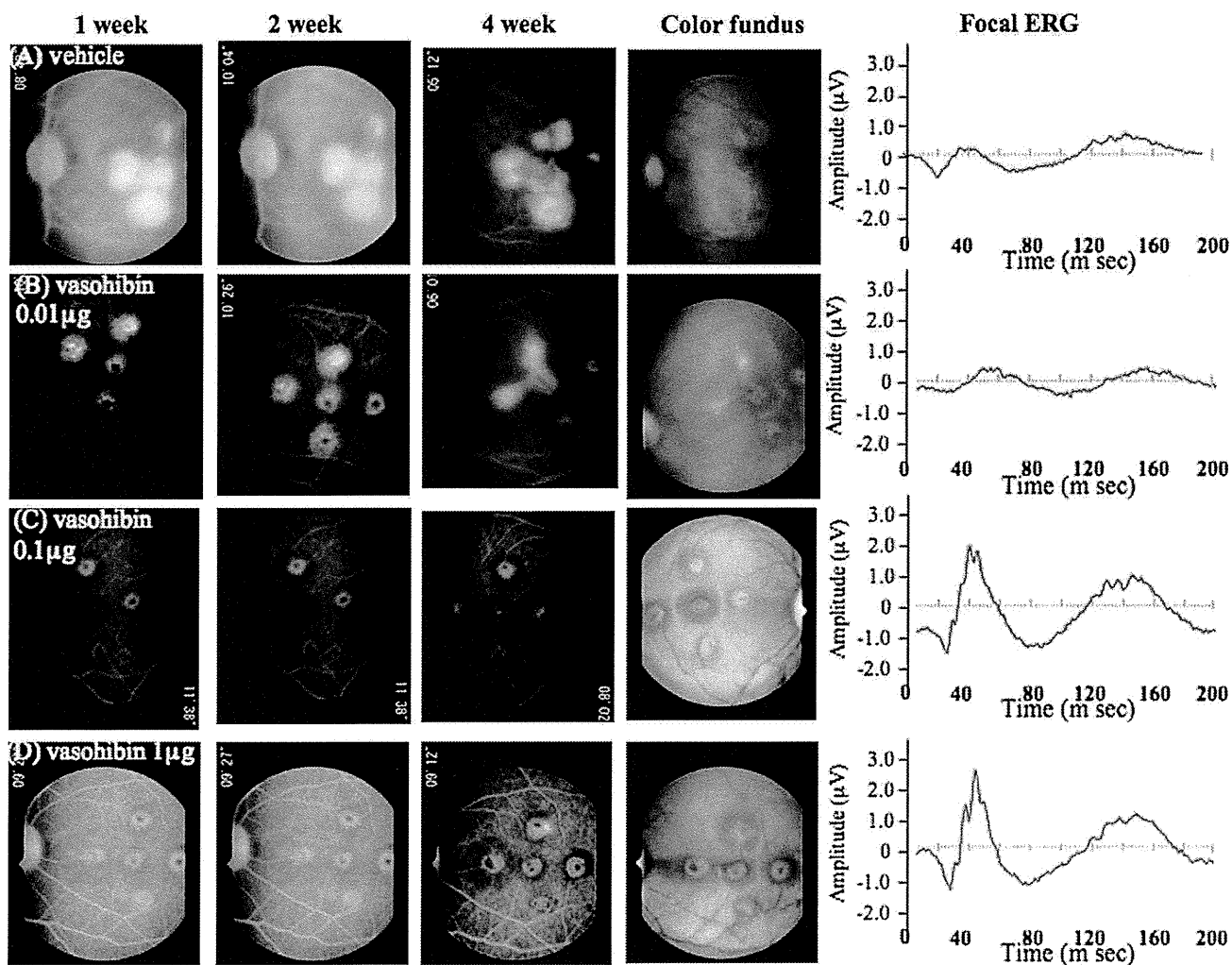
Fig. 1. Representative FAs, fundus photographs, and focal ERGs from 6 monkey eyes are shown. Vehicle or 0.01, 0.1, 1 μg of vasohibin-1/50 μL of vehicle was injected intravitreally, and representative results at 1, 2, and 4 weeks after laser treatment are shown (see quantitative values in Figure 2, A–D). The FA images are those at around 10 minutes after the fluorescein injection. Color fundus photographs were taken 4 weeks after the laser application. Focal ERGs recorded 4 weeks after the laser photocoagulation are shown in the right column for each eye.

The CNV activity was scored using the FA grading system[22] for all five laser spots in each eye. The FA score for each spot was summed and compared with each other (Figure 2A). Our findings showed that there was significantly less leakage after 0.1 μg of vasohibin-1 than that for vehicle ($P = 0.016$) and for 0.01 μg ($P = 0.035$) of vasohibin-1 at 1 week. Significantly less leakage after 0.1 μg of vasohibin-1 than that of vehicle was also observed at 4 weeks ($P = 0.0307$). Because 1 μg of vasohibin-1 showed mild inflammation in 1 eye, we did not analyze the CNV in these eyes. The percentage of eyes with FA scores of 4 is also listed in Figure 2B. Our results showed that 45% of vehicle-treated eyes had Grade 4 leakage, and it was 45% in 0.01 μg of vasohibin-1–treated eyes, 7% with 0.1 μg of vasohibin-1–treated eyes, and

none in the 1-μg vasohibin-1–treated eyes (only 2 eyes) at 1 week. Similarly, the percentage of eyes with Grade 4 leakage was 27%, 13%, 7%, and 0% at 2 weeks and 20%, 20%, 0% and 0% at 4 weeks after the vasohibin-1 injection (Figure 2B).

The amplitudes of the a-waves of the focal ERGs after 0.01 μg of vasohibin-1 were significantly smaller than those of the controls ($P = 0.041$) (Figure 2C). The amplitudes of the b-waves of the focal ERG b amplitudes in the vehicle-injected eyes ($P = 0.0085$) and in the 0.01-μg vasohibin-1–injected eye ($P = 0.0184$) were significantly smaller than those of the controls (Figure 2D). The results of inflammation, FA leakage, and ERG amplitudes led us to select 0.1 μg of vasohibin-1 as the optimal concentration for intravitreal injection to reduce the laser-induced CNV in our monkeys.
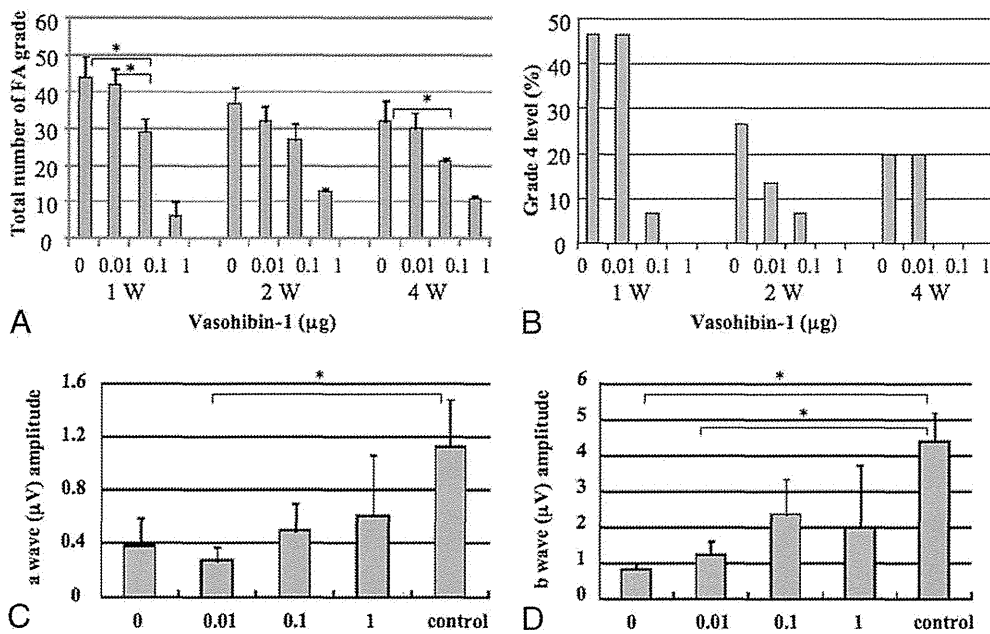
Fig. 2. Fluorescein angiographic scores for each of the 5 laser spots in each eye are plotted for each group, and the amplitudes of the a- and b-waves of the focal ERGs. **A.** Fluorescein angiographic scores for each of the five laser spots in each eye are plotted for each group. Statistically significant differences are shown as asterisks. **B.** Distribution of Grade 4 FA scores for each group is shown. **C** and **D.** Average amplitude of the a-waves (**C**) and b-waves (**D**) of the focal ERG recorded 4 weeks after intravitreal vasohibin-1. Vehicle (0) or 0.01, 0.10, or 1.00 μg of vasohibin-1 was injected in control eyes or eyes after the laser burns. Untreated controls show the effects before laser treatment. The averages $\pm$ standard deviations of the amplitudes of the a- and b-waves are plotted on the ordinate.

Effects of Repeated Injections of Vasohibin-1

Next, we examined the effects of repeated intravitreal injections of 0.1 μg of vasohibin-1/50 μL of vehicle in the right eyes on 0, 4, and 7 days after the laser application while the fellow eyes received an injection of the vehicle on the same days. We studied three eyes in each group. Representative fundus photographs, FAs, and OCT images after vehicle alone are shown in Figure 3 (A and B) and after 0.1 μg of vasohibin-1/50 μL of vehicle in Figure 3 (C and D). The FA scores were significantly lower in the vasohibin-1–injected eyes than in the vehicle-injected eyes at 4 weeks (P = 0.009; Figures 3 and 4A). At 1 week and 2 weeks after the vasohibin-1 injections, the FA scores were not significantly different (P = 0.07). The percentage of eyes scored as Grade 4 was 13.3% at 1 week, 26.7% at 2 weeks, and 26.7% at 4 weeks in the vehicle-treated eyes, whereas no Grade 4 eyes were observed in the 0.1 μg of vasohibin-1/50 μL of vehicle-treated eyes at any time (Figure 4B).

Although statistical significance was not observed in the a-wave amplitude of the focal ERGs, statistically significant larger b-wave amplitudes were observed in the vasohibin-1–treated eyes than that of vehicle (P = 0.039) (Figure 4, C and D).

Optical coherence tomography examinations showed that the retinal pigment epithelium and Bruch membrane were disrupted in the laser-treated eyes at 1 week and 2 weeks after the laser application (Figure 3, B and D) as was found in histologic preparations.[22] At 4 weeks, an retinal pigment epithelium–like membrane appeared over the CNV lesion (Figure 3, B and D).

This line was shown to be cytokeratin positive. The OCT images showed that the size of the CNV increased gradually especially in vehicle-treated eyes as was seen in the FA images.

Optical coherence tomography also showed that the macular thickness (Figure 4E) and volume (Figure 4F) of the CNV lesions after 0.1 μg of vasohibin-1/50 μL of treated eyes was ~30% less than the vehicle-treated eyes in the central 1 mm. When we examined the volume of the central 6 mm, no difference was observed between the vasohibin-1–treated and vehicle-treated eyes.

Histology and Immunostaining of Choroidal Neovascularization

Histopathologic analyses showed that the retina and choroid surrounding the CNV had normal architecture in both the vehicle and vasohibin-1–treated eyes as reported.[26] The vehicle-treated eyes after the laser application showed a disruption of the Bruch membrane and retinal pigment epithelium complex, and the eyes had different degrees of fibrous tissues and vessels (Figure 5, C and E). Eyes treated with vasohibin-1 tended to have smaller CNV than that of vehicle-treated eyes.

Cytokeratin labeling demonstrated that retinal pigment epithelial cells from the edges of the wound had proliferated and covered the laser wound to different degrees. Although a disruption of the cytokeratin labeling was present in the vehicle-treated eyes (Figure 5, D and F), we could not find any significant difference from that of the vasohibin-1–injected eyes. Different
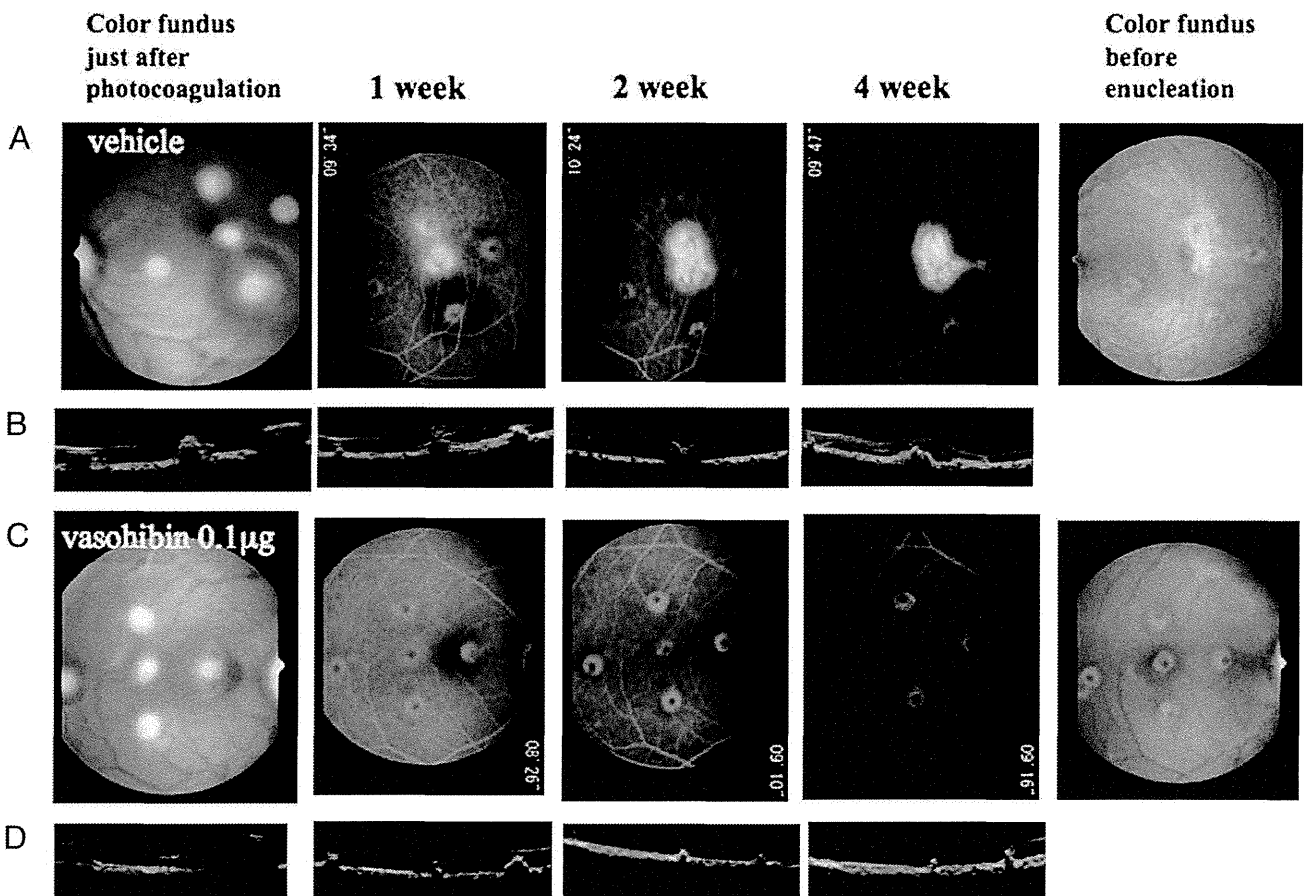
Fig. 3. Fluorescein angiograms, ocular coherence tomographic images, color fundus photographs, and focal ERGs are shown. Vasohibin-1 (0.1 μg/50 μL) was injected into the vitreous of the right eyes 3 times on 0, 4, and 7 days after laser application, and the same amount of vehicle was injected into left eyes on the same days. Photographs show the fundus just after the laser application and the day of enucleation. Fluorescein angiographs recorded 1, 2, and 4 weeks after laser application. Photographs of the right (A) and left (C) eyes are shown. The results of OCT on the indicated days are shown in the same vertical columns for the indicated day (B) and (D).

numbers of macrophage-like cells were also observed in the neural retina.[21]

In immunostained eyes, vasohibin-1 positivity was found mainly in the CNV especially on the ECs in the CNV (Figure 5B). The regions surrounding the CNV showed little vasohibin-1–positive staining. Some monkeys showed no vasohibin-1 expression by immunohistochemistry even in the CNV at 28 day after laser application. Positive staining for vasohibin-1 appeared to be greater in the more active CNVs (Figure 5A), and it was more obvious in nontreated monkey eyes, although we could not determine whether the staining was significantly greater because only 3 monkey eyes were studied.

Vascular Endothelial Growth Factor in Aqueous During Experiments

The level of VEGF was determined by enzyme-linked immunosorbent assay. The average VEGF level in the aqueous in the vasohibin-1–injected eyes was 15.3 pg/mL, and it was 20.6 pg/mL in the vehicle-treated eyes at 4 days after laser application. The average VEGF level in the vasohibin-1– and vehicle-treated eyes were 7.0 pg/mL and 8.9 pg/mL, respectively, at 4 weeks after laser application (Figure 6). For both times, the differences were not significant.

Discussion

Our results demonstrated that when 10 μg or 100 μg of vasohibin-1 was injected intravitreally into nontreated normal monkey eyes, a mild anterior chamber inflammation developed. No signs of inflammation or any adverse effects were found when <1 μg of vasohibin-1 was injected into nonlaser treated eyes, although we used only 1 eye for each dose. However when 1 μg of vasohibin-1 was injected into laser-treated eyes, a mild inflammation developed in 1 of the 3 eyes. Inflammation has also been reported in monkey
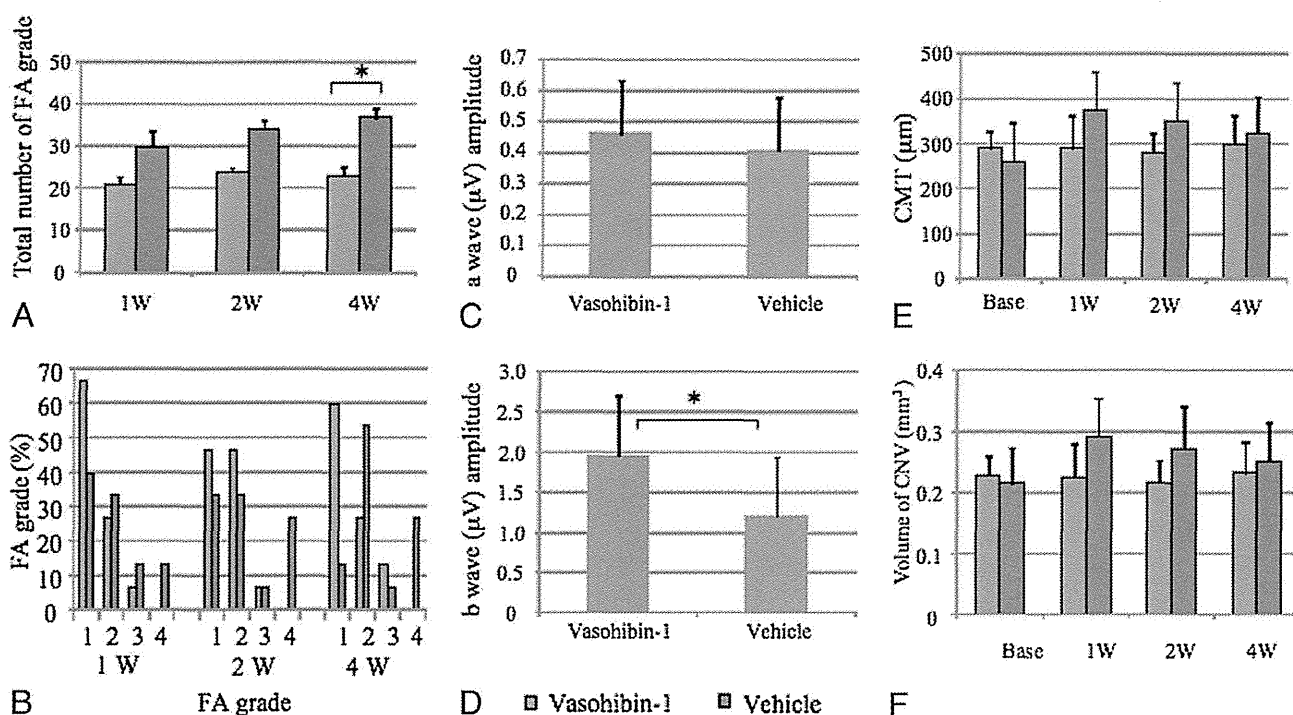
Fig. 4. Results of FA, focal ERG, and OCT are shown. **A.** Significantly less FA leakage was observed after 0.1 μg/50 μL of vasohibin-1 than after vehicle treatment at 4 weeks. **B.** Distribution of Grade 4 FA eyes for each group. **C** and **D.** Average amplitudes of the a-waves (**C**) and b-waves (**D**) of the focal ERGs. **E** and **F.** Average central macular thickness (CMT) and the central 3.4 mm and volume of area of either vasohibin-1–treated (blue) or vehicle-treated (red) eyes before (base) and 1, 2, and 4 weeks after laser application. Lower thickness and volumes were observed in the vasohibin-1–treated monkeys. Data are the standard deviations.

eyes after intravitreal injections of fragments of mouse and human chimera antibodies against VEGF.[22,27]

Fluorescein angiography examination after vasohibin-1 injection in laser-treated eyes showed significantly lower FA scores in eyes that received 0.1 μg and 1 μg of vasohibin-1 than the vehicle-injected eyes, although the number of eyes may have affected the statistics. Fluorescein leakage from the laser spots close to the macula was greater than that of the other laser spots. These results are compatible with the results of Shen et al,[28] who also found that the laser spot was larger and the leakage was greater for lesions closer to the macula. We also found that fluorescein leakage was different among monkeys, even though we applied the same amount of vasohibin-1.[22] This variability may be because the body weight ranged from 4.1 kg to 10.1 kg and age from 4 years to 6 years among the monkeys.

After we injected 0.1 μg of vasohibin-1 3 times in the right eyes and vehicle into the left eyes of 3 monkeys, we found significantly less fluorescein leakage in the vasohibin-1–treated right eyes than in the vehicle-treated eyes. The results of focal ERGs and OCT were well correlated with the results of FA findings, although the quantitative values were not significantly different.

Taken together, these results showed that intra-vitreal vasohibin-1 is able to reduce the activity of the laser-induced CNV in monkeys. With 3 injections of 0.1 μg of vasohibin-1, the results were not so different from that of only 1 injection at 4 days after the laser application. This may indicate that there may be an optimum time for the vasohibin-1 to affect the course of the laser-induced CNV. Alternatively, the results may be related to the half-life of vasohibin-1.

We found that vasohibin-1 was expressed on ECs especially those in the CNV lesions. Careful examinations showed that vasohibin-1 expression was limited to the CNV lesion and may not show extensive expression in other regions under normal physiologic conditions. Although we have not followed the expression of vasohibin-1 during the course of CNV development in monkeys, vasohibin-1 expression may be enhanced in the new vessels as was reported.[29] The vasohibin-1 expression appeared stronger in non-treated monkey eyes, although this could not be quantified. Vasohibin-1 has been reported to be present on the ECs only in the stroma of tumors and not in the noncancerous regions of the tissue in surgically resected tissues of the same patient.[29] These findings suggest that vasohibin-1 may be expressed mainly in the new vessels as it was in our laser-induced CNVs.
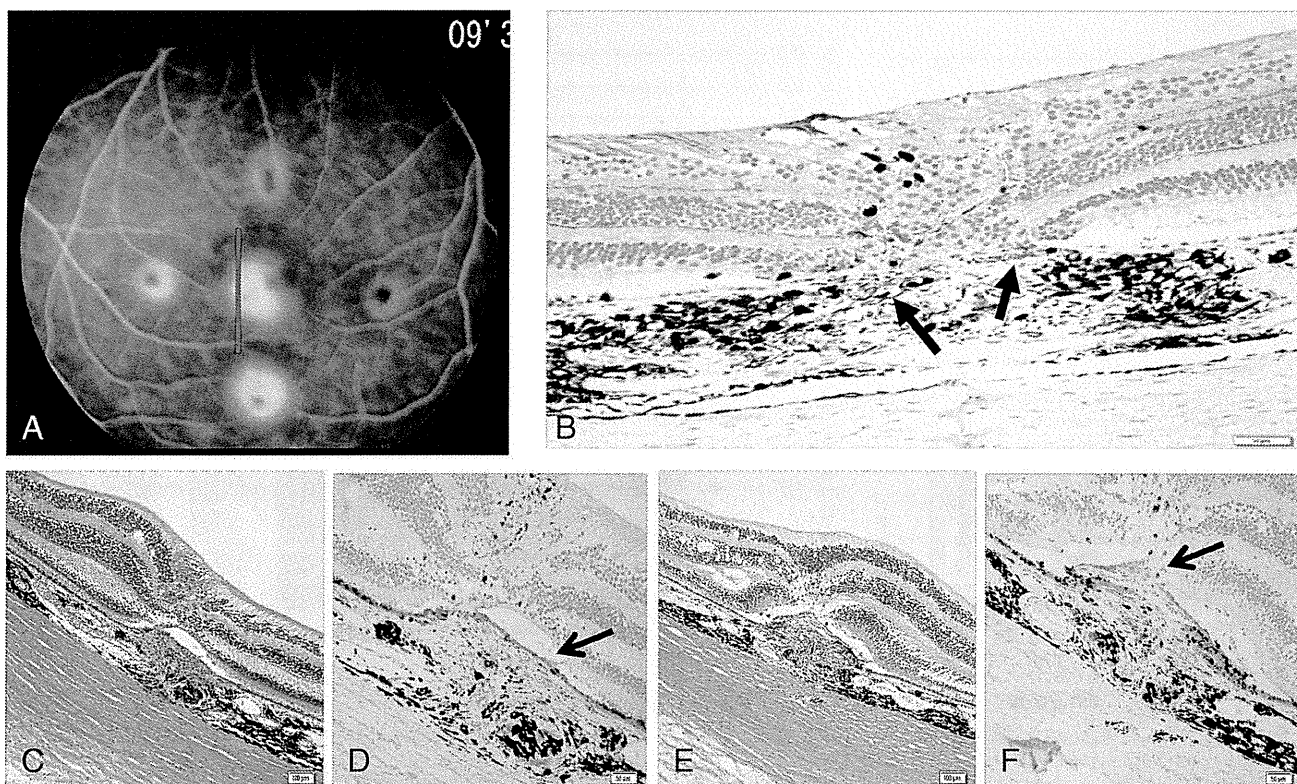
Fig. 5. Fluorescein angiograms 4 weeks after laser application or vehicle injection are shown. A. Immunohistochemistry for vasohibin-1 (B), the same eye as shown in (A) at the red line, is shown. Arrows indicate vasohibin-1 labeling. Vasohibin-1 expression is concentrated on the vessels around the CNV (arrows), but markedly less than in the CNV. Vaoshibin-1 expression was observed at active CNV (red line in A). The subretinal space is an artifact of histologic processing. Cytokeratin labeling is also shown with vasohibin-treated eye (D) and vehicle-treated eye (F). Arrows show labeling of cytokeratin. Bar = 50 μm. C and E. Hematoxylin and eosin staining of vasohibin-1–treated and vehicle-treated eyes, respectively, are shown. Bar = 100 μm. Cytokeratin labeling shows that retinal pigment epithelium covers CNV in the vasohibin-1–treated eyes (D), and a disruption of cytokeratin labeling is observed in vehicle-treated eye (F).

Hosaka et al[29] reported that exogenous vasohibin-1 blocked angiogenesis and maturation of not only the cancerous tissue but also the surrounding vessels and, thus, enhanced the antitumor effects of
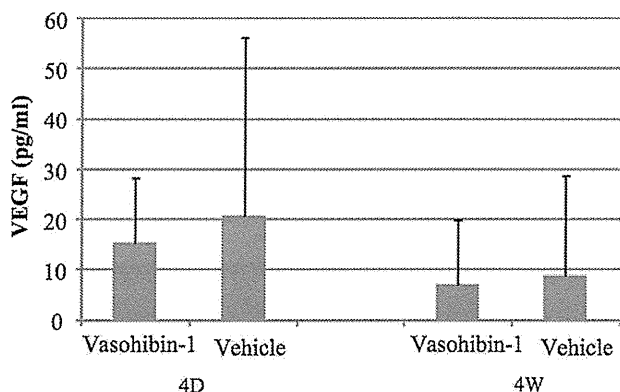

Fig. 6. Concentration of VEGF in aqueous in laser-treated monkey eyes 4 days and 4 weeks after laser application is shown. Vertical axis shows VEGF concentration in picograms per milliliter, and horizontal axis is the day of examination. Vascular endothelial growth factor in vasohibin-1–treated eyes (blue boxes) and vehicle-treated eyes (red boxes) show no significant difference at any times.

vasohibin-1. Intravitreal injection of vasohibin-1 may also suppress angiogenesis in CNVs by the same mechanism.

The amount of VEGF in the aqueous in the vasohibin-1–treated eyes did not differ from that in vehicle-treated eyes. Thus, Zhou et al[30] reported that external vasohibin-1 had no effect on the level of VEGF when they used adenovirus encoding human vasohibin-1 on mouse corneal neovascularization induced by alkali burn. They also reported that vasohibin-1 may downregulate the VEGF receptor 2 (VEGFR2). Shen et al[16] also reported a downregulation of VEGFR2 by vasohibin-1 during mouse ischemic retinopathy. Our previous studies have also shown a downregulation of VEGFR2 by external vasohibin-1 in laser-induced mouse CNVs.[19] Thus, vasohibin-1 may reduce the activity of a CNV by partially downregulating VEGFR2 in the eyes. If this is correct, vasohibin-1 may not affect the favorable aspects of VEGF such as its neuroprotective effect,[31] especially if VEGF works through VEGFR1 rather than VEGFR2. Vasohibin-1 also can be used with anti-VEGF antibody for CNV therapy.

The benefits of combined therapy, such as photodynamic therapy and anti-VEGF antibody, have been discussed.[27]

In conclusion, intravitreal vasohibin-1 in monkey eyes is safe and can reduce the activity of laser-induced CNVs and thus preserve the function of the macula.

Key words: choroidal neovascularization, laser-induced, monkey, vascular endothelial growth factor, vasohibin-1.

References

1. Klein R, Peto T, Bird AC, Vannewkirk MR. The epidemiology of age-related macular degeneration. Am J Ophthalmol 2004; 137:486–495.
2. Bressler NM, Bressler SB, Fine SL. Age-related macular degeneration. Surv Ophthalmol 1998;32:375–413.
3. Argon laser photocoagulation for neovascular maculopathy. Three-year results from randomized clinical trials Macular Photocoagulation Study Group. Arch Ophthalmol 1986;104: 694–701.
4. Thomas MA, Grand MG, Williams DF, et al. Surgical management of subfoveal choroidal neovascularization. Ophthalmology 1992;99:952–968.
5. Eckardt C, Eckardt U, Conrad HG. Macular rotation with and without counter-rotation of the globe in patients with age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol 1999;237:313–325.
6. Reichel E, Berrocal AM, Ip M, et al. Transpupillary thermotherapy of occult subfoveal choroidal neovascularization in patients with age-related macular degeneration. Ophthalmology 1999; 106:1908–1914.
7. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one year results of 2 randomized clinical trials-TAP report Treatment of Age-related Macular Degeneration with Photodynamic Therapy (TAP). Study Group. Arch Ophthalmol 1999;117:1329–1345.
8. Grisanti S, Tatar O. The role of vascular endothelial growth factor and other endogenous interplayers in age-related macular degeneration. Prog Retin Eye Res 2008;27:372–390.
9. Miller JW, Adamis AP, Shima DT, et al. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 1994;145:574–584.
10. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–346.
11. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
12. Pilli S, Kotsolis A, Spaide RF, et al. Endophthalmitis associated with intravitreal anti-vascular endothelial growth factor therapy injections in an office setting. Am J Ophthalmol 2008;145:879–882.
13. Lux A, Llacer H, Heussen FMA, Joussen AM. Non-responders to bevacizumab (Avastin) therapy of choroidal neovascular lesions. Am J Ophthalmol 2007;91:1318–1322.
14. Watanabe K, Hasegawa Y, Yamashita H, et al. Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. J Clin Invest 2004;114:898–907.
15. Shimizu K, Watanabe K, Yamashita H, et al. Gene regulation of a novel angiogenesis inhibitor, vasohibin, in endothelial cells. Biochem Biophys Res Commun 2005;327:700–706.
16. Shen J, Yang X, Xiao WH, et al. Vasohibin is up-regulated by VEGF in the retina and suppresses VEGF receptor 2 and retinal neovascularization. FASEB J 2006;20:723–725.
17. Sato H, Abe T, Wakusawa R, et al. Vitreous levels of vasohibin-1 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. Diabetologia 2009;52:359–361.
18. Wakusawa R, Abe T, Sato H, et al. Expression of vasohibin, an antiangiogenic factor, in human choroidal neovascular membranes. Am J Ophthalmol 2008;146:235–243.
19. Wakusawa R, Abe T, Sato H, et al. Suppression of choroidal neovascularization by vasohibin-1, vascular endothelium-derived angiogenic inhibitor. Invest Ophthalmol Vis Sci 2011;52:3272–3280.
20. Tobe T, Ortega S, Luna JD, et al. Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in a murine model. Am J Pathol 1998;153:1641–1646.
21. Heishi T, Hosaka T, Suzuki Y, et al. Endogenous angiogenesis inhibitor vasohibin1 exhibits broad-spectrum antilymphangiogenic activity and suppresses lymph node metastasis. Am J Pathol 2010;176:1950–1958.
22. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–346.
23. Miyake Y, Yanagida K, Yagasaki K, et al. Subjective scotometry and recording of local electroretinogram and visual evoked response. System with television monitor of the fundus. Jpn J Ophthalmol 1981;25:439–448.
24. Kondo M, Ueno S, Piao CH, et al. Comparison of focal macular cone ERGs in complete-type congenital stationary night blindness and APB-treated monkeys. Vision Res 2008;48: 273–280.
25. Hogan MJ, Kimura SJ, Thygeson P. Signs and symptoms of uveitis. I. Anterior uveitis. Am J Ophthalmol 1959;47:155–170.
26. Zhang M, Zhang J, Yan M, et al. Recombinant anti-vascular endothelial growth factor fusion protein efficiently suppresses choroidal neovascularization in monkeys. Mol Vision 2008;14:37–49.
27. Husain D, Kim I, Gauthier D, et al. Safety and efficacy of intravitreal injection of ranibizumab in combination with verteporfin PDT on experimental choroidal neovascularization in the monkey. Arch Ophthalmol 2005;123:509–516.
28. Shen WY, Lee SY, Yeo I, et al. Predilection of the macular region to high incidence of choroidal neovascularization after intense laser photocoagulation in the monkey. Arch Ophthalmol 2004;122:353–360.
29. Hosaka T, Kimura H, Heishi T, et al. Vasohibin-1 expression in endothelium of tumor blood vessels regulates angiogenesis. Am J Pathol 2009;175:430–439.
30. Zhou SY, Xie ZL, Xiao O, et al. Inhibition of mouse alkali burn induced-corneal neovascularization by recombinant adenovirus human vasohibin-1. Mol Vision 2010;16:1389–1398.
31. Alon T, Hemo I, Itin A, et al. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1995;1:1024–1028.

ORIGINAL RESEARCH ARTICLE

Macular function evaluated by focal macular electroretinograms after reduced fluence photodynamic therapy in eyes with polypoidal choroidal vasculopathy

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Abstract The purpose of this study was to evaluate the macular function by measuring the focal macular electroretinograms (ERGs) recorded before and after reduced fluence photodynamic therapy (RFPDT) in patients with polypoidal choroidal vasculopathy (PCV). Eleven eyes of 11 patients with PCV were studied. Their ages ranged from 62 to 85 years with a mean of 74.7 ± 6.9 years. The exposure time for the RFPDT was reduced to 42 s, so that the total energy of the laser was approximately one-half that of the standard PDT. We measured the visual acuity, foveal thickness, and focal macular ERGs before and after the RFPDT. The follow-up period ranged from 13 to 34 months with a mean of 26 months after the treatment. A significant recovery of vision was seen even at 1 week after the RFPDT ($P < 0.005$), and the visual acuities improved gradually thereafter ($P < 0.0005$). The foveal thickness was significantly reduced at 1 week after the treatment ($P < 0.005$) and then continued to become significantly thinner with time ($P < 0.0001$). A slight recovery of the a- and b-wave amplitudes was seen postoperatively without a transient reduction in the amplitudes. The b-wave amplitude was significantly larger at 3 months after the treatment than at baseline ($P < 0.05$). Choroidal hypoperfusion did not develop 3 months postoperatively in the indocyanine green angiograms. Exudative changes recurred in 4 (27%) eyes after 1 year and in 9 (82%) eyes during the follow-up period. RFPDT provided short-term benefits in selected patients with PCV with small lesions. The macular function was retained after RFPDT without a transient decrease in visual function. Further study is needed to determine the long-term efficacy of RFPDT for eyes with PCV.

Keywords Photodynamic therapy · Reduced fluence · Electroretinogram (ERG) · Focal ERG · Polypoidal choroidal vasculopathy (PCV)

Introduction

Polypoidal choroidal vasculopathy (PCV) is characterized by an abnormal choroidal vascular network with polypoidal dilatations of the choroidal vessels [1, 2]. PCV is the most prevalent subtype of age-related macular degeneration (AMD) in Japan and other Asian countries [3–5].

Photodynamic therapy (PDT) has been shown to have therapeutic benefit in patients with PCV especially in Japan [6, 7]. PDT is highly recommended in cases with smaller lesions, and good visual outcomes have been obtained after PDT [8].

One of the unfavorable side effects of PDT is a dysfunction of the macula associated with

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hypoperfusion of the choroids [9]. Ishikawa et al. [10] evaluated the macular function by the focal macular electroretinograms (ERGs) after PDT in patients with AMD including those with PCV. The focal macular ERGs were decreased at 1 week and 1 month after PDT. However, the focal responses recovered to the baseline level after 3 months. They showed a significant correlation between the decrease in the focal macular ERGs and choroidal hypoperfusion that developed after PDT. Lai et al. also demonstrated a transient reduction in the amplitudes of the multifocal ERGs after PDT [11].

Michells et al. reported on the use of reduced fluence PDT (RFPDT) in which the irradiation energy or exposure time was reduced to one-half of the standard energy for PDT [12]. They reported that RFPDT closed choroidal neovascularization (CNV) in patients with age-related macular degeneration (AMD) without appearing to disturb the choroidal circulation. Yamashita et al. [13] treated patients with PCV by RFPDT and reported good anatomical and visual outcomes after 1 year with an average of 1.3 treatments/year.

The purpose of this study was to evaluate the macular function following RFPDT in Japanese patients with PCV by measuring the focal macular ERGs.

Methods

Subjects

Eleven eyes of 11 patients (9 men and 2 women) with PCV were studied. The patients were treated in the Retinal Unit of the Iwate Medical University Hospital, and their ages ranged from 62 to 85 years with a mean of 74.7 ± 6.9 years. The clinical characteristics of all patients are shown in Table 1. All patients received comprehensive ophthalmological examinations including measurement of the Snellen visual acuity, indirect ophthalmoscopy, and slit-lamp examination at every visit. In addition, color fundus photographs, fluorescein angiography (FAG), indocyanine green angiography (ICGA), and optical coherence tomography (OCT) were recorded. The foveal thickness was measured from the inner limiting membrane (ILM) to outer border of the RPE. When a retinal pigment epithelial detachment was present, we measured from the ILM to Bruch's membrane instead of the RPE. The follow-up periods ranged from 13 to 34 months with a mean of 26 ± 6.7 months.

The diagnosis of PCV was made based on the diagnostic criterion proposed by the Japanese research group of PCV [14]. Patients with subretinal elevated orange lesions or polypoidal structures in the ICGA images were defined as having PCV. The greatest linear dimension (GLD) of the polypoidal structures and vascular network was measured in all eyes. We included patients with unilateral PCV whose GLD of the vascular lesions was less than 4,000 μm but more than 2,000 μm. So most of the treated area overlapped the retinal area from which the focal macular ERGs were elicited (Fig. 1d). The GLD ranged from 2,340 to 3,975 μm with a mean of 2,886 ± 528 μm.

This research was conducted in accordance with the Institutional Guidelines of the Iwate Medical

Table 1 Characteristics of patients

Age	Gender	GLD	BCVA (logMAR)		Foveal thickness (μm)		Follow-up (months)
			Baseline	3 months	Baseline	3 months	
62	M	3,113	0.60	0.60	413	301	34
65	M	2,744	0.70	0.30	360	185	30
70	M	3,975	1.00	0.80	266	191	25
74	F	3,479	0.90	0.30	454	256	26
75	M	2,340	0.30	0.16	287	178	20
76	M	2,653	0.80	0.70	443	287	30
76	F	2,459	0.60	0.40	454	252	29
78	M	2,588	0.16	0.20	447	347	13
79	M	3,375	0.90	0.90	575	307	30
82	M	2,396	0.16	0.10	283	239	18
85	M	2,620	0.60	0.30	400	290	34

GLD greatest linear dimension, *BCVA* best-corrected visual acuity, *MAR* minimum angle of resolution, *M* male, *F* female
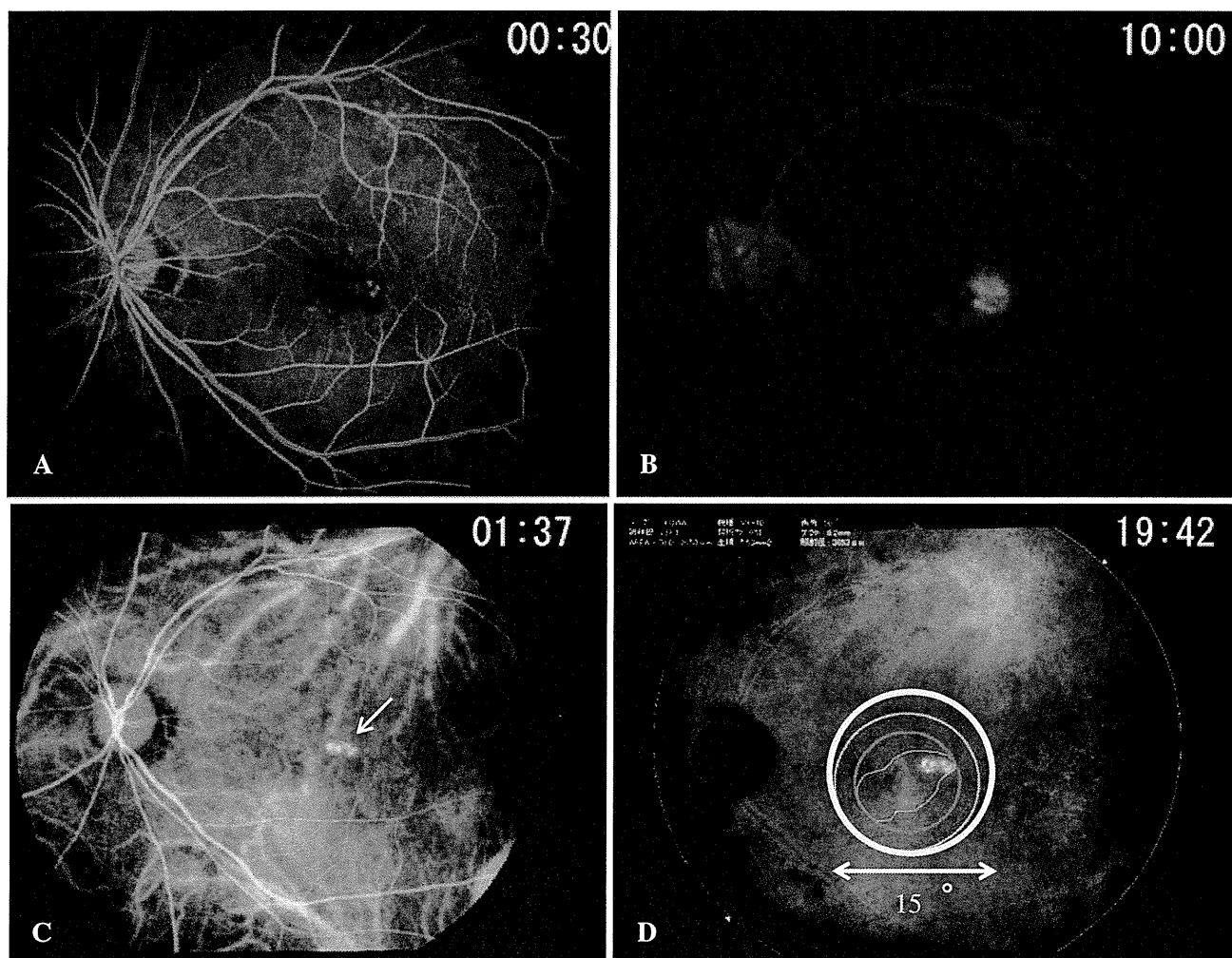
Fig. 1 Representative case of polypoidal choroidal vasculopathy. Early-phase fluorescein angiogram is shown in **a** and late phase in **b**. Indocyanine green angiograms at the early phase are shown in **c** and late phase in **d**. The *pink circle* has a diameter of the greatest linear dimension of the lesion (**d**). The *blue circle* represents the area treated by reduced fluence photodynamic therapy. The *white circle* represents the retinal area from which the focal macular electroretinograms were elicited

University, and the procedures conformed to the tenets of the Declaration of Helsinki. An informed consent was obtained from all subjects after a full explanation of the nature of the experiments.

Reduced fluence photodynamic therapy (RFPDT)

We performed a single RFPDT with veteporfin (Visdyne®) on all patients using standard parameters except for the exposure time. Briefly, the patients were given 6 mg of Visdyne®/meter^2 of body surface area in 5% glucose dilution intravenously over a period of 10 min. Fifteen minutes after the start of the infusion, the fundus was irradiated with a 689-nm diode laser at an intensity of 600 mW. The exposure time was reduced to 42 s, so that the total energy of the laser was approximately 25 J/cm^2 [12]. After the treatment, the patients were instructed to avoid sunlight and to wear sunglasses for 5 days.

Focal macular ERGs

Before the ERG recordings, the pupils were confirmed to be maximally dilated to approximately 8 mm diameter following a topical application of a mixture of 0.5% tropicamide and 0.5% phenylephrine HCL. The stimulus and observation system was integrated into an infrared fundus camera (Mayo Co., Nagoya, Japan). This system for recording focal macular ERGs under direct observation was developed by Miyake