Retrograde labeling of retinal ganglion cells (RGCs)

Seven days before the rabbits were killed, the optic nerves were exposed by lateral orbitotomy. Using a Hamilton syringe, 10 μl of a 0.1% fluorescent dye (Fast Blue; Polysciences, Warrington, PA, USA) was injected into the optic nerve 2 mm behind the eyeball. Care was taken not to injure any blood vessels, especially the ophthalmic artery that enters the sclera from the ventral margin of the optic nerve. After the surgery the eyes were treated topically with levofloxacin.

Assessment of RGC survival

Animals were killed by an overdose of ketamine and xylazine 1 week after the fast blue application. Whole, flat-mounted retinas were then assayed for the retinal ganglion cell density. The rabbit eyes were enucleated and fixed in 4% paraformaldehyde for 10 h at room temperature. After removal of the anterior segments, the resulting posterior eyecups were left in place. Subsequently, 4 radial cuts were made in the periphery of each eyecup, with the retina then carefully separated from the retinal pigment epithelium. To prepare the flat mounts, the retina was dissociated from the underlying structures, flattened by making 4 radial cuts, and then spread on a gelatin-coated glass slide. Labeled RGCs were visualized under a fluorescence microscope (Olympus BX-51/DP70; Olympus, Tokyo, Japan) by using a filter set (excitation filter 330–385 nm; barrier filter 420 nm; WU; Olympus). Fluorescence-labeled RGCs were counted in 12 microscopic fields of retinal tissue from 2 regions in each quadrant at 2 different eccentricities, central and peripheral. We counted the RGCs in each eye by using Image-Pro Plus software (Version 4.0; Media Cybernetics, The Imaging Expert, Bethesda, MD, USA). Cell counts were conducted by the same person in a masked fashion, with the identity of the original retinas unknown to the investigator until all cell counts from all the different groups were completed. Changes in the densities of the RGCs were expressed as the RGC survival percentage, which was based on comparison of the surgical and contralateral control eyes. The specimens that were compared came from different retinal regions of the same animal.

O_2^- analysis

O_2^- generation was determined by measuring the reduction of nitroblue tetrazolium (NBT) to a diformazan precipitate as previously described [10]. NBT (50 mg/ml; Research Organics, Cleveland, OH, USA) was dissolved in dimethylformamide (DMF) and distilled BSS. The final DMF concentration was 10%. Reduction was detected by intravitreal injection of 5-μl NBT solution in the period after vitrectomy before ischemia. For observation of the diformazan precipitate, a microscope (S5; Carl Zeiss, München, Germany) was used with a 3CCD digital camera (MKC-507; Ikegami Tsushinki, Tokyo, Japan) and recorded using a DVD recorder.

Statistical analysis

Image analysis was performed with Image-Pro Plus software (The Imaging Expert) to assess O_2^- generation area density. We determined the total blue-stained area indicated O_2^- generation area. Evaluation by Image-Pro Plus analysis of photographs was performed blind by two researchers not directly involved in this study and the generation area was automatically produced by the software. All statistical values are presented as mean ± standard deviation (SD). Data were analyzed by use of the unpaired Student's t test. Values of $p < 0.05$ were considered statistically significant.
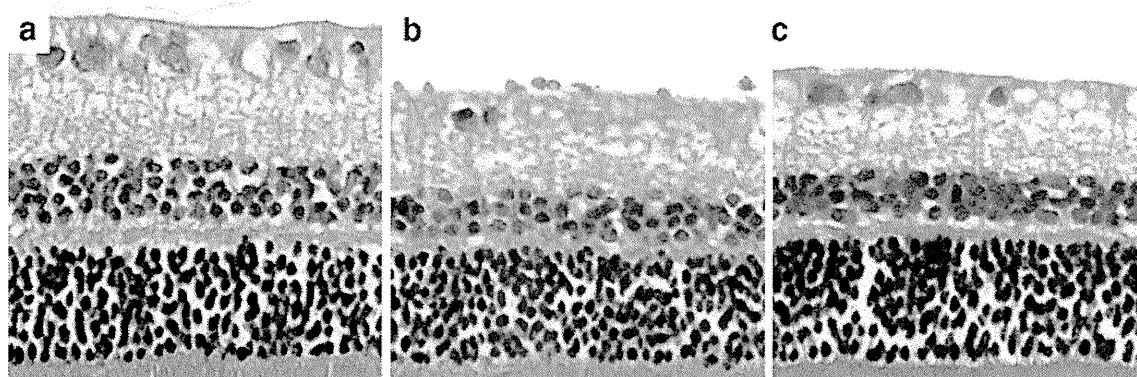

Fig. 1 Light micrographs of a cross-section of **a** hematoxylin and eosin histology through normal rabbit retina and **b** 7 days after ischemia with balanced salt solution (BSS) alone, or **c** BSS + D-allose. Cell loss in the ganglion cell layer (GCL) and reduced inner nuclear layer (INL) thickness were ameliorated in the D-allose group. *Bar* 10 μm
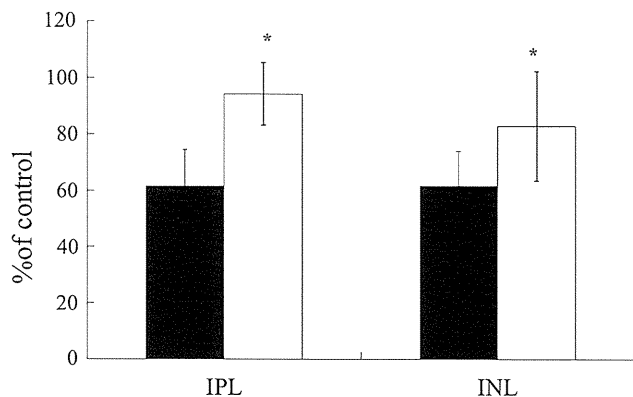
Fig. 2 Percentage changes relative to control values in the thicknesses of the IPL and INL 7 days after ischemia with BSS alone and BSS + D-allose. *Black bar* D-allose group, *white bar* vehicle group. Administration of 2% D-allose significantly prevented the reduction in the number of cells in the GCL and the thickness of the INL. Results are expressed as mean ± standard deviation (SD). *$P < 0.05$

Results

Histological change in the retina after ischemia with and without D-allose

Figure 1a shows a normal retina. Light-microscopic photographs were taken 7 days after ischemia (Fig. 1b, c). The retina of the untreated eye in the animals, was used as control. In the vehicle group, significant reductions in the thickness of the INL were observed. The thickness of the IPL was 61.6 ± 13.0% that of the control and the thickness of the INL was reduced to 61.8 ± 12.2% that of the control ($n = 6$; Fig. 2). In the D-allose group, the thickness of the IPL was 94.2 ± 11.1% that of the control and the thickness of the INL was 82.9 ± 19.4% that of the control ($n = 5$; Fig. 2). Reduced INL thickness was ameliorated in the D-allose group.

Fig. 3 Effects of D-allose on ischemia-induced retinal ganglion cell (RGC) death.
a Retrograde labeling of RGCs in nonischemic eyes, and
b 7 days after ischemic injury treated with vehicle or D-allose. *Bar* 100 μm. RGCs were counted in the central and peripheral areas. *Gray bar* D-allose group, *black bar* vehicle group. Graph depicts the mean ± SD for three rabbits treated with vehicle and three rabbits treated with D-allose. *$P < 0.05$
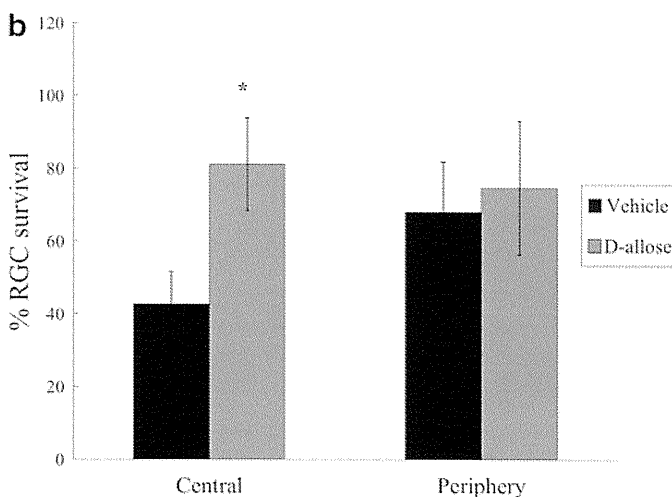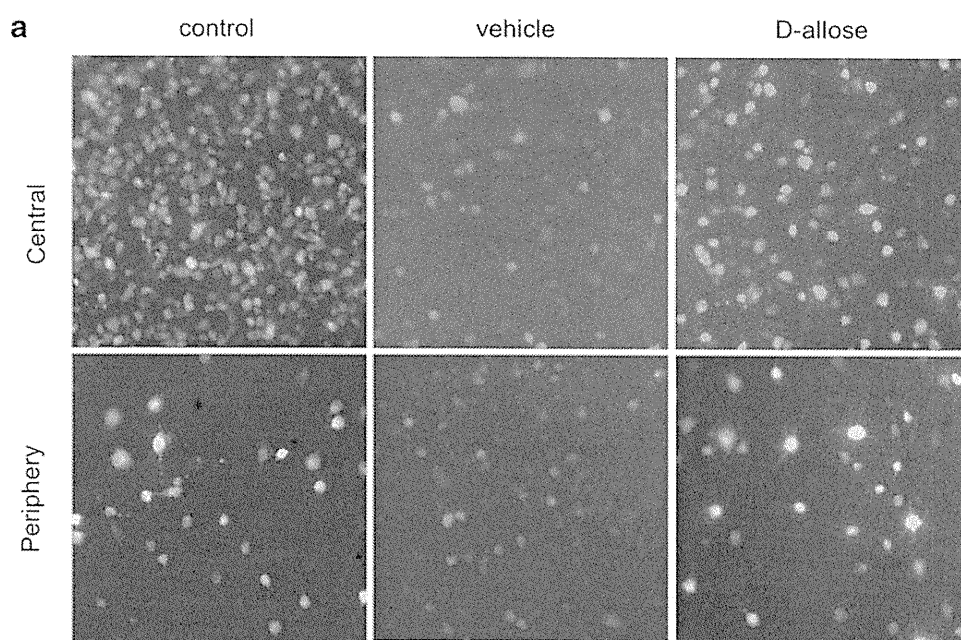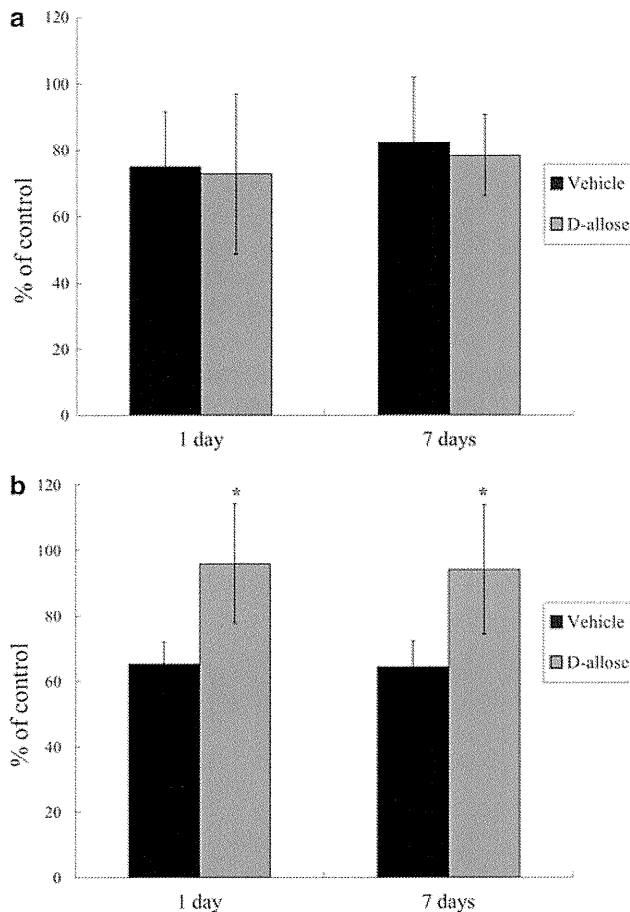
Fig. 4 a On postoperative day 7, the a-wave amplitude was 78.4 ± 12.3% in the D-allose group and 82.3 ± 19.6% in the vehicle group. *Gray bar* D-allose group, *black bar* vehicle group. b The b-wave amplitudes were 94.1 ± 19.8 and 64.3 ± 7.9%, respectively. On postoperative days 1 and 7, the mean amplitude of the b-wave for eyes treated with D-allose was significantly higher than for those treated with vehicle. *Gray bar* D-allose group, *black bar* vehicle group. Results are expressed as mean ± SD. *$P < 0.05$

Survival of RGCs

Figure 3a shows representative results of RGC labeling for both vehicle and D-allose-treated rabbits. RGC survival in the central retinas of the eyes with ischemia was 42.8 ± 8.8% in the vehicle-treated group ($n = 4$) and 81.2 ± 12.7% in the D-allose-treated group ($n = 4$, $P = 0.01$; Fig. 3b). In the peripheral retina, RGC survival in eyes with ischemia was 68.0 ± 13.8% in the vehicle-treated group and 74.7 ± 18.3% in the D-allose-treated group ($P = 0.64$; Fig. 3b).

ERGs

The mean amplitudes of both the a-wave and b-wave are shown in Fig. 4. On postoperative day 7, the a-wave amplitude was 78.4 ± 12.3% in the D-allose group ($n = 5$) and 82.3 ± 19.6% in the vehicle group ($n = 5$; Fig. 4a). The b-wave amplitudes were 94.1 ± 19.8 and 64.3 ± 7.9%, respectively (Fig. 4b). The mean amplitude of the b-wave in eyes treated with D-allose was significantly higher than in those treated with vehicle. Likewise, no significant differences in the mean amplitude of a-waves were identified between the D-allose and vehicle groups. Both a-wave and b-wave amplitudes in the non-operated eyes were stable and essentially equal both before and after surgery.

Effects of D-allose on released O_2^-

Light-microscopic photographs were taken after treatment without D-allose, i.e., BSS alone (Fig. 5a), and with D-allose (Fig. 5b). Without D-allose treatment, the blue color was observed after ischemia and became stronger over time. However, in the presence of D-allose, the blue color
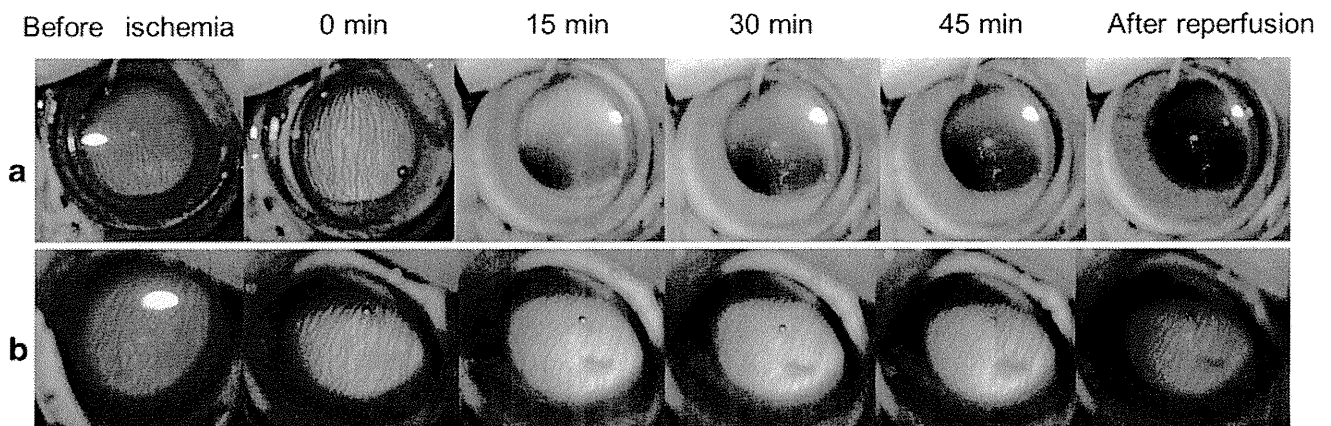

Fig. 5 Effects of D-allose on the release of superoxide anion (O_2^-). *Blue color* indicates release of O_2^-. Ischemia was induced for 45 min. Color photographs were taken before ischemia induction, then 0, 15, 30 and 45 min after starting ischemia, then immediately after reperfusion. a Vehicle group and b D-allose group
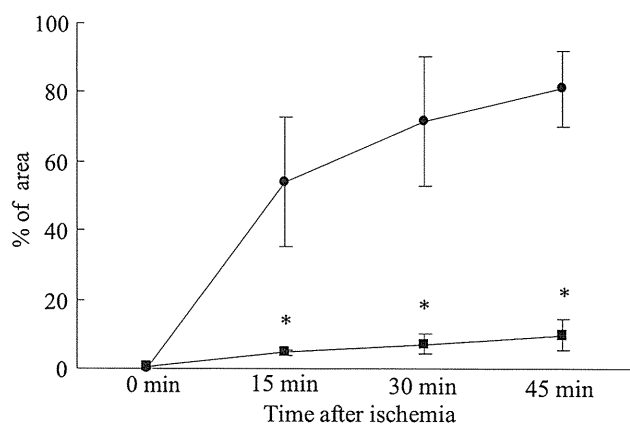
Fig. 6 Measured area O_2^- expression (%). *Filled squares* D-Allose group, *filled circles* vehicle group. Comparison of mean values of O_2^- expression between the vehicle group and the D-allose group at each time point. Mean O_2^- expression was significantly different 15, 30, and 45 min after ischemia. Data are mean ± SD. $*P < 0.05$

decreased compared with that without D-allose. Compared with the peripheral area, the blue color intensity was stronger in the central area. Figure 6 shows results from quantification of the color levels expressed as a percentage change. Mean O_2^- expression was significantly different between the vehicle group and the D-allose group 15, 30, and 45 min after ischemia.

Discussion

These findings show that intraocular irrigation with D-allose during vitrectomy protects the morphology and function of the retina against ischemia injury.

Because D-allose may inhibit hexose transport [11], co-injection of 200 mg/kg glucose with 200 mg/kg D-allose has been shown to have no protective effect against retinal ischemia reperfusion injury [3]. BSS contains 5.11 mM glucose; i.e., approximately 0.1% glucose. Because 2% D-allose in BSS was used in this study, the glucose in BSS was not sufficient to abolish the protective effects of D-allose.

Both clinically and under experimental conditions, the functional status of the retina can be monitored continuously by recording ERGs. The b-wave of the ERG has been identified as a particularly sensitive index of retinal ischemia both in humans [12] and in experimental models of retinal ischemia in vitro [13]. Glutamate acts as a mediator of neuronal injury under ischemic conditions [14] and extracellular glutamate has been found to increase in ischemic eyes [3, 15, 16]. Reperfusion injury is thought to be mediated in part by relative hyperglycemia and high oxygen levels, leading to oxygen radical formation. D-Allose may be a down-regulation agent of hexose transport [11]. D-Allose could reduce the production of ROS by modulating the glycolytic response. Because D-allose can

suppress glutamate release and the production of ROS after ischemia [3], D-allose protects both the morphology and function of the retina. Because there were no morphological changes in the outer retina in this study (data not shown), recovery of the a-wave amplitudes in the vehicle group was not suppressed. Therefore, we could not confirm any differences in the recovery of a-wave amplitudes between the vehicle and D-allose groups.

We recently reported that D-allose suppresses the production of H_2O_2 as determined by diaminobenzidine solution without hydrogen peroxide [3]. NBT is an electron acceptor that can be reduced by accepting electrons from various reductants, including superoxide [17] and other reductants, for example those generated from dehydrogenase systems [18, 19]. Studies have previously shown that NBT staining in normal rat retina is affected by inhibition of free radical-related enzyme systems, suggesting that NBT might be useful in the study of free radicals. During and after ischemic episodes, univalent reduction of oxygen in the mitochondrial respiratory chain is thought to be a major source of O_2^- [20]. O_2^- can be reduced to H_2O_2, a reaction catalyzed by superoxide dismutase (SOD) [7]. H_2O_2 has been identified as a potent inducer of apoptosis [21]. D-Allose may exert neuroprotective effects by reducing the production of not only H_2O_2, but also O_2^-.

Ocular perfusion pressure would be especially important in eyes with diabetes if, as has been suggested, autoregulation in the retina and optic nerve head is impaired [22, 23]. Because high infusion pressure during PPV is useful in preventing bleeding, we must also consider protecting the retina against damage caused by pressure-induced ischemia in cases of diabetic retinopathy [24]. Levels of glutamate potentially toxic to retinal ganglion cells have been found in the vitreous of patients with PDR [25]. This glutamate could then initiate a forward cascade of further neuronal ischemia.

Our findings suggest that intraocular irrigation with D-allose during vitrectomy may protect both the morphology and function of the retina against ischemia-induced damage.

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Morphologic and Functional Advantages of Macular Hole Surgery with Brilliant Blue G–Assisted Internal Limiting Membrane Peeling

Removal of the internal limiting membrane (ILM) is an effective additional treatment in macular hole (MH) surgery.[1-3] The transparency of the ILM requires high skill to peel the membrane. In 2000, a technique using indocyanine green (ICG) to stain and peel the ILM was reported.[4,5] However, some investigators[6,7] have reported retinal toxicity of the residual ICG. Other investigators have shown the toxicity of ICG to the retinal pigment epithelium in vitro[8-10] and in vivo.[11-13] These reports indicate that surgeons have to be very careful not to allow ICG to remain subretinally at the end of MH surgery because it can cause postoperative complications such as retinal pigment epithelial changes[14] and subsequent visual field loss.[15,16]

In 2006, Enaida et al[17] initially reported that brilliant blue G (BBG) stains the ILM while having low retinal toxicity in their morphologic study using electron microscopy. In rapid succession, they also reported the clinical possibility of using BBG for ILM staining and peeling in MH and epiretinal membrane cases with no adverse events.[18] Compared with ICG, the toxicity of BBG to cultured retinal ganglion cells was significantly lower based on evaluation of retinal ganglion cell apoptosis.[19] Ueno et al[20] injected ICG and BBG in clinical concentrations into the subretinal space of rats. They found that ICG caused retinal degeneration and retinal pigment epithelium cell atrophy, while BBG had no detectable toxic effects. After confirmation of the safety of BBG, Cervera et al[21,22] reported their experience with ILM peeling using BBG and concluded that dyeing with BBG appeared to be an interesting alternative to ICG.

Much improvement in the resolution of optical coherence tomography has enabled us to observe microstructure of the macula in MHs before and after surgery. Recent studies have revealed the correlation between visual recovery and the presence of the inner and outer segments of the photoreceptor (IS/OS) junction after MH surgery.[23,24] The IS/OS junction can be observed in the normal eye as the continuous line located in the outer retina. Another investigator has reported the importance of the external limiting membrane (ELM) compared with the IS/OS in visual recovery after MH surgery.[25] Thus, continuity of the IS/OS junction and the ELM has been well known as an important factor for postoperative recovery of visual acuity. In the present study, the results, including macular microstructure and visual acuity, of MH surgery using BBG and ICG were compared.

Materials and Methods

This was a nonrandomized, retrospective, interventional case series. Between September 2007 and April 2009, 53 eyes of 53 consecutive patients with idiopathic full-thickness MH underwent MH surgery with ILM peeling using ICG (n = 22) (between September 2007 and August 2008) or BBG (n = 31) (between September 2008 and April 2009) at Kagawa University Hospital. In all patients, the surgery was performed as soon as possible after an initial visit to our hospital. Best-corrected visual acuity (BCVA), optical coherence tomography examination using spectral-domain optical coherence tomography (Carl Zeiss Meditec, Inc, Dublin, CA), and slit-lamp fundus examinations using a 78-diopter lens were performed before and 1, 3, and 6 months after surgery. Optical coherence tomography reading was performed by one of the authors (F.S.) in a masked fashion without knowledge of the staining dye used in ILM peeling or the surgical outcomes. The optical coherence tomography reader evaluated the ELM or IS/OS junction as reconstructed or restored when continuity of the ELM or the IS/OS line was observed at the fovea after

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The authors report no conflicts of interest.

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surgery. The presence or absence of continuity of the ELM or IS/OS line could be clearly determined (Figures 1 and 2).

All cases underwent 25-gauge, transconjunctival, sutureless vitrectomy. Cataract surgery was performed simultaneously in patients aged 50 years. After posterior vitreous detachment creation in eyes with Stage 2 or 3 holes and removal of the posterior hyaloid, 0.125% ICG or 0.25 mg/mL of BBG was sprayed onto the posterior retina around the MHs. The ICG solution (Ophthagreen, Santen Pharmaceutical Co Ltd, Osaka, Japan) was prepared at a concentration of 0.125% using dilution in BSS plus (Alcon Lab, Fort Worth, TX). The BBG solution (Coomassie BBG 250; Sigma–Aldrich, St. Louis, MO) was prepared at a concentration of 0.25 mg/mL using dilution in BSS plus. Three surgeons (K.F., F.S., and H.Y.) performed the MH surgeries using ICG (between September 2007 and August 2008) or BBG (between September 2008 and April 2009).

Immediately after the injection of both dyes, the dye solution in the vitreous cavity was aspirated using a vitreous cutter. The ILM was incised using a 25-gauge microvitreoretinal blade and carefully peeled from the underlying retina in a circumferential manner within about a 1.5-disk diameter radius around an MH, using a microforceps. If stain solution remaining within MHs was observed, it was aspirated with a soft-tipped needle. An air–fluid exchange was performed, and 20% sulfur hexafluoride was infused. Strict face-down positioning was maintained for 3 days after surgery. This study was approved by the Institutional Review Board of Kagawa University Faculty of Medicine.
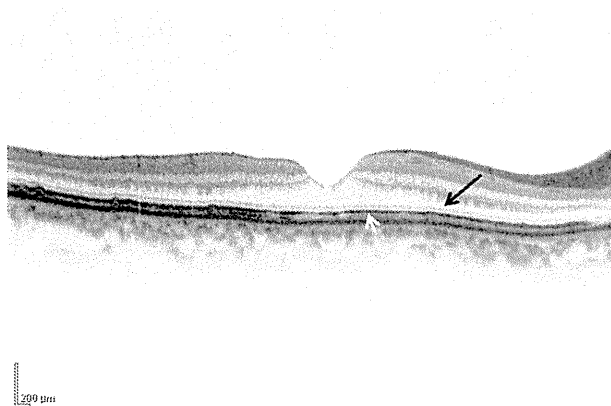

Fig. 1. The reconstructed ELM line (a large arrow) and the restored IS/OS junction line (a small arrow) are clearly observed at the fovea 1 month after surgery.
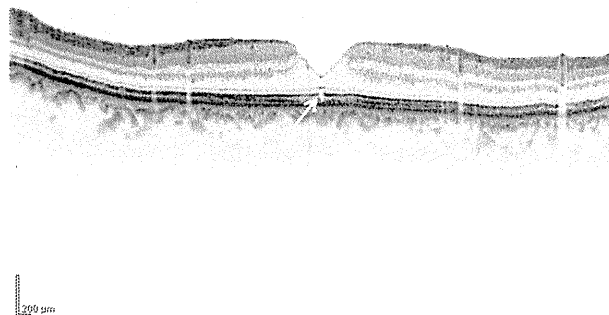

Fig. 2. The lack of continuity of the IS/OS junction line (an arrow) is observed. The IS/OS junction is not restored in this case.

Results

Baseline Characteristics

Baseline characteristics for all patients are shown in Table 1. The BBG group included 31 eyes of 31 patients (14 men and 17 women). Median age at the time of surgery was 67 years (range, 56–80 years). Stage 2, 3, and 4 MHs were present in 14, 13, and 4 eyes, respectively. Preoperative mean BCVA ± SD was 0.61 ± 0.29 logarithm of the minimal angle of resolution (logMAR). The ICG group included 22 eyes of 22 patients (12 men and 10 women). Median age at time of surgery was 68 years (range, 54–79 years). Stage 2, 3, and 4 MHs were present in 10, 8, and 4 eyes, respectively. Preoperative mean BCVA ± SD was 0.59 ± 0.27 logMAR. No significant differences were noted between the groups in age ($P = 0.59$, Mann–Whitney U test), sex ($P = 0.18$, Fisher exact probability test), disease duration ($P = 0.98$, Mann–Whitney U test), stage of MHs ($P = 0.84$, chi-square test), and preoperative mean logMAR visual acuity ($P = 0.77$, unpaired t-test).

Best-Corrected Visual Acuity Results and Macular Hole Closure Rates

Best-corrected visual acuity results and MH closure at 6 months after surgery are shown in Tables 2 and 3. In both BBG and ICG groups, the MH was successfully closed in all cases at 6 months postoperatively. Table 3 shows the visual results after surgery. In the BBG group, the mean BCVA ± SD improved significantly from 0.61 ± 0.29 logMAR preoperatively to 0.10 ± 0.20 logMAR at 6 months postoperatively ($P < 0.001$, paired t-test). Best-corrected visual acuity improved by ≥0.3 logMAR in 27 eyes (87%) and stabilized in 4 eyes (13%) at 6 months after surgery. Best-corrected visual acuity was 20/20 or better at 6 months after surgery in 20 of 31 eyes

Table 1. Baseline Characteristics

	BBG Group (n = 31)	ICG Group (n = 22)	P
Patient age (years)			
Median	67	68	0.59, Mann–Whitney U test
Range	56–80	54–79	
Disease duration (months)			
Median	3	3	0.98, Mann–Whitney U test
Range	1–8	1–8	
Stage (n)			
2	14	10	0.84, chi-square test
3	13	8	
4	4	4	
BCVA at baseline, logMAR	0.61 ± 0.29	0.59 ± 0.27	0.77, paired t-test

(65%). In the ICG group, the mean BCVA ± SD improved significantly from 0.59 ± 0.27 logMAR preoperatively to 0.14 ± 0.17 logMAR at 6 months postoperatively ($P < 0.001$, paired t-test). Best-corrected visual acuity improved by ≥0.3 logMAR in 18 eyes (82%) and stabilized in 4 eyes (18%) at 6 months after surgery. Best-corrected visual acuity was 20/20 or better at 6 months after surgery in 7 of 22 eyes (32%). No significant differences between the 2 groups were seen in mean BCVA ($P = 0.39$, unpaired t-test) and change in BCVA by ≥0.3 logMAR ($P = 0.71$, chi-square test) at 6 months after surgery. However, for a BCVA of 20/20 or better, the BBG group showed a significantly higher rate than the ICG group ($P = 0.03$, Fisher exact probability test). Figure 3 shows the changes in logMAR visual acuity of both groups.

Microstructural Results After Macular Hole Surgery

The ELM reconstruction rates at 1, 3, and 6 months after surgery were 65%, 90%, and 94%, respectively, in the BBG group and 68%, 91%, and 100%, respectively, in the ICG group (Table 2, Figure 4). The rates of IS/OS junction restoration at 1, 3, and 6 months after surgery were 32%, 61%, and 87%, respectively, in the BBG group, compared with 5%, 50%, and 91%, respectively, in the ICG group (Table 2,

Figure 5). A significant difference in the rate of IS/OS junction restoration at 1 month after surgery was found between the 2 groups ($P = 0.02$, Fisher exact probability test; Table 2, Figure 5).

Proportion of Simultaneous Cataract Surgery and Adverse Effects

In the BBG group, 24 eyes (excluding 4 eyes that were pseudophakic before surgery) underwent combined phacoemulsification and posterior chamber intraocular lens implantation, because progression of nuclear sclerotic cataracts is not preventable in patients >50 years of age. In the 3 eyes without combined cataract surgery, cataract surgery was not performed after vitrectomy, and 28 eyes (90%) were pseudophakic at 6 months after surgery. For the 22 eyes in the ICG group, because 2 eyes were pseudophakic preoperatively and cataract surgery was combined in 18 eyes, 20 eyes (90%) were pseudophakic at 6 months after surgery. No significant difference in the proportion of pseudophakic eyes at 6 months after surgery between the 2 groups was identified.

No significant adverse effects related to either dye were observed in the present study. In both groups, neither retinal detachment nor MH reopening was observed.

Table 2. Best-Corrected Visual Acuity Results and MH Closure at 6 Months After Surgery

	BBG Group (n = 31)	ICG Group (n = 22)	P
Anatomical results			
MH closure, n (%)	31 (100)	22 (100)	—
Recovery of ELM line, n (%)			
At 1 month	20 (65)	15 (68)	1.00, Fisher exact probability test
At 3 months	28 (90)	20 (91)	1.00, Fisher exact probability test
At 6 months	29 (94)	22 (100)	0.51, Fisher exact probability test
Recovery of IS/OS line, n (%)			
At 1 month	10 (32)	1 (5)	0.02, Fisher exact probability test
At 3 months	19 (61)	11 (50)	0.57, Fisher exact probability test
At 6 months	27 (87)	20 (91)	1.00, Fisher exact probability test

Table 3. Visual Results After Surgery

	BBG Group (n = 31)	ICG Group (n = 22)	P
Visual results			
Mean BCVA at 6 months, logMAR	0.10 ± 0.20	0.14 ± 0.17	0.39, paired t-test
Changes in BCVA ≥0.3 logMAR, n (%)			
Improved	27 (87)	18 (82)	0.71, chi-square test
Stable	4 (13)	4 (18)	
Worsened	0 (0)	0 (0)	
Eyes with BCVA of 20/20 or better	20/31 (65)	7/22 (32)	0.03, Fisher exact probability test

Discussion

Indocyanine green is the first adjuvant clinically used to stain the ILM.[4] This procedure of staining the ILM has spread quickly and is still now performed by vitreoretinal surgeons around the world. However, several reports[8–10] have noted the retinal toxicity of ICG. Alternative stains have been tried to stain the ILM, such as infracyanine green, trypan blue, Patent blue, Bromophenol blue, and BBG.[17,26–29] Of these stains, BBG shows a high ability to stain the ILM and, more importantly, a low possibility of cytotoxicity.[17–20]

Internal limiting membrane peeling procedures with any stains have achieved almost 100% postoperative MH closure rates. In a previous report,[25] the ELM reconstruction rate was 80% at 3 months postoperatively. In the present study, almost 90% of ELMs were reconstructed at 3 months in both groups, and almost 100% of ELMs were reconstructed at 6 months

postoperatively. There were no significant differences between the BBG and ICG groups in the ELM reconstruction rates. The rate of IS/OS junction restoration has been reported as 4% at 1 month after surgery.[30] The dye they used in their operation for ILM peeling was 0.25% ICG, and their result was very close to the results of the present study's ICG group. As with ELM reconstruction, the rates of IS/OS junction restoration increased to almost 90% with time. At 1 month postoperatively, the IS/OS junction had restored in 32% of the BBG group and 5% of the ICG group; the difference was significant ($P = 0.02$; Fisher exact probability test). Because of the features of MHs, direct exposure of the bare retinal pigment epithelium and retina inside MHs to dyes is unavoidable. Because ICG injected into the subretinal space induces retinal cell degeneration,[20] this lag in restoration might be reasonable. The postoperative microstructural change of the IS/OS junction in the present
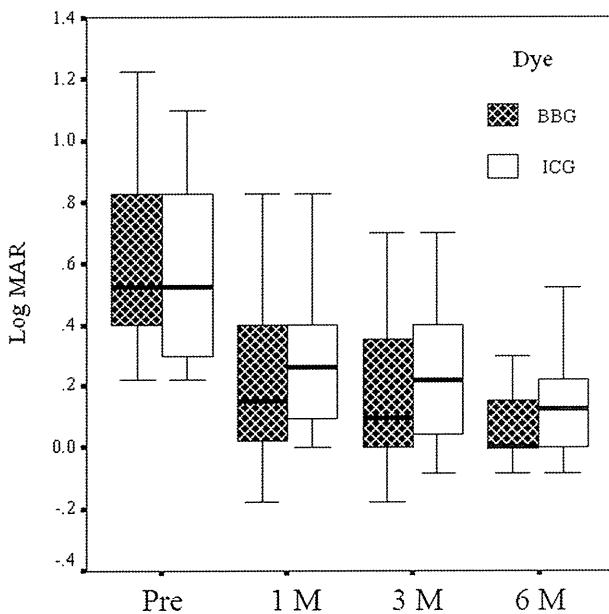

Fig. 3. Preoperative and postoperative BCVA within 6 months after MH surgery in both groups. There are no significant differences in mean logMAR BCVA between the two groups at any visit.
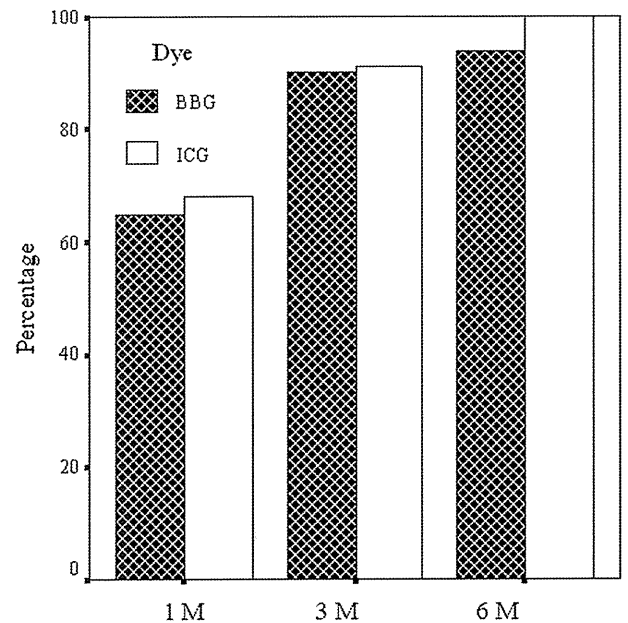

Fig. 4. External limiting membrane reconstruction rates at 1, 3, and 6 months after surgery. There are no significant differences in the rates between the two groups at any visit.
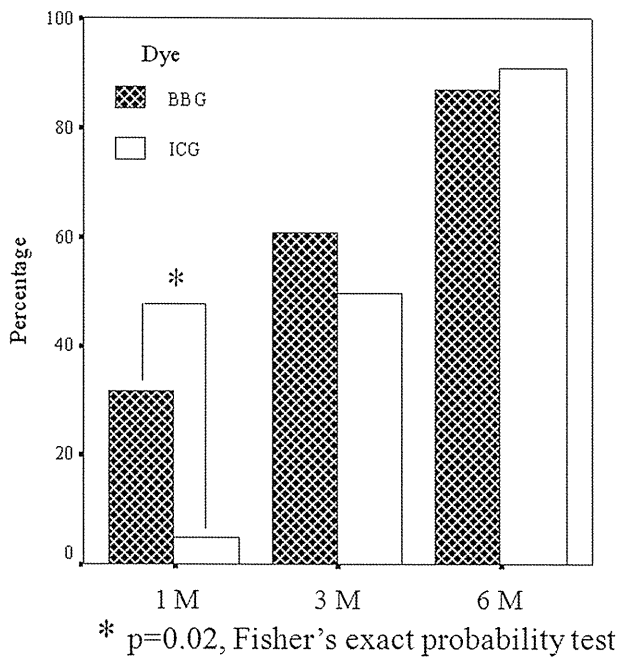
* p=0.02, Fisher's exact probability test

Fig. 5. The rates of IS/OS junction restoration at 1, 3, and 6 months after surgery. There are no significant differences in the rates between the 2 groups at 3 and 6 months postoperatively. The rate at 1 month after surgery is significantly higher in the BBG group than in the ICG group ($P = 0.02$; Fisher exact probability test).

study seems to indicate that BBG is more useful than ICG, though the mechanism of the restoration of the IS/OS junction is not well known.

Overall changes in BCVA in both groups were similar to those in previous reports about MH surgery. There were no significant differences between the two groups at any time points postoperatively. Although the lag in visual acuity improvement as expected by the morphologic restoration lag observed in the ICG group was not found in the current study, the rate of visual acuity of 20/20 or better at the final visit was significantly higher in the BBG group than that in the ICG group. This fact indicates that a restored IS/OS line, which indicates the presence of photoreceptors, may not work well at an early stage of restoration, so that the visual results at the 1-month visit did not show any significant difference between the 2 groups. Because the BBG group showed a significantly higher rate than the ICG group for a BCVA of 20/20 or better at the final visit, early restoration of the IS/OS junction can be important for the long-term visual outcome.

In contrast with earlier reports[19,20] confirming the safety of BBG, Yuen et al[31] reported the toxicity of BBG in an in vitro study. They evaluated the toxicity of several dyes including BBG and ICG using a human retinal pigment epithelial cell line (ARPE-19) and a murine retinal ganglion/Muller glial cell primary cell

culture. A viability assay of ARPE-19 cells after 30 minutes of exposure to 4 different concentrations (10, 2.5, 0.25, and 0.125 mg/mL) of BBG was used. Every concentration of BBG resulted in a significantly lower viability than control, though every concentration (1, 0.5, 0.25, and 0.125 mg/ml) of ICG showed absolutely no toxicity in exactly the same study. As they noted in their report, 30-minute exposure is unlikely to occur in regular MH surgery, but it could occur in cases of MH with retinal detachment. In contrast, the influence of 0.25 mg/mL of BBG on cultured retinal ganglion cells was negligibly small after 30 minutes of exposure, and this result agrees with a similar previous report,[19] though the predetermined exposure time was shorter. Yuen et al[31] also studied a short exposure time of 3 minutes, and both dyes showed no toxicity in the concentrations used in the current study. All the cases in which we performed MH surgery with 0.25 mg/mL of BBG in the current study did not develop any adverse effects, such as retinal pigment epithelium atrophy inside MHs or retinal degeneration around MHs. Because a high dye concentration facilitates apoptosis of cultured retinal pigment epithelium within 72 hours, we infer that the 6-month observation period of the current study is long enough to conclude that 0.25 mg/mL of BBG has no toxicity.

In conclusion, BBG is useful as an adjuvant for easy ILM peeling in MH surgery. No apparent retinal toxicity was observed in both the ICG and BBG groups. The early restoration of the IS/OS junction observed in the BBG group seems important for a better long-term visual outcome. Further clinical investigations focused on the early restoration of the IS/OS junction observed in the BBG group are needed.

Key words: brilliant blue G, indocyanine green, internal limiting membrane, macular hole, vitrectomy, spectral-domain optical coherence tomography.

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Efficacy and Safety of Switching from Topical Latanoprost to Bimatoprost in Patients with Normal-Tension Glaucoma

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Abstract

Purpose: The aim of this study was to evaluate the efficacy and safety of bimatoprost in Japanese patients with normal-tension glaucoma (NTG) who showed insufficient response to latanoprost.

Methods: A prospective, nonrandomized study was conducted in patients with NTG, with ≤20% intraocular pressure (IOP) decrease from pretreatment baseline with latanoprost monotherapy who had been switched to bimatoprost. The IOP was measured at 4, 8, and 12 weeks after the switch to bimatoprost. In 12 weeks after the switch to bimatoprost, efficacy and safety were evaluated.

Results: Postswitch to bimatoprost, IOP was significantly reduced at every visit. Bimatoprost produced significantly greater mean% IOP reduction rate from pretreatment than that of latanoprost at week 12 ($P < 0.01$). There was a significant correlation between% IOP reduction of bimatoprost and that of latanoprost (Pearson $r^2 = 0.374$; $P = 0.007$). No significant difference was observed in the mean scores of conjunctival hyperemia and corneal epithelial disorder between bimatoprost-treated eyes and latanoprost-treated eyes.

Conclusions: Significant additional IOP lowering was achieved by switching to bimatoprost in Japanese patients with NTG with insufficient response to latanoprost. Bimatoprost treatment was safe and well tolerated.

Introduction

PROSTAGLANDIN ANALOGS have gained widespread clinical use for treatment of glaucoma because of their efficacy at lowering intraocular pressure (IOP).[1–3] Latanoprost is a prodrug of the naturally occurring prostaglandin (PG) $F_{2\alpha}$ and is endowed with a strong IOP-reducing effect.[4–6] Bimatoprost is an analog of $PGF_{2\alpha}$-1-ethanolamide (prostamide $F_{2\alpha}$). Prostamides are derived from an endocannabinoid anandamide by COX-2,[7] and have pharmacological and biochemical properties distinct from PG $F_{2\alpha}$.[7,8] Similar to $PGF_{2\alpha}$ analogs, the IOP lowering mechanism of bimatoprost is likely to be attributed to the increase in uveoscleral outflow,[9] which is associated with extracellular matrix remodeling.[10] In addition, in subjects with ocular hypertension (OH) and glaucoma, the increase of both the pressure-sensitive (trabecular) outflow and the pressure-insensitive (uveoscleral) outflow by bimatoprost could be ascribed to the changes in the trabecular meshwork or in the sclera, or both.[9,11–13] Although the pharmacological mechanisms of actions of latanoprost and bimatoprost have been thought to be similar, there is a possibility that with patients for whom 1 agent is neither fully effective nor tolerable, another agent may be useful.[14]

The Tajimi study, which is one of the largest glaucoma epidemiology studies in Japan, showed that the glaucoma prevalence rate in Japanese older than 40 years of age is 5.0%, and the rate of open-angle glaucoma is 3.9%.[15,16] The study also reported that almost 90% of the open-angle glaucoma consisted of normal-tension glaucoma (NTG). The NTG is a clinical entity characterized by glaucomatous optic nerve damage and visual field defects with an IOP in the statistically normal range. The IOP is, however, a part of the pathogenic process in NTG, and IOP lowering is effective in reducing the progression of glaucomatous damage.[17] Although latanoprost is commonly used as first-line therapy in the treatment of NTG, there are some cases that show insufficient response to latanoprost.[18]

The purpose of this study was to evaluate the efficacy and safety of bimatoprost in eyes with insufficient response to latanoprost in Japanese patients with NTG.

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Methods

This clinical trial was conducted at the following 3 investigational sites. December 2009 to December 2010: Department of Ophthalmology, Kagawa University Faculty of Medicine, Social Insurance Ritsurin Hospital, and Ueda Eye Clinic (Kagawa, Japan). All the aspects of the study were in compliance with the Declaration of Helsinki, and all the patients gave their consent on being sufficiently informed by an investigator.

Examinations of visual acuity, refraction, both central and peripheral fields, slit-lamp examination, and gonioscopy were performed on all the patients. The eligibility criteria were age ≥ 20 years; bilateral or unilateral NTG: glaucomatous optic disc abnormalities and corresponding glaucomatous visual field defects, normal open angle, and IOP (measured using Goldmann applanation tonometer) of 21 mmHg or lower without medication; $\leq 20\%$ IOP decrease from pretreatment baseline at least 12 weeks of treatment with latanoprost 0.005% (Xalatan[®]; Pfizer, New York, NY) monotherapy. Exclusion criteria were the subjects being with active ocular diseases in either eye except glaucoma; with retinal disease that has a potential risk of progression; with experience of ocular surgery or lazer treatment; with regimen for systemic or local administration of steroid during this study; with corneal disease in either eye that poses a problem for veracious IOP measurement.

A total of 18 patients who fit the study criteria were enrolled in this study. The study consisted of 4 scheduled visits over 12 weeks (day 0 and weeks 4, 8, and 12). At day 0 (preswitch), eligible patients who had used latanoprost 0.005% were switched to bimatoprost 0.03% (Lumigan[®]; Allergan, Inc., Irvine, CA) treatment. The administration time of bimatoprost had been set to just around the same time before administration of latanoprost.

Measurements of IOP, best-correlated visual acuity, and biomicroscopic examinations were conducted at each visit. The IOP was measured at the same time period during the administration of latanoprost with Goldmann applanation tonometer by using the same procedure at all centers. The outcome due to primary efficacy was the main change in IOP at week 12 from preswitch.

Biomicroscopy was performed by using a slit-lamp examination without pupil dilation. The examination included an assessment of the lid/lashes, conjunctiva, anterior chamber, cornea, iris, and lens. Conjunctival hyperemia was assessed by a single observer by using a 5-point hyperemia grading scale using 5 different photographs for hyperemia matching: 0 = none, 0.5 = trace, 1 = mild, 2 = moderate, and 3 = severe. Corneal epithelial disorders were recorded by using an A (area) D (density) grading scale by a slit-lamp examination.[19]

The study outcome for efficacy was based on the conditions of the patients' eyes with the higher IOP at the eligibility visit. If IOP was same in both eyes, we analyzed the right eye. Descriptive statistics for mean IOP, mean IOP change, and% IOP change from pretreatment were calculated. Statistical significance was assessed by using paired t test. The degrees of conjunctival hyperemia and corneal epithelial disorder were analyzed by using an averaged score of both eyes' values. Evaluation of the degrees of conjunctival hyperemia and corneal epithelial disorder was analyzed by using a Wilcoxon signed-rank test. The correlation mean%
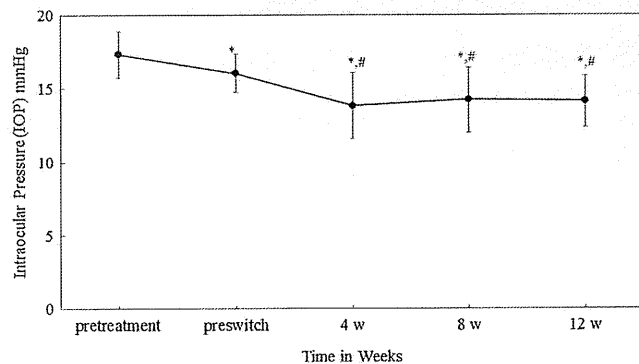

FIG. 1. Reduction in mean IOP after a switch to bimatoprost. Data express the mean $\pm$ SD. $*P < 0.05$ versus pretreatment (paired t test). $^{\#}P < 0.05$ versus preswitch (paired t test). IOP, intraocular pressure; SD, standard deviation.

IOP change from pretreatment between eyes treated with latanoprost and eyes treated with bimatoprost was analyzed by using a Pearson's correlation coefficient test. All the statistical analyses were performed by using SPSS for Windows, Version 11.5 (SPSS, Inc., Chicago, IL). A P value of 0.05 or less was considered statistically significant. Data are presented as mean $\pm$ standard deviation.

Results

There were 4 men and 14 women (mean age, 68.2 ± 15.3 years), who had the mean refractive error of -2.3 ± 4.9 diopters. All subjects completed the study. No significant changes in visual acuity were detected throughout follow-up (data not shown).

The IOP data were as follows: pretreatment = 17.3 ± 1.6 mm Hg; preswitch = 16.0 ± 1.3 mm Hg; 12 weeks = 14.1 ± 1.7 mm Hg. At week 12, IOP was significantly lower than both the pretreatment IOP ($P < 0.01$) and the preswitch IOP ($P < 0.01$) (Fig. 1). Although the mean% IOP reduction from pretreatment to preswitch (latanoprost) was $-7.5\% \pm 5.6\%$, the mean% IOP reduction from pretreatment to 12 weeks (bimatoprost) was $-18.7\% \pm 8.9\%$ (Fig. 2). At week 12, 7 patients showed $\geq 20\%$ IOP decrease, and 2 patients showed $\geq 30\%$ IOP decrease from pretreatment (Fig. 3). There was a
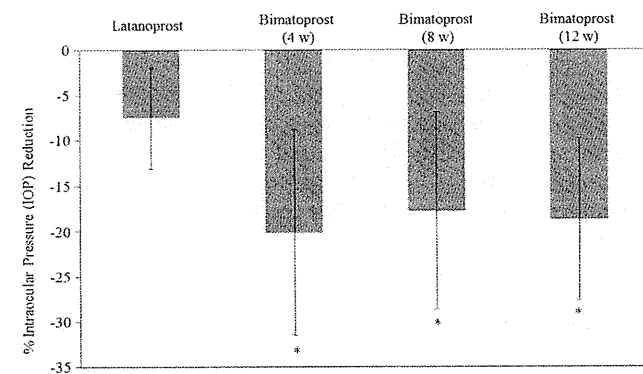

FIG. 2. Mean% IOP reduction from pretreatment to preswitch (Latanoprost) and at week 4, 8, and 12 (Bimatoprost). The mean% IOP reduction rate of bimatoprost was significantly greater than that of latanoprost ($*P < 0.01$, paired t test). Data express the mean $\pm$ SD.
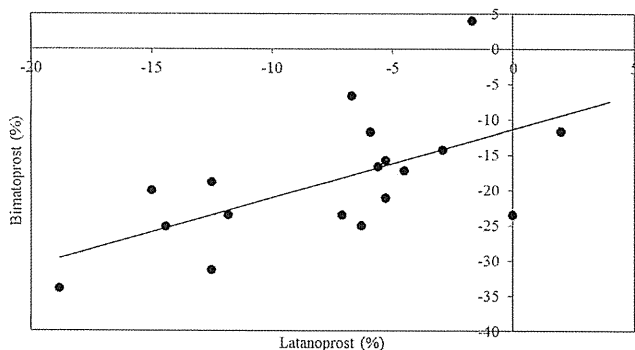
FIG. 3. Correlation between% IOP reduction rate of bimatoprost and latanoprost. % IOP reduction rate of bimatoprost was significantly correlated with that of latanoprost. (Pearson correlation coefficient $R^2 = 0.374$; $P = 0.007$).

significant correlation between% IOP reduction of bimatoprost and that of latanoprost (Pearson correlation coefficient $r^2 = 0.374$; $P < 0.01$) (Fig. 3).

The mean hyperemia scores at preswitch and week 12 were 0.31 ± 0.35 and 0.56 ± 0.54 ($P = 0.27$), respectively (Table 1). The mean corneal epithelial disorder scores at preswitch and week 12 were 0.67 ± 0.97 and 0.67 ± 0.97 ($P > 0.99$), respectively (Table 1).

Discussion

The incidence rate of latanoprost nonresponders is reported at 28.1% and was highest in patients with NTG in Japan.[18] The reduction of IOP in patients with lower baseline IOP may be more difficult.[20] Several studies revealed that the IOP-lowering effect of bimatoprost was even equal to or higher than that of latanoprost.[2,11–14]

Bimatoprost was difficult to be converted to its free acid form in human eyes, and free acid was slightly detected at the site of action in the eye.[8,21,22] In contrast, latanoprost is a prodrug that needs de-esterification to yield a pharmacologically active free fatty acid. Due to this, the pharmacological effect of bimatoprost is difficult to be attenuated because of its metabolism compared with latanoprost. Gandolfi and Cimino[14] previously reported that most of the subjects with glaucoma or OH who showed no significant IOP response to latanoprost were successfully treated with bimatoprost. They speculated that the lack of response to latanoprost was associated with poor de-esterification of the prodrug to the pharmacologically active free fatty acid. In early studies, the additional IOP-lowering effect of bimotoprost was seen in patients who responded poorly to latanoprost, thus suggesting a superior IOP-lowering effect of bimatoprost, compared with latanoprost.[14,23] Mean IOP be-

fore the switch to bimatoprost, however, was approximately 23 mm Hg in their study.[14,23] Our study suggests that the decrease of IOP also occurred with switching to bimatoprost in Japanese patients who are insufficient responders to latanoprost even though pretreatment IOP is low.

In this study, we showed that the mean IOP reduction rate of bimatoprost was significantly correlated with the mean IOP reduction rate of latanoprost. Bimatoprost showed a trend to enhance the potency of latanoprost. Prostaglandin $F_{2\alpha}$ (FP) prostanoid receptors are G-protein coupled receptors that mediate the actions of PG $F_{2\alpha}$, which is confirmed to be an alternative splice variant of the human FP (altFP) prostanoid receptor gene.[24] Since bimatoprost interacts not with PG FP receptor but with prostamide receptor, bimatoprost is likely to have a pharmacologically inherent receptor.[7,8] It has been reported that bimatoprost may interact with the FP-altFP receptor heterodimer to induce alterations in second-messenger signaling.[25] FP-altFP complexes may represent the underlying basis of bimatoprost pharmacology.[25] Since prostamide and FP receptors may be encoded by the same gene, the lowering effects on IOP of bimatoprost might correlate with those of latanoprost.

Conjunctival hyperemia was the most commonly reported side effect of bimatoprost and the most frequently observed biomicroscopic finding in several studies.[2,3,14] Conjunctival hyperemia occurs more frequently with bimatoprost than with latanoprost.[26] There were, however, no significant differences in the mean score of conjunctival hyperemia between bimatoprost-treated eyes and latanoprost-treated eyes in this study. The switch from latanoprost to bimatoprost in the glaucoma therapy was associated with less conjunctival hyperemia than that measured in patients in whom bimatoprost was used as first-line therapy.[27] One of the limitations of this study is that there was no control group. There is the possibility that some patients who had conjunctival hyperemia caused by latanoprost may continue after withdrawal. Ocular surface hyperemia occurs by endothelial-derived nitric oxide-mediated vasodilatation and is not associated with intraocular inflammation.[28] Even though there is a trend toward exacerbation during the switching phase, no patients withdrew from the treatment. In addition, the deepening of eyelid sulcus due to bimatoprost has been reported.[29] Although 1 patient complained of the deepening of eyelid sulcus, the patient continued the treatment.

Low ocular perfusion pressure is an established risk factor in glaucoma.[30] Quaranta et al.[31] recently reported that in previously untreated patients with NTG, both latanoprost and bimatoprost reduced the IOP from untreated baseline, to a similar extent, over a 24-h curve. Latanoprost was associated with slightly improved ocular diastolic perfusion pressure over 24-h but similar absolute perfusion levels to those of bimatoprost.[31]

In conclusion, bimatoprost provided a significant reduction in IOP for at least 12 weeks for Japanese patients with NTG who showed insufficient response to latanoprost.

Author Disclosure Statement

No competing financial interests exist.

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TABLE 1. MEAN CONJUNCTIVAL HYPEREMIA AND AREA DENSITY GRADING SCALE SCORES

	Mean conjunctival hyperemia	Mean area density grading scale score
Preswitch	0.31 ± 0.35	0.67 ± 0.97
At 4 weeks	0.47 ± 0.41 ($P = 0.60$)	0.94 ± 1.20 ($P = 0.78$)
At 8 weeks	0.50 ± 0.54 ($P = 0.46$)	0.78 ± 1.00 ($P = 0.98$)
At 12 weeks	0.56 ± 0.54 ($P = 0.27$)	0.67 ± 0.97 ($P > 0.99$)

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厚生科学研究費補助金　障害者対策総合研究事業（感覚器障害分野）
分担研究報告書

未熟児網膜症の治療に使用する抗 VEGF 剤と無灌流領域

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研究要旨：未熟児網膜症（ROP）に対する新たな治療法として、抗 VEGF 剤の硝子体内注射が注目されている。これまでの報告では、抗 VEGF 剤の硝子体内注射は眼内新生血管の活動性を低下させることによって ROP の治療として有用であるとされている。しかし最近になり、抗 VEGF 剤の注射によって網膜での無灌流領域（NPA）が拡大するのではないかという報告がなされた。もしそうであれば、この治療法は ROP の治療薬として望ましくない一面があることになる。そこで今回我々は、網膜静脈分枝閉塞（BRVO）の患者 58 名 58 眼において抗 VEGF 剤であるベバシズマブ（アバスチン®）の硝子体内注射（IVB）と注射後 1 か月における NPA の面積を計測した。その結果、NPA の面積は IVB によって拡大していないことがわかった。今回の研究は ROP の網膜で計測したものではないが、少なくとも BRVO においては IVB は網膜の NPA を拡大させることはないことがわかった。

A．研究目的

血管内皮増殖因子（Vascular endothelial growth factor: VEGF）はROPにおける新生血管発生と増殖変化の主たる因子と考えられている。このVEGFを抑制する抗VEGF療法は、未熟児網膜症（ROP）に対する新たな治療法として注目されており、実際に良好な臨床成績が報告されてきている[参考文献1,2]。これまでの報告では、抗VEGF剤の硝子体内注射は眼内新生血管の活動性を低下させることによってROPの治療として有用であるとされている。

しかしながら最近になり、主に血管閉塞性疾患に使用した場合、抗VEGF剤の注射によって網膜での無灌流領域（NPA）が拡大するのではないかという報告がなされた [参考文献3-7]。もしそうであれば、この治療法はROPの治療薬として望ましくない一面があることになる。

そこで今回我々は、網膜静脈分枝閉塞（BRVO）の患者58名58眼において抗VEGF剤であるベバシズマブ（アバスチン®）の硝子体内注射（IVB）前と注射後1か月におけるNPAの面積を計測した。

B．研究方法

BRVOによる黄斑浮腫による治療目的でIVBを施行した58名58眼（男性25名、女性55名：年齢41-89歳、平均66.2歳）を対象とした。IVB施行前と施行後1か月の時点で蛍光眼底造影を施行し、NPAの面積をImageNet 1024®（Topcon）を使用して測定した。NPAの面積は、視神経乳頭の面積で除することによって乳頭面積（DA）で表した。出血によるブロックとNPAを鑑別するために、常に眼底写真と対比してNPAの領域のみを計測するようにした（図1）。
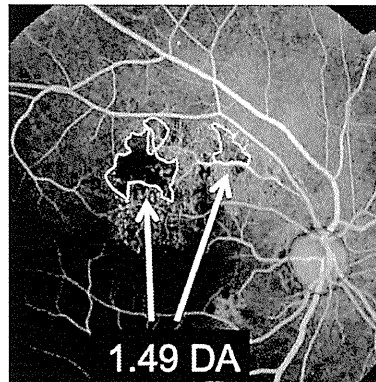
図1：NPAの計測方法。NPAの面積をデジタル計測し、乳頭面積で除してDAで表した。

（倫理面への配慮）

本研究は、名古屋大学医学部の倫理委員会の承認を得て行った。患者には今回の研究について十分説明の後に書面による承諾を得て行った。

C．研究結果

58眼中37眼はIVB前にNPAが存在しなかった。この37眼では、IVB後にNPAが出現したものが3眼あった。それらの面積は、0.13, 0.47, 0.60 DAであった。

58眼中21眼ではIVB前にNPAが存在しており、その面積は3.45±4.66 DAであった。これら21眼のIVB後1か月のNPA面積は、3.45±5.19 DAであった。両者には統計学的に有意な差はなかった（P=0.36）。

この21眼のIVB前後のNPA面積の変化を図2に示す。21眼で、IVB前後でNPA面積が1DA以上増加したものは1眼のみ（赤線）であった。一方で、IVB前後でNPA面積が1DA以上低下したものは4眼（青線）であった。
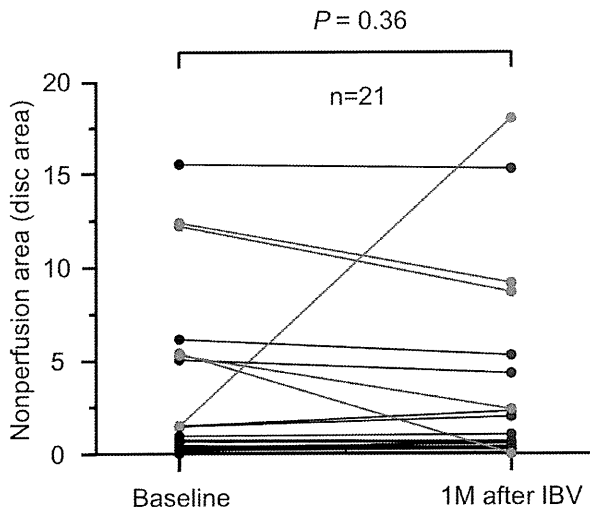
図2：BRVOでIVB施行前にNPAが存在した21眼における IVB前後のNPA面積の変化

図3にNPAがIVB前後に減少した症例の蛍光眼底造影検査の結果を示す。この症例は61歳の男性であり、NPA面積が12.4 DAから1か月の間に9.18 DAに減少した。
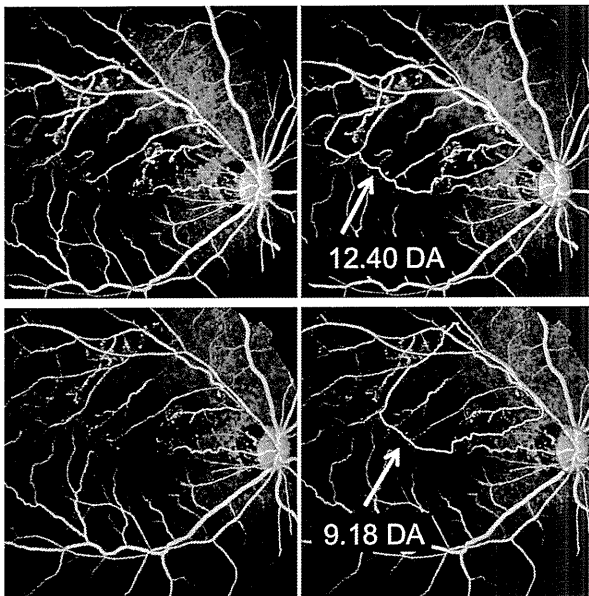

図3：BRVOでIVB施行前にNPAが減少した症例。61歳の男性で、NPA面積が12.4 DAから1か月の間に9.18 DAに減少した。

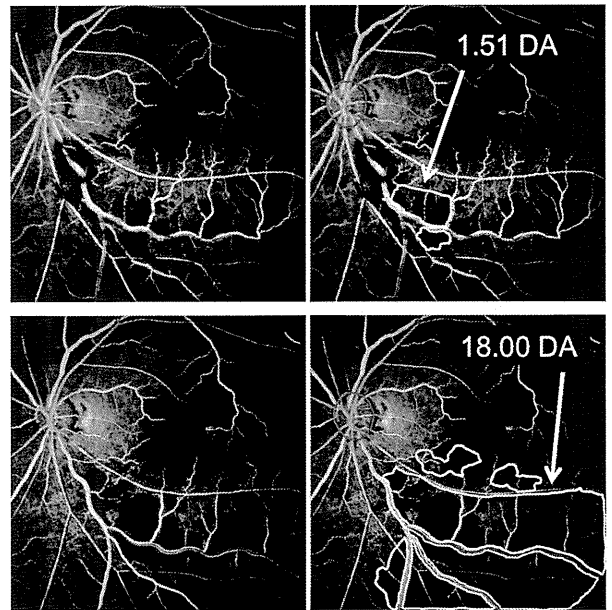次に、図4にNPAがIVB前後で急激に増加した1眼（赤線）の蛍光眼底造影検査の結果を示す。この症例は67歳の女性であり、NPA面積が3.0 DAから18.0 DAに1か月の間に急激に増加した。

図4：BRVOでIVB施行前にNPAが増加した症例。67歳の女性で、NPA面積が1.51 DAから1か月の間に18.00 DAに増加した。

D．考察

最近になり、IVB後に網膜に虚血性変化が生じたという報告がいくつかみられる。Kimらは、CRVOの症例でIVB後に非虚血型が虚血型に変化した1例を報告している[参考文献6]。また、Papadopoulouらは、抗VEGF剤注射後に網膜血管径が減少したことを報告している[参考文献4]。

その一方で、IVBは網膜における虚血性変化をおこさないという報告もある。CRVOとBRVOの29眼における定性的検討を行ったPragerらは、NPA面積はIVB前後で変化なかったと報告しているし、Kookらも糖尿病網膜症の129眼におけるNPAの検討により、その面積は増加していないことを報告している。

もしもIVBによって網膜に急激な虚血変化が引き起こされるのであれば、IVB後1か月の時点でNPAが増加する症例が多くみられるはずであると仮定して今回の研究を行った。その結果今回の我々のBRVO58眼の結果では、IVB前とIVB後で有意なNPA面積の変化はみられなかった。しかも、58眼中、IVB後にNPAが1DA以上増加したのはたったの1眼のみであった。以上により、IVBがNPAを促進させる可能性は低いと結論した。

それでは、なぜ我々の1眼で急激なNPAの増加がみられたのであろうか。Hayrehは、65歳以上のBRVOでは、発症から6か月の経過観察中に約16%の症例において、非虚血型から虚血型に移行することを報告している[参考文献7]。図3に示した症例は、そのような非虚血型から虚血型への移行症例であり、IVBとの関連で生じたものではなかっ

た可能性があると考えられた。

E．結論
　今回の我々の研究結果により、少なくとも成人のBRVO症例においては、抗VEGF抗体の硝子体内注射は網膜のNPAを拡大させることはないということがわかった。しかしながら、今回の結果は実際のROPやROPモデル動物で行った実験ではない。ROPモデル動物を使った実験報告の中では完全なVEGFブロックは網膜の虚血性変化を促進させるという結果もあり、今後はさらに多くの疾患や動物実験による証拠の集積が必要であると考えられた。

F．健康危険情報
　該当する危険あり（詳細）/なし

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H．知的財産権の出願・登録状況
　なし
1．特許取得
　なし
2．実用新案登録
　なし
3．その他（参考文献）
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PLoS one

Identification of Autoantibodies against TRPM1 in Patients with Paraneoplastic Retinopathy Associated with ON Bipolar Cell Dysfunction

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Abstract

Background: Paraneoplastic retinopathy (PR), including cancer-associated retinopathy (CAR) and melanoma-associated retinopathy (MAR), is a progressive retinal disease caused by antibodies generated against neoplasms not associated with the eye. While several autoantibodies against retinal antigens have been identified, there has been no known autoantibody reacting specifically against bipolar cell antigens in the sera of patients with PR. We previously reported that the transient receptor potential cation channel, subfamily M, member 1 (TRPM1) is specifically expressed in retinal ON bipolar cells and functions as a component of ON bipolar cell transduction channels. In addition, this and other groups have reported that human TRPM1 mutations are associated with the complete form of congenital stationary night blindness. The purpose of the current study is to investigate whether there are autoantibodies against TRPM1 in the sera of PR patients exhibiting ON bipolar cell dysfunction.

Methodology/Principal Findings: We performed Western blot analysis to identify an autoantibody against TRPM1 in the serum of a patient with lung CAR. The electroretinograms of this patient showed a severely reduced ON response with normal OFF response, indicating that the defect is in the signal transmission between photoreceptors and ON bipolar cells. We also investigated the sera of 26 patients with MAR for autoantibodies against TRPM1 because MAR patients are known to exhibit retinal ON bipolar cell dysfunction. Two of the patients were found to have autoantibodies against TRPM1 in their sera.

Conclusion/Significance: Our study reveals TRPM1 to be one of the autoantigens targeted by autoantibodies in at least some patients with CAR or MAR associated with retinal ON bipolar cell dysfunction.

Introduction

Paraneoplastic retinopathy (PR) is a progressive retinal disorder caused by an autoimmune mechanism and is associated with the presence of anti-retinal antibodies in the serum generated against neoplasms not associated with the eye [1–4]. The retinopathy can develop either before or after the diagnosis of a neoplasm. Patients with PR can have night blindness, photopsia, ring scotoma, attenuated retinal arteriole, and abnormal electroretinograms (ERGs). The diagnosis of PR is usually made by the identification of neoplasms and anti-retinal autoantibodies in the sera.

PR includes two subgroups: cancer-associated retinopathy (CAR) [5,6] and melanoma-associated retinopathy (MAR) [7–10]. Although CAR and MAR share similar clinical symptoms, the ERG findings

are very different. Both a- and b-waves are severely attenuated in CAR, indicating extensive photoreceptor dysfunction, whereas only the b-wave is severely reduced while the a-wave is normal in MAR, suggesting bipolar cell dysfunction [8,9]. However, it was recently reported that cancers other than melanoma can cause bipolar cell dysfunction [11,12]. Several autoantibodies against retinal antigens have been identified, but a specific antigen associated with bipolar cells has not been identified in patients with CAR and MAR [1–10].

In the current study, we identified autoantibodies against the transient receptor potential cation channel, subfamily M, member 1 (TRPM1) [13–15] in the serum of one patient with lung cancer. The ERG findings in this patient indicated a selective ON-bipolar cell dysfunction. We also investigated the sera of 26 MAR patients and found that two contained autoantibodies against TRPM1. Our results suggest that TRPM1 is one of the retinal autoantigens in at least some patients with CAR or MAR and may cause retinal ON bipolar cell dysfunction.

Results

Case report of CAR associated with ON bipolar cell dysfunction

A 69-year-old man visited the Nagoya University Hospital with complaints of blurred vision, photopsia and night blindness in both eyes of three months duration. At this point he was not diagnosed as suffering from any eye disease or systemic disease, including a malignant tumor, and his family history revealed no other members suffering from any eye diseases. On initial examination, his best-corrected visual acuity was 0.9 in the right eye and 0.6 in the left eye. Humphrey static perimetry revealed a severe decrease in sensitivity within the central 30 degrees of the visual field in both eyes (Fig. 1A). Dark-adaptometry of this patient showed a loss of the rod branch. The cone threshold was within normal range. Ophthalmoscopy showed a nearly normal fundus appearance except for slight hypopigmentation at the macula of the left eye, which may be due to age-related changes in the retinal pigment epithelium (Fig. 1B), but fluorescein angiography demonstrated periphlebitis of the retinal vessels (arrows, Fig. 1C). Spectral-domain optical coherence tomography (SD-OCT) showed that the morphology of the retina was normal in both eyes (Fig. 1D).

Electrophysiological examinations

Recordings of the full-field ERGs from this patient showed that the rod responses were undetectable (Fig. 2). The rod- and cone-mixed maximal response was a negative-type with an a-wave of normal amplitude and a b-wave that was smaller than the a-wave. The a-wave of the cone response had a wide trough, and the b-wave was reduced by 40%. The amplitude of the 30-Hz flicker ERG was reduced by 50%. The photopic long-flash ERG showed severely reduced ON response and normal OFF response. These ERG findings indicated that there was a defect in the signal transmission from photoreceptors to ON bipolar cells both in both rod and cone pathways.

Based on these ophthalmological and electrophysiological tests, we suspected that this patient might have PR and referred him to an internist. The general physical examination including positron emission tomography and computed tomography revealed two abnormal masses in the right lung. Biopsy of these masses confirmed that the masses were small cell carcinomas of the lung.

Detection of autoantibodies against TRPM1 in the serum of the CAR patient

Based on our ERG examination results, we hypothesized that the serum of this CAR patient may contain autoantibodies against
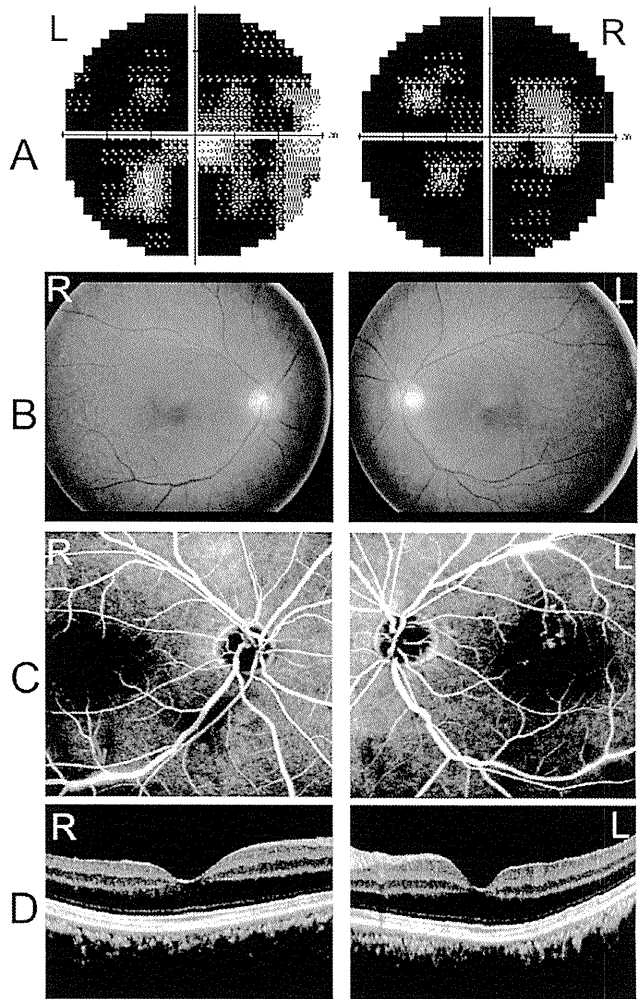

Figure 1. Ophthalmological findings from a patient with paraneoplastic retinopathy (PR) associated with lung cancer. (A) Threshold of static visual field (Humphrey, 30-2 program) plotted on a gray scale showing severely decreased sensitivities within the central 30 degrees of the visual field. (B) Fundus photographs of the patient showing a nearly normal fundus. (C) Fluorescein angiograms showing periphlebitis of the retinal vessels (arrows). (D) Spectral-domain optical coherence tomographic (SD-OCT) image of a 9 mm horizontal scan of the retina of our patient. The retinal structure in each retinal layer is normal.
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TRPM1. To test this hypothesis, we examined whether or not this CAR patient's serum could recognize human TRPM1 protein by Western blot analysis. We transfected an expression plasmid containing human TRPM1 cDNA with the C-terminal 3xFlag-tag (TRPM1-3xFlag) into HEK293T cells, and carried out a Western blot analysis using whole cell extracts harvested after 48 hrs cell growth. We first confirmed that TRPM1-3xFlag was expressed by cell using Western blot analysis and an anti-Flag antibody. We detected the ~200 kDa TRPM1-3xFlag band in the cell lysates (Fig. 3A).

Next, we performed Western blot analysis on the same lysates using the serum from our CAR patient and a healthy control person. We detected immunostaining of the same size protein, which was confirmed with the anti-Flag antibody, and with CAR serum. The control serum did not present a significant band (Fig. 3B, C). This result showed the presence of autoantibodies against TRPM1 in this CAR patient's serum.