Japan) examinations. A microscopic image of each section within 0.5–1 mm superior of the optic disc was scanned. In each computer image, the thickness of the inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) were measured.

1.5. Retrograde labeling of retinal ganglion cells

At 7 days prior to sacrifice, hydroxystilbamidine (Molecular Probes Inc., Eugene, OR) was injected bilaterally into the superior colliculi of anesthetized rats. The skull was exposed and kept dry and clean. After identifying and marking the bregma, a small window was drilled in the scalp in both the right and left hemispheres. The windows were drilled to a depth of 3.6 mm from the surface of the skull and located at 6.8 mm behind the bregma on the anteroposterior axis, and 1.5 mm lateral to the midline. Using a Hamilton syringe, 1.5 μl of 2% hydroxystilbamidine was slowly injected into the bilateral superior colliculi. After suturing the skin over the wound, antibiotic ointment was applied.

1.6. Tissue preparation and assessment of RGC survival

Animals were sacrificed using an overdose of Nembutal at 1 week after 2% hydroxystilbamidine (Molecular Probes Inc.) application. Whole, flat-mounted retinas were then assayed for retinal ganglion cell density. Rat eyes were enucleated and fixed in 4% paraformaldehyde for 10 h at room temperature. After removal of the anterior segments, the resultant posterior eyecups were left in place. Subsequently, four 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 four radial cuts, and then spread on a gelatin-coated glass slide. Labeled retinal ganglion cells (RGCs) were visualized under a fluorescence

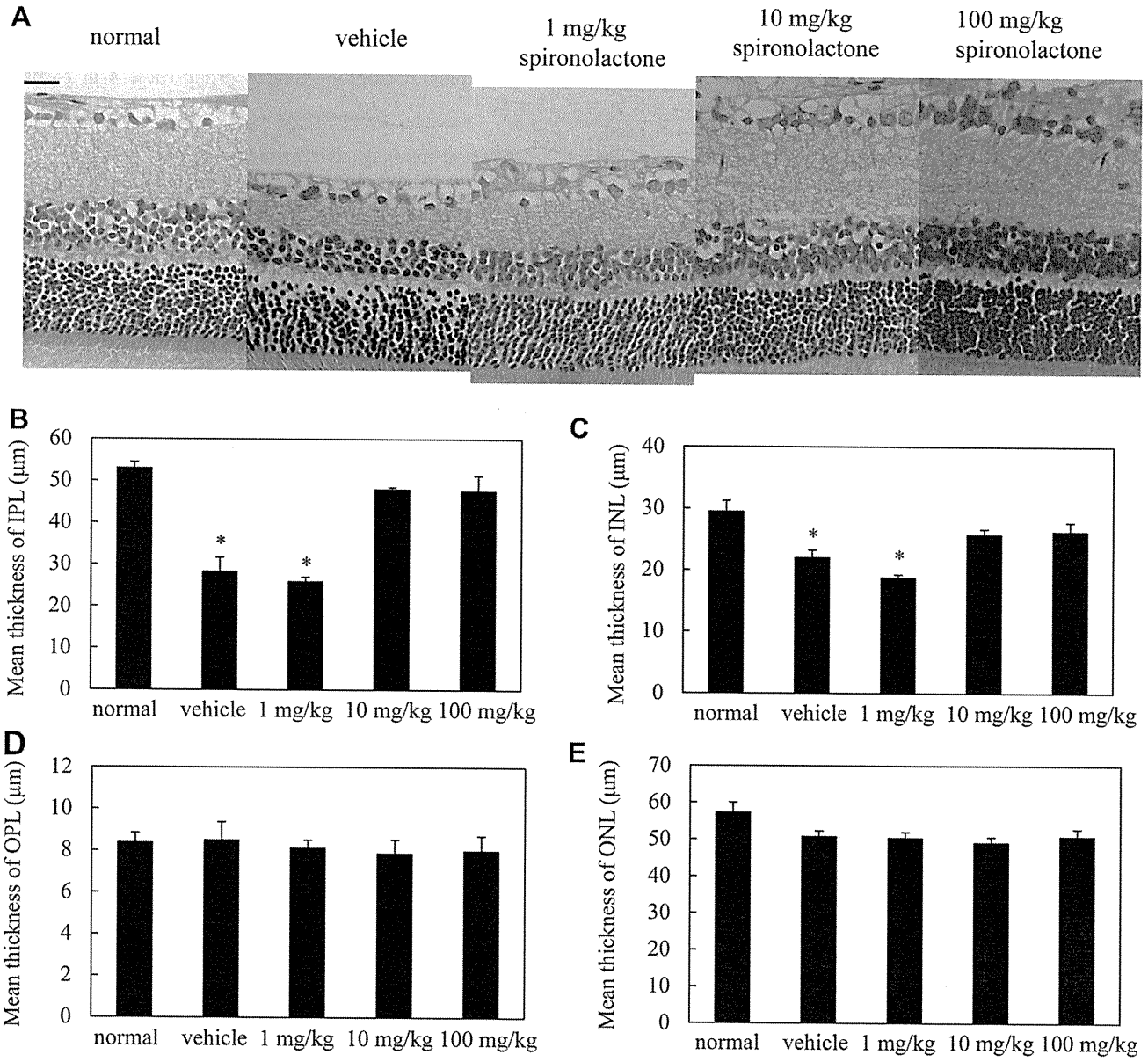

Fig. 3. Change in mean thickness of the IPL, INL, OPL, and ONL at 7 days after ischemia without spironolactone or with 1, 10 and 100 mg/kg spironolactone. Data express the mean ± SEM ($n = 4$ in each group). *$P < 0.05$ versus normal (Dunnett's multiple comparison test). Scale bar, 10 μm.

microscope (Olympus BX-51/DP70, Olympus, Tokyo, Japan) with an ultraviolet filter (blue-violet: 395–440 nm). Fluorescence-labeled RGCs were counted in 12 microscopic fields of retinal tissue from two regions in each quadrant at two different eccentricities, 1 mm (central) and 4 mm (peripheral) away from the optic disc. Image-Pro Plus software (Version 4.0, Media Cybernetics, Bethesda, MD) was used to count the total number of RGCs in each eye.

1.7. Statistical analysis

All data are presented as the mean ± SEM. Data were analyzed using an independent Student's *t*-test or Dunnett's multiple comparison test, as appropriate. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL). A P value of less than 0.05 was considered statistically significant.

2. Results

2.1. Histologic changes in the retina after ischemia with aldosterone

Thicknesses in the normal retina were 48.0 ± 2.5 μm for IPL, 27.2 ± 2.3 μm for INL, 8.2 ± 0.4 μm for OPL, and 54.8 ± 2.7 μm for ONL ($n = 5$) (Fig. 1). Thickness measurements in animals pretreated with distilled water were 27.6 ± 2.7 μm ($P < 0.001$) for IPL, 17.0 ± 0.9 μm ($P < 0.001$) for INL, 8.4 ± 0.5 μm ($P = 0.980$) for OPL, and 51.6 ± 1.3 μm ($P = 0.405$) for ONL ($n = 5$) (Fig. 1). Thicknesses in animals pretreated with 1 mg/kg candesartan were 41.8 ± 1.8 μm ($P = 0.129$) for IPL, 25.4 ± 1.0 μm ($P = 0.681$) for INL, 8.3 ± 0.7 μm ($P = 0.999$) for OPL, and 51.8 ± 1.0 μm ($P = 0.454$) for ONL ($n = 5$). However, in animals pretreated with 1 mg/kg candesartan and 80 μg/kg/day aldosterone, the thicknesses were 24.5 ± 1.0 μm ($P < 0.001$) for IPL, 17.0 ± 0.7 μm ($P < 0.001$) for INL, 7.8 ± 0.2 ($P = 0.760$) for OPL, and 55.0 ± 1.1 μm ($P > 0.999$) for ONL ($n = 5$). Thus, treatments combining aldosterone with candesartan provided no protective effect against retinal ischemia-reperfusion injury.

2.2. Effect of aldosterone on RGC survival

Fig. 2A shows representative results for the RGC labeling in the vehicle-, in the candesartan-, and in the candesartan and aldosterone-treated rats. While RGC death was mild in the candesartan-treated rats, treatments that also included aldosterone blocked the neuroprotective effects of the candesartan. In the central retina, the number of RGCs in the eyes with ischemia were 1394 ± 120 in the vehicle-treated group ($P = 0.005$), 1865 ± 176 ($P = 0.145$) in the candesartan-treated group, and 1652 ± 187 ($P = 0.031$) in the candesartan plus aldosterone-treated group ($n = 4$ in each group) (Fig. 2B). The number of RGCs in the peripheral retinas of the eyes with ischemia were 781 ± 36 in the vehicle-treated group ($P = 0.001$), 1069 ± 83 ($P = 0.137$) in the candesartan-treated group, and 925 ± 31 ($P = 0.012$) in the candesartan plus aldosterone-treated group ($n = 4$ in each group) (Fig. 2C).

2.3. Effect of spironolactone on the retina after ischemia

Thicknesses in the normal retina were 53.0 ± 1.5 μm for IPL, 29.5 ± 1.8 μm for INL, 8.4 ± 0.5 μm for OPL, and 56.0 ± 3.2 μm for ONL ($n = 4$) (Fig. 3). Thicknesses in the animals orally administered

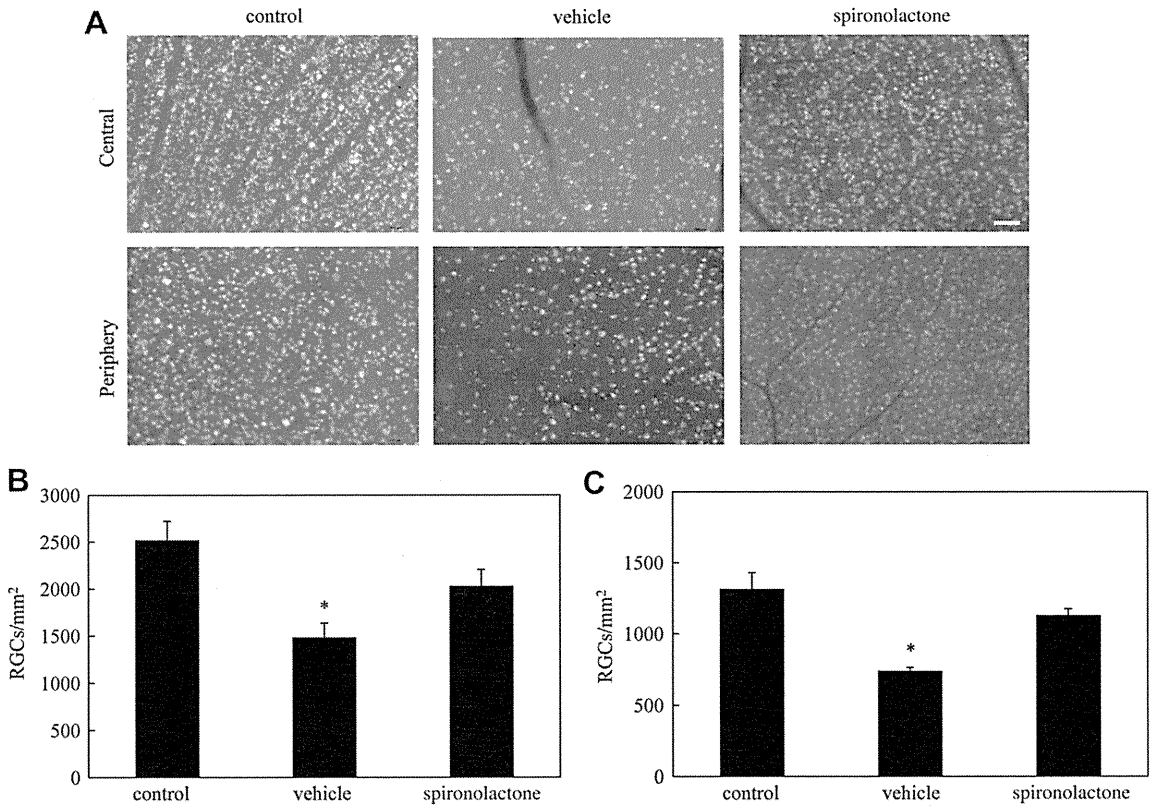

Fig. 4. Effect of spironolactone on ischemia-induced retinal ganglion cell death. (A) Retrograde labeling of RGCs in nonischemic eyes and at 7 days after ischemic injury after administration of vehicle or 10 mg/kg spironolactone. Micrographs of the central and peripheral areas were taken approximately 1 and 4 mm from the optic nerve head. Scale bar, 100 μm. RGCs were counted in the central (B) and peripheral (C) areas at approximately 1 and 4 mm from the optic nerve head. Graph depicts the mean ± SEM for the vehicle-treated animals with or without ischemia or spironolactone ($n = 4$ in each group). *$P < 0.05$ versus control (Dunnett's multiple comparison test).

1, 10, or 100 mg/kg/day dose of spironolactone were 25.9 ± 1.1 μm ($P < 0.001$), 48.0 ± 0.5 μm ($P = 0.403$) and 47.6 ± 3.6 μm ($P = 0.344$) for IPL, 18.8 ± 0.5 μm ($P < 0.001$), 25.8 ± 0.9 μm ($P = 0.16$) and 26.3 ± 1.5 μm ($P = 0.253$) for INL, 8.1 ± 0.4 μm ($P = 0.996$), 7.9 ± 0.7 μm ($P = 0.948$) and 8.0 ± 0.7 μm ($P = 0.981$) for OPL, and 50.4 ± 1.6 μm ($P = 0.211$), 50.1 ± 1.5 μm ($P = 0.101$) and 50.8 ± 2.0 μm ($P = 0.259$) for ONL, respectively ($n = 4$ in each group) (Fig. 3).

2.4. Effect of spironolactone on RGC survival

Fig. 4A shows representative results of the RGC labeling in both the vehicle- and the spironolactone (10 mg/kg/day)-treated rats. As seen in Fig. 4B, the number of RGCs in the central retinas of the eyes with ischemia were 1480 ± 156 in the vehicle-treated group ($P = 0.006$) and 2025 ± 179 ($P = 0.159$) in the spironolactone-treated group. In the peripheral retina, the number of RGCs in the eyes with ischemia were 738 ± 28 in the vehicle-treated group ($P = 0.001$) and 1128 ± 48 ($P = 0.188$) in the spironolactone-treated group ($n = 4$ in each group).

2.5. Renin-angiotensin-aldosterone system in the retina

As shown in Fig. 5, there was no protective effect against retinal ischemic damage after pretreatment with aldosterone ($n = 4$). However, a protective effect against retinal ischemic damage was seen after pretreatment with spironolactone and candesartan (Fig. 5) ($n = 4$).

2.6. Effect of local administration of aldosterone

Although treatment with local administration of aldosterone without ischemia did not affect the retinal thickness in the normal rat, a significant decrease in the number of RGCs was observed (Fig. 6) ($n = 4$). Results for treatment with local administration of aldosterone 30 min before ischemia were similar to those found after systemic administration of aldosterone (Fig. 6) ($n = 4$). No protective effects against retinal ischemia-reperfusion injury were noted after the treatments that combined local administration of aldosterone with candesartan (Fig. 6) ($n = 5$).

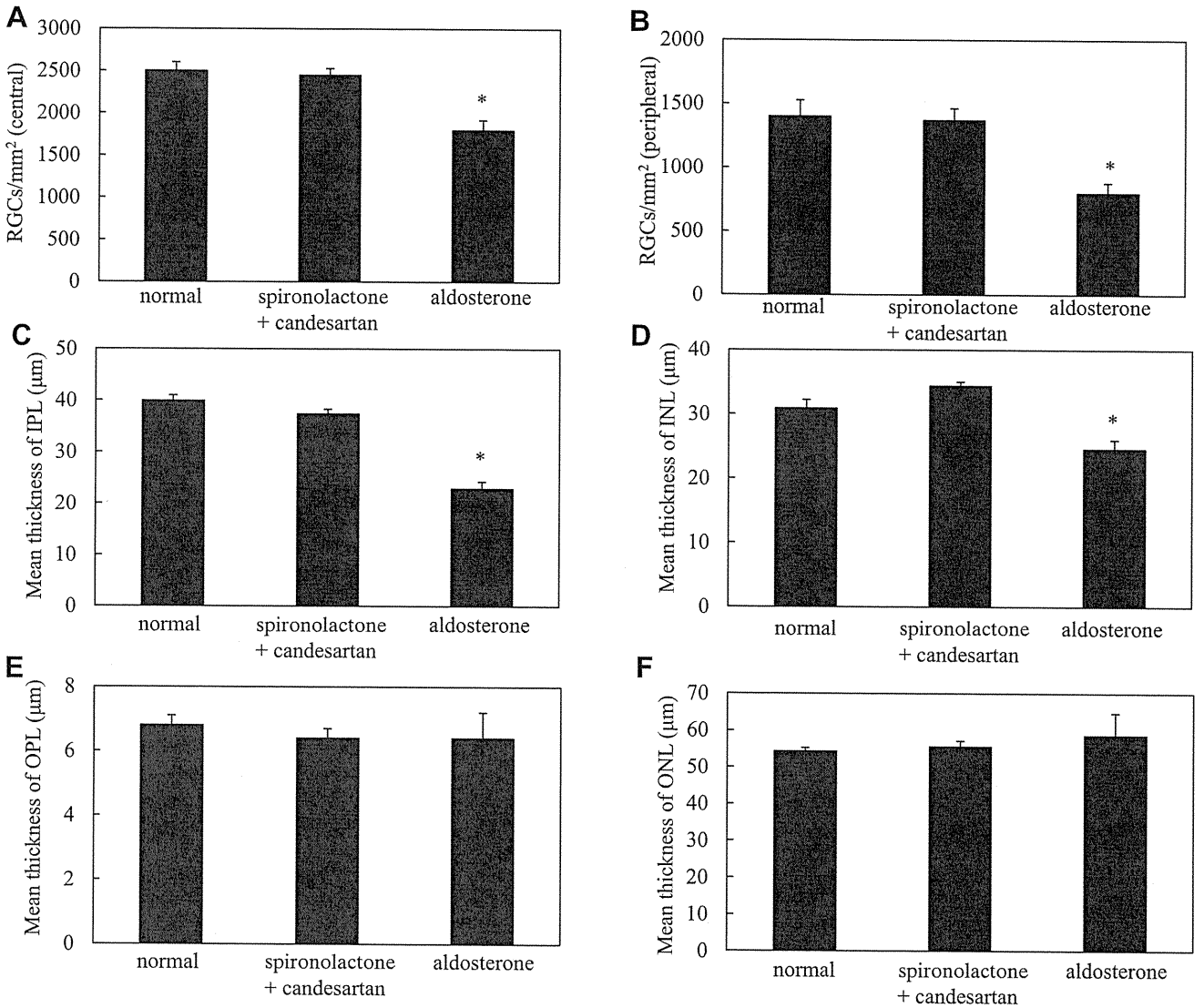

Fig. 5. RGC survival and change in mean thickness of the IPL, INL, OPL, and ONL at 7 days after ischemia and treatments with either aldosterone, or spironolactone and candesartan. Data express the mean ± SEM ($n = 4$ in each group). *$P < 0.05$ versus normal (Dunnett's multiple comparison test).

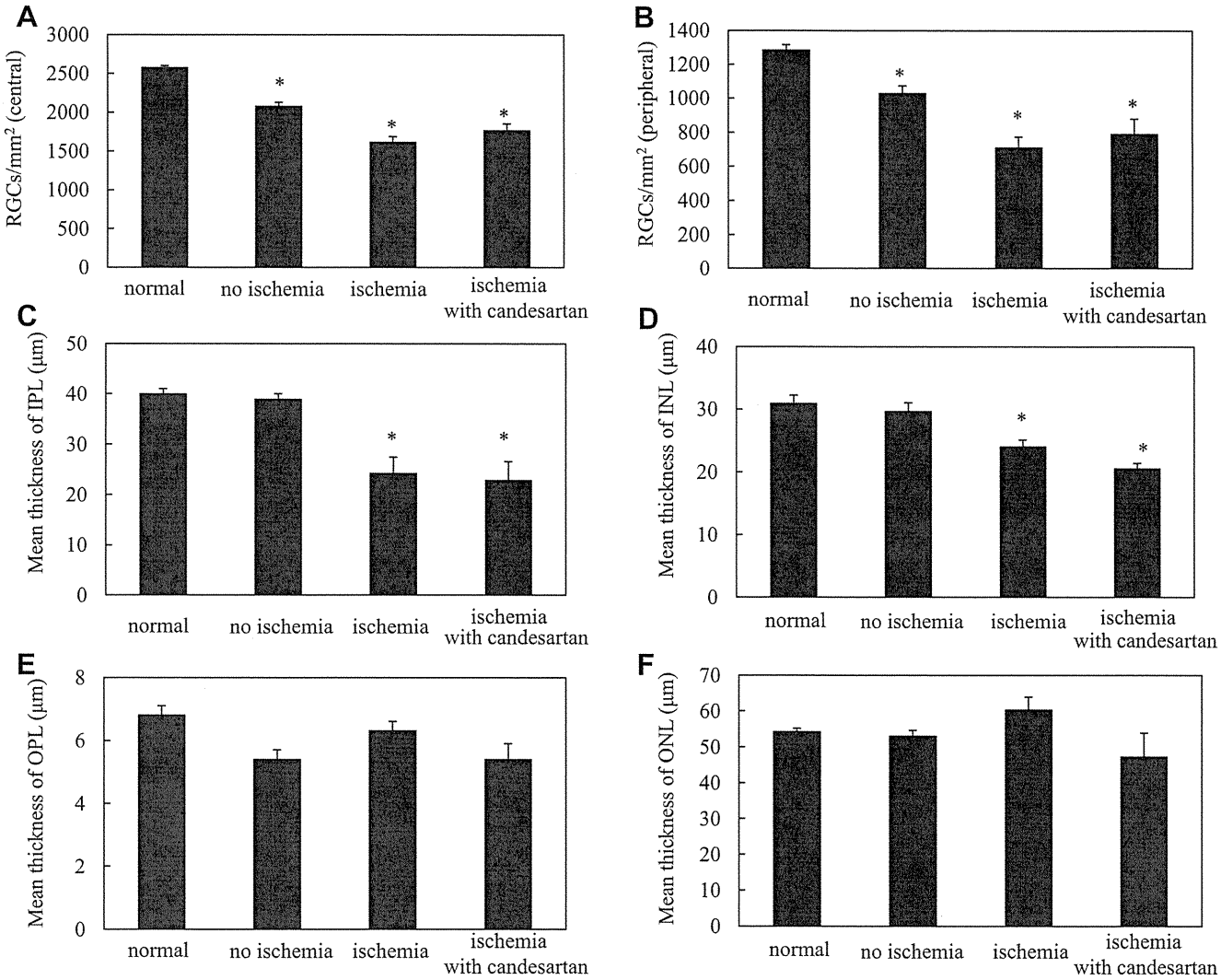

Fig. 6. RGC survival and change in mean thickness of the IPL, INL, OPL, and ONL at 7 days after local administration of aldosterone. Data express the mean ± SEM. *$P < 0.05$ versus normal (Dunnett's multiple comparison test).

3. Discussion

The present study demonstrated that the protective effect of candesartan was lost when retinal ischemic rats were administered aldosterone. Similarly, aldosterone antagonism with spironolactone also decreased the retinal ischemia-reperfusion injury.

In a recent study that used a rat model of oxygen-induced retinopathy, it was shown that the eye expresses MRs and that these receptors are involved in retinal vascular pathology (Wilkinson-Berka et al., 2009). In addition, the authors also noted that MR antagonism led to a reduction of the pathological angiogenesis normally associated with the inflammation and oxidative stress in this rat model (Wilkinson-Berka et al., 2009). Therefore, it is conceivable that the beneficial effects on the neurologic outcome of ischemia-reperfusion injury that are observed after spironolactone administration may be related to MR inhibition.

Aldosterone synthase is the rate-limiting enzyme in aldosterone production. In the adrenal gland and cardiovascular tissues, Ang II stimulates aldosterone synthase via the AT1-R (Rogacz et al., 1990) and AT1-R blocker (ARB), which causes a reduction in the aldosterone-related pathology (Harada et al., 2001; Fukuda et al., 2010). A previous study has reported finding enhanced MR signaling in the kidney and heart (Williams et al., 2004). Additionally, this study also demonstrated that eplerenone, which is an aldosterone antagonist, was able to dramatically retard the progression of renal and cardiac diseases. Thus, as was pointed out by the authors of this previous study, plasma renin–aldosterone profiles are not always predictive of the antihypertensive efficacy of MR blockade. Overall, these findings raise the possibility that molecules other than circulating aldosterone may be involved in the activation of the MR.

We also have recently reported that candesartan, which is an ARB, had a neuroprotective effect against retinal ischemia-reperfusion injury because of its ability to reduce oxidative stress (Fukuda et al., 2010). When aldosterone was administered to rats receiving candesartan, there was a complete reversal of the RAAS suppression-induced neuroprotective effect against the retinal ischemia-reperfusion injury. In addition, we have also demonstrated that spironolactone had a neuroprotective effect against retinal ischemia-reperfusion injury. When taken together, these findings suggest that MR and aldosterone are able to influence ischemic damage in the retina and thus, these MR antagonists could potentially be used for therapeutic application in cases of retinal ischemic damage.

AT1-R is known to be expressed primarily by RGCs, including Müller cells and astrocytes (Downie et al., 2009). We previously reported that AT1-R was present in RGCs and the INL of the ischemic retina (Fukuda et al., 2010). Additionally, ARBs reduce aldosterone synthase mRNA in oxygen-induced retinopathy (Wilkinson-Berka et al., 2009). This indicates that aldosterone synthase should be produced by AT1-R-positive cells. It has also been demonstrated that MRs are present in RGCs, the INL, retinal pigment epithelium, and in the vasculature (Wilkinson-Berka et al., 2009). Since a decrease occurred in both the INL thickness and the number of RGCs after ischemic-reperfusion injury, we assumed that this could be caused by the death of AT1-R- or MR-positive cells. In the present or previous (Fukuda et al., 2010) study, we showed that systemic administration of candesartan was able to inhibit this retinal ischemic damage. While it is possible that plasma candesartan might reach the inner retina and play a part in this inhibition, as of yet, we do not know if the plasma Ang II is also capable of reaching the inner retina. Future studies that definitively clarify the mechanism of this inhibition will need to be conducted.

The present study demonstrated that a local aldosterone/MR system exists within the retina and thus, MR blockade might be of potential therapeutic benefit in retinal ischemic insult cases.

Acknowledgments

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Effects of Intravitreally Injected Bevacizumab on Vascular Endothelial Growth Factor in Fellow Eyes

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Abstract

Purpose: Whether an intravitreal injection of bevacizumab in 1 eye will have any effect on the fellow eye has been discussed. The aim of this study was to determine the level of vascular endothelial growth factor (VEGF) in the fellow eyes after an intravitreal injection of bevacizumab in 1 eye with proliferative diabetic retinopathy.
Methods: Eight patients who had similar findings of proliferative diabetic retinopathy in both eyes were studied. Four patients had rubeosis (rubeosis group), and 4 patients did not have rubeosis (no-rubeosis group) in the anterior chamber. All patients received an intravitreal injection of bevacizumab (1.25 mg) in 1 eye. Samples of aqueous humor were collected from the injected eyes just before the injection of bevacizumab and 1 day after the first injection just before vitrectomy. Samples of aqueous humor from the fellow eyes were collected just before a second injection of bevacizumab in the fellow eye at 7 days after the first injection. The concentration of VEGF in the aqueous humor was measured by enzyme-linked immunosorbent assay.
Results: After 1 day, the concentration of VEGF in injected eyes was significantly reduced from 3,230.3 ± 2,136.8 to 3.1 ± 3.6 pg/mL ($P < 0.05$) in eyes with rubeosis and 465.0 ± 78.8 to 0 pg/mL ($P < 0.05$) in those without rubeosis. After 7 days, the VEGF level of the fellow eyes was still significantly lower than that in the injected eye just before the injection of bevacizumab (688.5 ± 443.1 pg/mL) in the rubeosis group, and it was 7.8 ± 13.2 pg/mL in the no-rubeosis group ($P < 0.05$).
Conclusions: A single intravitreal injection of bevacizumab significantly reduced the VEGF concentrations in the aqueous humor of the fellow untreated eye. Thus, we need to be observant of the fellow eyes after a unilateral injection and also examine the patients for systemic changes.

Introduction

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) plays a key role in the development of human eye diseases characterized by neovascularizations, for example, proliferative diabetic retinopathy (PDR). Because the level of VEGF in the vitreous is highly correlated with the severity of diabetic retinopathy,[1,2] intravitral bevacizumab (Avastin; Genentech, Inc., San Francisco, CA), a recombinant human monoclonal IgG1 antibody that binds to all isoforms of human VEGF, has been used to treat PDR. Although bevacizumab has been approved by the United States Food and Drug Administration for intravenous use for metastatic colorectal cancer, its beneficial effects on eyes with neovascular age-related macular degeneration,[3] diabetic retinopathy,[4] and retinal vein occlusion have been reported.[5] Intravitreal bevacizumab has been shown to lead to a significant decrease in the VEGF levels in the aqueous of patients with diabetic retinopathy.[6–8]

However, warnings have been published on adverse systemic complications of bevacizumab.[9,10] This is relevant, because we have shown that the blood level of VEGF is decreased after an intravitreal injection of bevacizumab, which indicated that an effective concentration of bevacizumab had entered the general circulation.[11] The question then arises whether an intravitreal injection of bevacizumab in 1 eye will have any effect on the VEGF level in the fellow eye.

Avery et al. reported that some patients with bilateral PDR had a regression of neovascularization in both eyes when bevacizumab was intravitreally injected in only 1 eye.[4] Yoon et al. described the rapid progression of subclinical age-related macular degeneration in an untreated fellow eye after

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intravitreal bevacizumab[6] and Sawada et al. reported that, after a unilateral intravitreal injection of bevacizumab in eye with diabetic retinopathy, the VEGF concentration in the aqueous humor of the contralateral eye was not decreased at 7 days after the first injection.[7] A recent study reported that there were no pharmacological indications for a significant concentration of unbound bevacizumab.[8] Whether an intravitreal injection of bevacizumab in 1 eye will have any effect on the fellow eye remains undetermined.

Thus, the purpose of this study was to determine the level of VEGF in the fellow eyes of patients with diabetic retinopathy who had received an intravitreal injection of bevacizumab in 1 eye.

Methods

Subjects

The procedures used in this study conformed to tenets of the Declaration of Helsinki and were performed after receiving approval from the Institutional Review Committee of Kansai Medical University. An informed consent was obtained from all patients. Patients with aggressive PDR were studied.

Sixteen eyes of 8 patients with type 2 diabetes mellitus (age range: 44–68, average: 55.8 years) were studied (clinical data of patients are shown in Table 1). The clinical findings of PDR were very similar in both eyes of all patients. Of the 8 patients, 4 patients had aggressive PDR with rubeosis of the iris and/or angle structures (rubeosis group, cases 1–4 in Table 1) and 4 patients did not have rubeosis of the iris and angle structures (no-rubeosis group, cases 5–8 in Table 1). The clinical findings indicated that all patients would benefit from vitrectomy, and all received an intravitreal injection of bevacizumab as a preoperative adjunctive therapy. Because the clinical findings of PDR were similar in both eyes of all patients, the first treated eye was selected by the visual acuity, severity of vitreous hemorrhage, and the intraocular pressure. Otherwise, the right eye was selected if these clinical data were all the same.

Injection technique

The intravitreal injection of bevacizumab was performed as previously described in detail.[11–14] Briefly, under sterile conditions in the operating room, 1.25 mg/0.05 mL of bevacizumab (Avastin) was injected into the vitreous by a 30-gauge needle that was inserted into the eye 4.0 mm posterior to the limbus. The needle was carefully removed, and a sterile cotton applicator was used to prevent reflux. The postoperative medications included topical antibiotics 4 times/day. Vitrectomy was performed on the following day.

Collecting ocular samples

All patients received an intravitreal injection of bevacizumab (1.25 mg) in 1 eye and underwent vitrectomy on the following day. The fellow eye received an intravitreal injection of bevacizumab at 7 days after the first injection of bevacizumab.

Samples of the aqueous humor were collected from the first eye just before the injection of bevacizumab and 1 day after the injection just before the vitrectomy. The samples of the aqueous humor from the fellow eyes were collected at

TABLE 1. CLINICAL DATA OF THE PATIENTS

Case	Age	Sex	Severity of DR, ETDRS severity scale[20] (right eye)	Severity of DR, ETDRS severity scale (left eye)	First eye	Treatment after the injection of bevacizumab	Concentration of VEGF before the injection (pg/mL)	Concentration of VEGF at 1 day after the injection (pg/mL)	Rubeosis after the injection	Concentration of VEGF in the fellow eye (pg/mL)	Rubeosis of the fellow eye after the injection
1	59	M	75	75	R	PPV+PC	568.0	0	—	0	—
2	44	M	71	71	R	PPV+PEA+IOL+PC	376.0	0	—	0	—
3	59	M	71	71	R	PPV+PEA+IOL+PC	456.0	0	—	1.5	—
4	55	F	75	71	R	PPV+PEA+IOL+PC	460.2	0	—	29.7	—
5	68	F	85	85	R	PPV+PEA+IOL+PC	1,248.5	6.3	Not detectable	89.1	Remain unchanged
6	48	M	81	85	L	PPV+PEA+IOL+PC	1,849.9	6.2	Not detectable	628.2	NVR was decreased
7	54	M	81	85	L	PPV+PEA+IOL+PC	5,963.0	0	Decreased	953.6	NVD was decreased
8	59	F	81	81	R	PPV+PC+SO	3,859.8	0	Decreased	1,083.3	Remain unchanged

Cases 1–4: Rubeosis(−) group; cases 5–8: Rubeosis(+) group.
DR, diabetic retinopathy; ETDRS, early treatment diabetic retinopathy study; VEGF, vascular endothelial growth factor; PPV, pars plana vitrectomy; PEA, phacoemulsification and aspiration; IOL, intraocular lens implantation; PC, photocoagulation; NVD, neovascularization from the disc; NVR, neovascularization from the retina; SO, silicon oil injection.

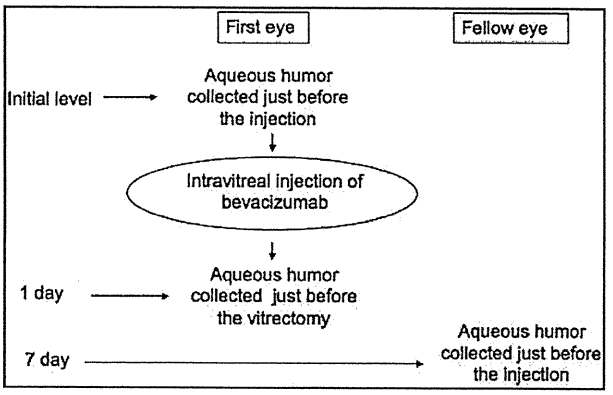

FIG. 1. Flow chart of the experimental protocol. Bevacizumab (1.25 mg/mL) was injected into the vitreous cavity of the first eye as a preoperative adjunctive therapy. Samples of the aqueous humor from the injected eyes (first eyes) were collected just before the injection of bevacizumab and at 1 day after the injection just before the vitrectomy. The samples of the aqueous humor from the fellow eyes were collected at 7 days after the first injection just before the injection of bevacizumab.

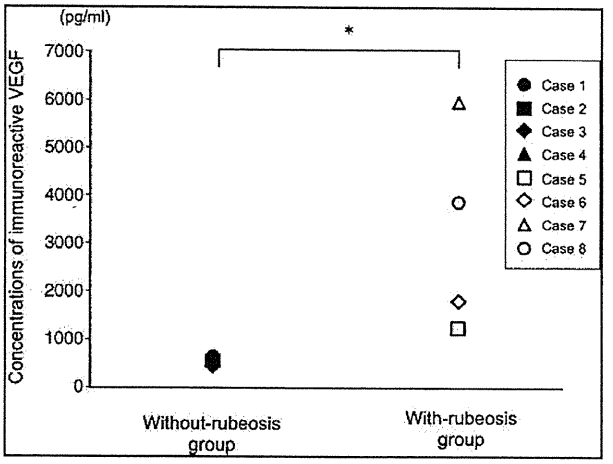

FIG. 2. VEGF concentration in the aqueous humor before the injection. Mean VEGF concentration in the aqueous humor was 465.0±78.8 pg/mL (mean±standard deviation; range: 376–568 pg/mL) in the rubeosis group and 3,230.3±2,136.8 pg/mL (range: 1,248.5–5,963.0 pg/mL) in the no-rubeosis group. The higher level of VEGF in the rubeosis group is significant (*$P<0.05$). VEGF, vascular endothelial growth factor.

7 days after the first injection just before the injection of bevacizumab (Fig. 1). All samples were collected in tubes containing EDTA and were immediately placed on ice and stored at −80°C before use.

VEGF assay

The samples were thawed on ice, and the concentration of VEGF in the plasma was determined with an ELISA Kit (Quantikine® VEGF ELISA Kit; R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. This assay employs a quantitative sandwich enzyme immunoassay technique. The concentrations of VEGF were determined by measuring the absorbance at 450 nm with a microplate reader (Titertek, Multiscan; Flow Laboratories, Inc., Helsinki, Finland), and the values were obtained from a standard curve that was determined with standard dilutions (range: 0–2,000 pg/mL). The background absorbance was subtracted from all values.

Statistical analyses

Results are expressed as the means±standard deviation, and the significance of any differences was determined by repeated-measure analysis of variance by ranks with Dunn's multiple comparison test. A P value of <0.05 was accepted as significant.

Results

Injected eyes

The mean VEGF concentration in the aqueous humor was 465.0±78.8 pg/mL (mean±standard deviation; range: 376–568 pg/mL) in the no-rubeosis group and 3,230.3±2,136.8 pg/mL (range: 1,248.5–5,963.0 pg/mL) in the rubeosis group. The higher level in the rubeosis group was highly significant ($P<0.05$; Fig. 2). After 1 day, the VEGF concentration of the injected eyes significantly decreased to 0 pg/mL ($P<0.05$; Fig. 3) in the no-rubeosis group and to 3.1±3.6 pg/mL with a range of 0–6.27 pg/mL in the rubeosis group ($P<0.05$; Fig. 4). The decrease of ocular neovascularization was observed in all eyes at 1 day after the injection.

Fellow eyes

Seven days later, the average VEGF level in the no-rubeosis group was 7.8±13.2 pg/mL with a range of 0–29.7 pg/mL, which was significantly lower than the pre-injection level of the first eye ($P<0.05$; Fig. 3). In the rubeosis group, the average VEGF level in the rubeosis group was 688.5±443.1 pg/mL with a range of 89.1–1,083.2 pg/mL ($P<0.05$; Fig. 4). This was also significantly lower than the

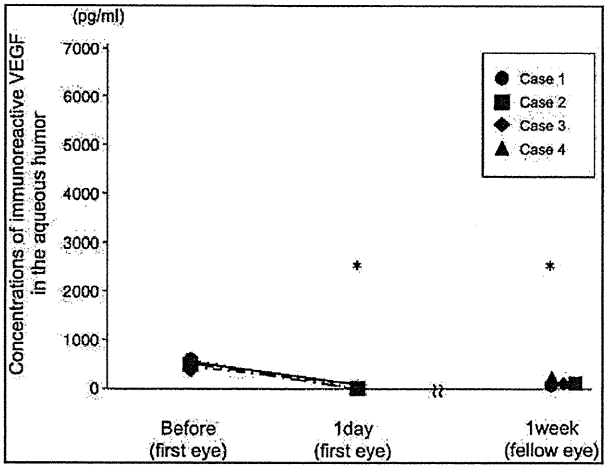

FIG. 3. No-rubeosis group. The VEGF concentration of the injected eyes significantly decreased to 0 pg/mL (*$P<0.05$) after 1 day. After 7 days, the VEGF levels of fellow eyes (7.8±13.2 pg/mL) were significantly lower than that before the injection in the first eye (*$P<0.05$).

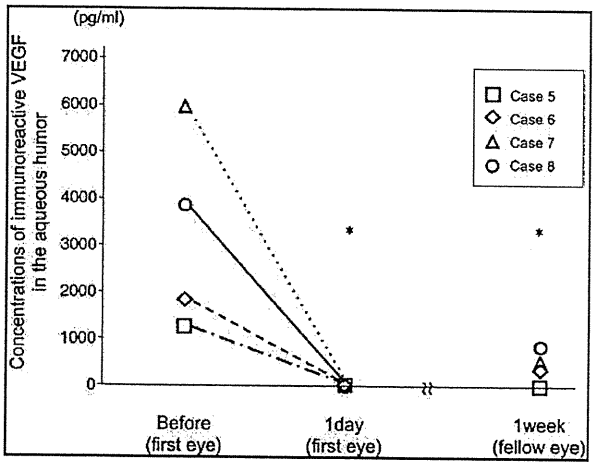

FIG. 4. Rubeosis group. The VEGF concentration of injected eyes significantly decreased to 3.1±3.6 pg/mL with a range of 0–6.27 pg/mL ($P<0.05$) after 1 day. It was 688.5± 443.1 pg/mL with a range of 89.1–1,083.2 pg/mL after 7 days in the fellow eyes. The VEGF levels in the aqueous humor of fellow eyes were significantly lower than that before the injection in the first eye (*$P<0.05$).

preinjection level of the first eye. The decrease of retinal neovascularization was observed in cases 4, 6, and 7 and the decrease of rubeosis was observed in cases 6 and 7.

Discussion

The mean VEGF concentration in the aqueous humor in the eyes with rubeosis was significantly higher than that in the no-rubeosis eyes. This is in agreement with the higher VEGF level in the aqueous humor, which is significantly correlated with the VEGF level in the vitreous and the vitreous level of VEGF is correlated with the severity of diabetic retinopathy and the activity of the PDR.[1,2]

With the increasing use of bevacizumab for VEGF-mediated eye diseases, it is important to know the adverse systemic effects of intravitreal injection. Although experimental data on primates suggested that the full-length antibody might not penetrate the internal limiting membrane of the retina,[15] recent studies have shown that the full-length antibody did penetrate into the rabbit[16] and monkey retinas[17] within 24 hours. In addition, we have shown that the blood level of VEGF was markedly reduced as early as 1 day after the intravireal injection of bevacizumab and it was significantly lower than that of the initial level for as long as 1 month.[11] These results indicated that bevacizumab injected into the vitreous cavity enters the general circulation, where it acts to decrease the level of free VEGF rapidly and the effects lasted for at least 1 month.

Whether an intravitreal injection of bevacizumab in 1 eye will have any effect on the fellow eye has been discussed,[6–8] but it remains undetermined. Miyake et al. examined the effects of an intravitreal injection of bevacizumab in monkey eyes and reported that the aqueous VEGF concentrations in the fellow eyes did not change throughout the experiment.[18] However, they used only 3 normal monkeys. It is known that the VEGF levels in the blood and eyes are quite different in diabetics and normal individuals. Because they used normal monkeys without diabetes, they might not have detected significant differences in the VEGF levels. They also measured aqueous bevacizumab concentrations and reported that it peaked at 3 days, although the levels were low.[18] Even in normal animals, bevacizumab injected into the vitreous was detected in the untreated eye. Thus, it is quite possible that higher levels of aqueous bevacizumab concentrations would be detected in the fellow eyes with diabetic retinopathy because of the higher permeability of the vessels.

The VEGF concentrations in the aqueous humor of the fellow untreated eyes were significantly decreased at 7 days after the injection of bevacizumab, compared with the initial levels of the first eyes both in the no-rubeosis group and the rubeosis group.

Because we did not measure the VEGF concentration in the aqueous humor of the fellow eyes before the injection of bevacizumab into the first eye, we do not know the exact decrease of VEGF concentrations in the fellow untreated eye. However, we selected the patients who had similar clinical findings of PDR in both eyes. Thus, it is quite reasonable to consider that both eyes would have similar VEGF concentrations in the aqueous humor before the first injection of bevacizumab. Therefore, the low level of VEGF in the aqueous humor of the fellow untreated eyes indicates that bevacizumab injected into the vitreous cavity entered the general circulation rapidly and entered the fellow untreated eyes.

In addition, Avery et al. reported that some patients with bilateral PDR had a regression of neovascularization in both eyes when bevacizumab was intravitreally injected in only 1 eye.[4] Our findings support their observations. Intravitreally injected bevacizumab can pass into the general blood circulation and enter the fellow eyes and then can regress neovascularization.

Recently, the adverse systemic complication events after bevacizumab have been discussed,[9,10,19] and the elevation of blood pressure, severe gastrointestinal bleeding leading to hypotension, and myocardial ischemia have been reported.[10,19] A single intravitreal injection of bevacizumab 1.25 mg/0.05 mL was enough to reduce the VEGF levels significantly in the blood[11] and also affects the fellow untreated eye. We do not know how much of the intravitreally injected bevacizumab passes through the eye to the general circulation in patients with diabetic retinopathy. Thus, we need to be more careful about adverse systemic events. Further studies would be necessary for an appropriate dose of intravitreal injection of bevacizumab.

In conclusion, 1.25 mg/0.05 mL of a single intravitreal injection of bevacizumab significantly reduces the concentrations of VEGF in the aqueous humor of the fellow untreated eye. Thus, we need to be more careful about the fellow untreated eyes and examine patients for systemic changes.

Author Disclosure Statement

No competing financial interests exist.

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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; ≤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%

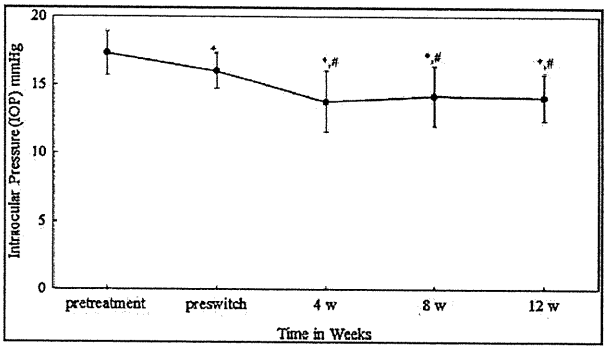

FIG. 1. Reduction in mean IOP after a switch to bimatoprost. Data express the mean±SD. $^{*}P<0.05$ versus pretreatment (paired t test). $^{\#}P<0.05$ versus preswitch (paired t test). IOP, intraocular pressure; SD, standard deviation.

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±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%±5.6%, the mean% IOP reduction from pretreatment to 12 weeks (bimatoprost) was −18.7%±8.9% (Fig. 2). At week 12, 7 patients showed ≥20% IOP decrease, and 2 patients showed ≥30% IOP decrease from pretreatment (Fig. 3). There was a

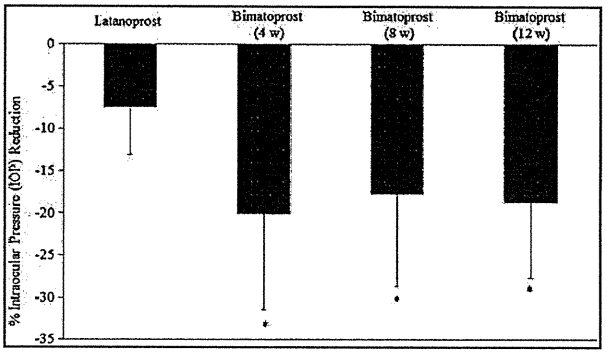

FIG. 2. Mean% IOP reduction from pretreatment to preswitch (Latanoprost) and at week 4, 8, and 12 (Bimatoprost). The mean% IOP reduction rate of bimatoprost was significantly greater than that of latanoprost ($^{*}P<0.01$, paired t test). Data express the mean±SD.

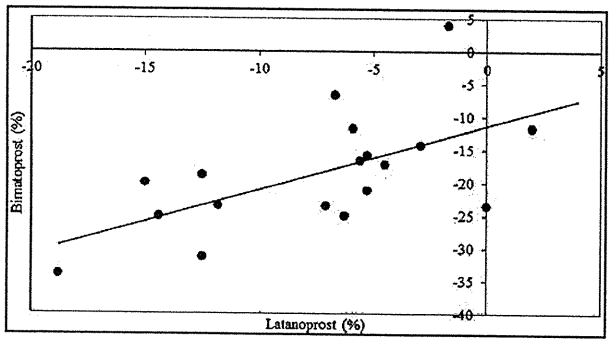

FIG. 3. Correlation between% IOP reduction rate of bimatoprost and latanoprost. % IOP reduction rate of bimatoprost was significantly correlated with that of latanoprost. (Pearson correlation coefficient $R^2=0.374$; $P=0.007$).

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 before 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.

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 ($P>0.99$)

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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RESEARCH ARTICLE

Increased Expression of Tight Junctions in ARPE-19 Cells Under Endoplasmic Reticulum Stress

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ABSTRACT

Purpose: To investigate the effects of endoplasmic reticulum (ER) stress on the tight junctions of the retinal pigment epithelial (RPE) cells *in vitro*.

Materials and Methods: ER stress was induced in cultured ARPE-19 cells, a human RPE cell line, by exposure to tunicamycin (TM) or to thapsigargin (TG). After 6, 12, 24 and 48 hours of exposure, the expressions of GRP78/Bip (Bip), C/EBP-homologous protein (CHOP), vascular endothelial growth factor (VEGF), zonula occludens (ZO)-1, occludin and claudin-1 were determined by real-time RT-PCR. Immunoblot analysis and/or immunohistochemistry for proteins of tight junctions and ER stress markers, viz., Bip, activating transcription factor (ATF) 6, CHOP, and caspase-4, were performed at 48 hours after the exposure. Enzyme-linked immunosorbent assay was used to determine the concentration of VEGF165. Transepithelial electrical resistance (TER) of the ARPE-19 cells was determined to measure the permeability.

Results: The expressions of the mRNAs and/or proteins of Bip, CHOP, ATF6 and caspase-4 were significantly increased in ARPE-19 cells under ER stress induced by TM and TG. The mRNAs of VEGF were also increased by both TM and TG. However, the concentration of VEGF165 was not significantly increased after 48 hours exposure to TM and TG compared to that of the control in the apical chamber medium. The proteins and mRNAs of occludin and claudin-1 were significantly increased by TM and TG, and that of ZO-1 was significantly increased by TG. Immunohistochemistry showed that the staining of ZO-1, occludin and claudin-1 under ER stress was stronger than that of the control. A significant increase of TER was observed after exposure to TM and TG.

Conclusions: The increased expressions of tight junction molecules by TM– or TG-exposed ARPE-19 cells indicate that ER stress can alter the function of RPE cells and may be involved in the pathogenesis of age-related macular degeneration.

Keywords: Endoplasmic reticulum (ER) stress, Tight junction, Age-related macular degeneration (AMD), Retinal pigment epithelium (RPE), Transepithelial electrical resistance (TER)

INTRODUCTION

The endoplasmic reticulum (ER) is an important intracellular organelle responsible for the biosynthesis and folding of proteins.[1] It is also an early signal transduction site that responds to different cellular stressors, such as hypoxia, oxidative stress, glucose starvation and upset of the calcium homeostasis.[2] ER stress results from an accumulation of unfolded or misfolded proteins in the ER which leads to the activation of ER-localized transmembrane proteins, and activating transcription factor 6 (ATF6). ATF6 can then initiate the unfolded protein response (UPR).[3] While transient and low grade ER stress can be overcome by the UPR, persistent and severe ER stress results in the expression of inflammatory genes and apoptosis.[4–6]

ER stress has been linked to the pathogenesis of several diseases, including vascular and

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neurodegenerative diseases such as stroke,[7] diabetes mellitus,[8] Alzheimer disease[9] and Parkinson disease.[10] In the eye, recent studies have shown that ER stress is associated with diabetic retinopathy and age-related macular degeneration (AMD).[11–13] However, little is known about the role of ER stress in retinal damage.

The retinal pigment epithelial (RPE) cell is a highly specialized epithelium that has multifunctional and essential roles in the vertebrate eye.[14] The RPE forms the outer blood–retinal barrier (BRB), and thus controls the flow of solutes and fluid from the choroidal vasculature into the outer retina.[15] Therefore, RPE plays an important role in the pathogenesis of retinal diseases.

The strict control of fluid and solutes across the BRB is achieved through well developed tight junctions. Over 40 proteins have been found to be associated with tight junctions.[16] Zonula occludens (ZO)-1, claudins and occludin are the most studied of these proteins, especially how they are related to the BRB. However, to the best of our knowledge the effect of ER stress on the barrier functions of the RPE has still not been reported. Thus, the purpose of this study was to determine how ER stress affects the expression of the tight junction molecules in the RPE. To do this, we exposed cultured ARPE-19 cells, a human RPE cell line, with two ER stress inducers, tunicamycin (TM) and thapsigargin (TG). TM, a glucosamine-containing nucleoside antibiotic, is an inhibitor of N-linked glycosylation.[17] It inhibits dolichol pyrophosphate-mediated glycosylation of asparaginyl and residues of glycoproteins,[18] which causes an accumulation of unfolded proteins in the ER and induces ER stress.[19] TG, a highly lipophilic sesquiterpene lactone, is the most widely used inhibitor of the sarcoplasmic reticulum calcium-ATPase, which pumps Ca^{2+} into the ER of mammalian cells. The TG-mediated inhibition of the Ca^{2+}ATPases in the ER elevates the intracellular Ca which leads to an accumulation of unfolded protein.[20,21] This then induces ER stress.

We investigated how ER stress affected the expression of ZO-1, claudin-1 and occludin and also the expression of vascular endothelial growth factor (VEGF). We also tested whether ER stress affected the barrier function of ARPE-19 cells. We shall show that ER stress altered the tight junctions and increased the barrier function of RPE.

MATERIALS AND METHODS

RPE Cell Cultures

ARPE-19 cells were purchased from the American type culture collection (ATCC, Manassas, VA) and were cultured in Dulbecco modified Eagle medium/F-12 human amniotic membrane nutrient mixture (DMEM/F-12; Gibco, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone, Thermo Scientific, Rockford, IL). The cultures were grown in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

Preparation of Reagents

TG (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a concentration of 3 mM to produce stock solutions. The stock solutions were diluted with DMEM/F-12 medium to obtain 1 μM TG-containing culture medium. To evaluate the effect of DMSO, medium containing only DMSO (1:5000 of total volume) was also used for each study. For TM (Sigma-Aldrich), 1 μg/ml was used for each study. The concentrations of TG and TM used to treat ARPE-19 cells were based on the results of earlier studies.[12,22]

cDNA Preparation and Real-Time PCR

Real-time PCR was performed after 0, 6, 12, 24 and 48 hours of exposure to TM or TG to detect the mRNA expression of ER stress markers, viz., GRP78/Bip (Bip), C/EBP-homologous protein (CHOP) and VEGF. In addition, the mRNAs of tight junctions, ZO-1, occludin and claudin-1 in ARPE-19 cells, were also determined at the same times.

After exposure to each agent, cDNAs were prepared from the ARPE-19 cells with Fast SYBR® Green Cells-to-CT™ Kit (Ambion, Austin, TX) according to the manufacture's protocol. Briefly, ARPE-19 cells were lysed with DNase I (1:100), reaction was placed in a stop solution, then enrolled to reverse transcription (RT) using 10 μl of cell lysate in a RT Master Mix at 37°C for 60 min and 95°C for 5 min.

Real-time RT-PCR was performed using 4 μl of the prepared cDNA samples with PCR Master Mix with SYBR green (Fast SYBR® Green Cells-to-CT™ Kit, Ambion) and PCR primers on a thermal cycler (Opitcon2, MJ Research, California, USA) according to the manufacturer's instructions. After a denaturation step at 95°C for 20 seconds, amplification by two steps of 40 cycles for 3 seconds at 95°C and 30 seconds at 60°C followed. The concentrations of the PCR products were measured by fluorescence associated with the binding of the double-stranded DNA to the SYBR green dye in the reaction mixture. All experiments were repeated independently four times in triplicate. The sequences of the primers used are listed in Table 1. Quantification of each PCR product was expressed relative to GAPDH.

Western Blot Analysis

Western blot analysis was performed to detect the ER stress markers, viz., Bip, ATF6, CHOP, and caspase-4, and the tight junction proteins, viz., ZO-1, occludin and claudin-1. After exposure to TM or TG for 48 hours, cells were lysed in RIPA buffer (product No. R0278, Sigma-Aldrich) containing protease inhibitors (cocktails

TABLE 1 Primer sequences.

Genes	Oligo names	Sequences
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA
Bip/GRP78	Forward	GGCGTGGTAGTGCAAGCTGA
	Reverse	CCTATCCTTGGGCAGTATTGGATTC
CHOP	Forward	GCGCATGAAGGAGAAAGAAC
	Reverse	TCACAATTCGGTCAATCAGA
ZO-1	Forward	GACCAATAGCTGATGTTGCCAGAG
	Reverse	TGCAGGCGAATAATGCCAGA
Occludin	Forward	AAGAGTTGACAGTCCCATGGCATAC
	Reverse	ATCCACAGGCGAAGTTAATGGAAG
Claudin-1	Forward	CTGACATGTTTGTGCTCTGTTCCA
	Reverse	GCAGCCAAATGCCTTGCTC
VEGFA	Forward	TCACAGGTACAGGGATGAGGACAC
	Reverse	TCCTGGGCAACTCAGAAGCA

of product No. P2850 and 5726, Sigma-Aldrich). The protein concentration was determined by the bicinchoninic acid protein assay (Pierce Biotechnology Inc., Rockford, IL). Ten micrograms of protein were solubilized in by SDS–PAGE sample buffer (Nacalai tesque, Osaka, Japan), separated on SDS-PAGE, then transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). After blocking, the membrane was incubated with specific antibodies, viz., mouse anti-Bip/GRP78 antibody (BD Biosciences, CA, USA, 1:1000), mouse anti-GADD153/CHOP antibody (Santa cruz biotechnology, CA, USA, 1:400), mouse anti-caspase-4 antibody (MBL, Nagoya, Japan, 1:1000), rabbit anti- ATF6 antibody (Abcam, Cambridge, MA, USA, 1:2000), rabbit anti- ZO-1 antibody (Invitrogen, Camarillo, CA, 1:1000), rabbit anti-occludin antibody (Invitrogen, 1:1000), and rabbit anti-claudin-1 antibody (Invitrogen, 1:1000) overnight at 4°C. After washing, the secondary antibodies, viz., goat anti-rabbit HRP-conjugated antibody (Santa cruz biotechnology, 1:2000) or goat anti-mouse HRP-conjugated antibody (Santa cruz biotechnology, 1:2000) were applied for 15 min at room temperature.

The specific protein bands were made visible with an enhanced chemiluminescence detection system (super signal west femto maximum sensitivity substrate, Thermo Scientific) and were measured using a digital image analyzer (LAS4000mini, FujiFilm, Tokyo, Japan). The same membranes were blotted with HRP-conjugated goat anti-actin polyclonal IgG antibody (Santa cruz biotechnology, 1:10,000) to normalize the protein levels. All experiments were repeated independently four times in triplicate.

Immunohistochemistry

Immunohistochemistry was performed in cells grown on a circular culture cover glass (13 mm of diameter) coated with poly-L-lysin in 24-well plates (Matsunami Glass Ind., Ltd., Osaka, Japan). ER stress was induced by exposure of the cells to TM or TG for 48 hours. The cells were then washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, washed with 50 mM glycine in PBS three times, permeabilized by 0.1% TritonX-100 in PBS for 10 min, washed with 50 mM glycine in PBS again and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature.

The cover slips were exposed to rabbit anti-ZO-1, occludin, or claudin-1 antibodies (Invitrogen, 1:250) for 30 min at 37°C. After washing with 0.1% BSA in PBS, cells were further incubated with goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Invitrogen) for 30 min at 37°C, then washed with 0.1% BSA in PBS. Immunohistochemistry was performed in four independent experiments. The cells were mounted on microscope slides with Aqua-Poly/Mount (Polysciences, Inc, Warrington, PA, USA). Photographs were taken with a confocal laser scanning fluorescence microscope (BX51, Olympus, Tokyo, Japan).

Measurement of Transepithelial Electrical Resistance (TER)

ARPE-19 cells were grown on microporous filter membranes (0.4 μm pore size and 6.5 mm in diameter; Transwell, Costar, Corning Inc., Corning, NY) of apical chambers. The membrane was supported in 24-well culture plates. The cultures were started at a concentration of 3×10^4 cells/well and grown to confluence with exposure to TM or TG for 48 hours. The TER was measured with an epithelial voltmeter (EVOM2, World Precision Instruments, Sarasota, FL, USA) with electrodes (STX2, World Precision Instruments) according to the manufacturer's instructions.

The TER values were determined by subtracting the resistance of the filter alone (background) from the values obtained with the filters and the RPE cells. The resistance/unit area was equal to the resistance (ohm) × effective membrane area (cm^2). Measurements were repeated at least twice for each well in four independent experiments, and each experiment was repeated for at least five different wells.

Enzyme-Linked Immunosorbent Assay (ELISA)

ARPE-19 cells were seeded in 24-well culture plates in the same way as that used to measure the TER. The supernatants of the apical and basolateral chambers were collected and stored at −20°C. ELISA was performed using a human VEGF ELISA kit (Quantikine; R&D Systems, Mineapolis, MN, USA), according to the manufacturer's instructions. ELISA was performed in triplicate in three independent experiments.

Statistical Analyses

Data were statistically analyzed by a one-way ANOVA, and results are expressed as means ± standard error of the means (SEMs). A P value < 0.05 was accepted as significant.

RESULTS

Induction of ER stress markers, Bip, ATF6, CHOP, and caspase-4, after exposure to TM or TG

Bip is an established ER-resident chaperon, which binds to the ER stress sensors, but binds more stably to misfolded or unfolded proteins.[3] Thus, an up-regulation of Bip is one of the most commonly used markers of ER stress. CHOP, a commonly used marker of ER stress, is a member of the CCAAT/enhancer-binding protein family that is induced by ER stress. It is a sign of an activation of the UPR and participates in ER-mediated apoptosis.[23]

Therefore, we first investigated the expressions of Bip and CHOP in the ARPE-19 cells exposed to TM or TG. The expressions of Bip and CHOP mRNA were significantly up-regulated by TM exposure after 6, 12, 24, and 48 hours (Bip, 4.1-fold, 4.5-fold, 4.1-fold, and 5.1-fold; $P<0.001$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively: CHOP, 44.2-fold, 47.2-fold, 52.1-fold, and 88.4-fold; $P<0.001$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively) compared to that of the initial levels (Figure 1A).

The expressions of the mRNAs of Bip and CHOP were also significantly up-regulated by TG exposure after 6, 12, 24, and 48 hours (Bip, 5.4-fold, 7.2-fold, 6.7-fold, and 9.5-fold; $P<0.001$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively; CHOP, 44.2-fold, 82.6-fold, 84.4-fold, and 83.0-fold; $P<0.001$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively) compared to that of the initial levels (Figure 1B). These results indicate that ER stress was significantly up-regulated by TM and TG.

The results of Western blot analyses are shown in Figure 1C. The expressions of Bip, ATF6, CHOP, and caspase-4 proteins were increased by after exposure to TM and TG compared to that of the controls (Figure 1C). The level of Bip was increased to 2.0-fold by TM ($P<0.05$) and to 2.3-fold by TG ($P<0.01$, Figure 1D). ATF6 was increased to 3.6-fold by TM ($P<0.01$) and to 2.2-fold by TG ($P<0.05$, Figure 1D), CHOP was increased to 40.1-fold by TM ($P<0.001$) and to 46.8-fold by TG ($P<0.05$, Figure 1D), and cleaved caspase-4 was increased to 6.9-fold by TM ($P<0.05$) and to 14.7-fold by TG ($P<0.01$, Figure 1D).

VEGF Induction Under ER Stress-Induced by TM or TG

After 6, 12, 24, and 48 hours exposure to TM or TG, the expression of the mRNA of VEGF was up-regulated in time dependent way. It was significantly increased at 24 and 48 hours (TM, 2.1-fold at 24 hours, 3.8-fold at 48 hours; $P<0.001$, $P<0.001$, respectively; TG, 4.2-fold at 24 hours, 4.6-fold at 48 hours; $P<0.001$, $P<0.001$, respectively, Figure 2A and 2B).

The results of ELISA are shown in Figure 2C and 2D. In culture medium of the apical chamber, the concentration of VEGF165 was significantly increased after 24 hours exposure to TM and TG compared to that of the control (control, 327 pg/ml; TM, 904 pg/ml, $P<0.01$; TG 1100 pg/ml, $P<0.001$, respectively, Figure 2C) but the increase was not significant after 48 hours (control, 1541 pg/ml; TM 1897 pg/ml; TG 1886 pg/ml, respectively, Figure 2C). In culture medium of basolateral chamber, the concentration of VEGF165 was significantly increased after 24 and 48 hours exposure to TG but the increase was not significant after 24 hours exposure to TM (control, 55 pg/ml at 24 hours; TM, 61 pg/ml at 24 hours; TG 199 pg/ml at 24 hours, $P<0.05$; Control 285 pg/ml at 48 hours; TM 839 pg/ml at 48 hours, $P<0.001$; TG 1173 pg/ml at 48 hours, $P<0.001$, respectively, Figure 2D).

Induction of Tight Junction Components Under ER Stress Induced by TM or TG

The expression of the mRNA of ZO-1 was increased under ER stress induced by TM, but the increase was not significant (Figure 3A). However, the expressions of the mRNA of occludin and claudin-1 were significantly up-regulated by TM at 6, 12, 24, and 48 hours (occuldin, 2.1-fold, 3.5-fold, 4.7-fold, and 5.9-fold; $P<0.01$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively; claudin-1, 3.1-fold, 3.2-fold, 3.9-fold, and 3.8-fold; $P<0.001$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively) compared to that of the initial levels (Figure 3A).

The ER stress-induced by TG increased the mRNA expression of ZO-1 and it was significantly up-regulated after 24 and 48 hours (2.6-fold, 3.1-fold, $P<0.01$, $P<0.001$, respectively; Figure 3B). The mRNA expressions of occludin and claudin-1 were also up-regulated by TG exposure, and they were significantly up-regulated after 12, 24 and 48 hours compared to that of the initial levels (occuldin, 4.0-fold, 8.8-fold, 8.4-fold; $P<0.01$, $P<0.001$, $P<0.001$, respectively; claudin-1, 4.1-fold, 6.8-fold, 7.4-fold; $P<0.05$, $P<0.001$, $P<0.001$, respectively; Figure 3B).

The results of Western blot analyses are shown in Figure 3C and 3D. The expression of the protein of ZO-1 under ER stress was significantly increased by TG (2.8-fold, $P<0.05$) but the increase was not significant after TM (1.7-fold, $P>0.05$, Figure 3C and 3D). The protein levels of occludin and claudin-1under ER stress were significantly increased by TM and TG compared to that of the control (Figure 3C). The level of occludin was increased to 2.8-fold by TM ($P<0.05$) and to 2.7-fold by TG ($P<0.05$, Figure 3C and 3D), and claudin-1 was

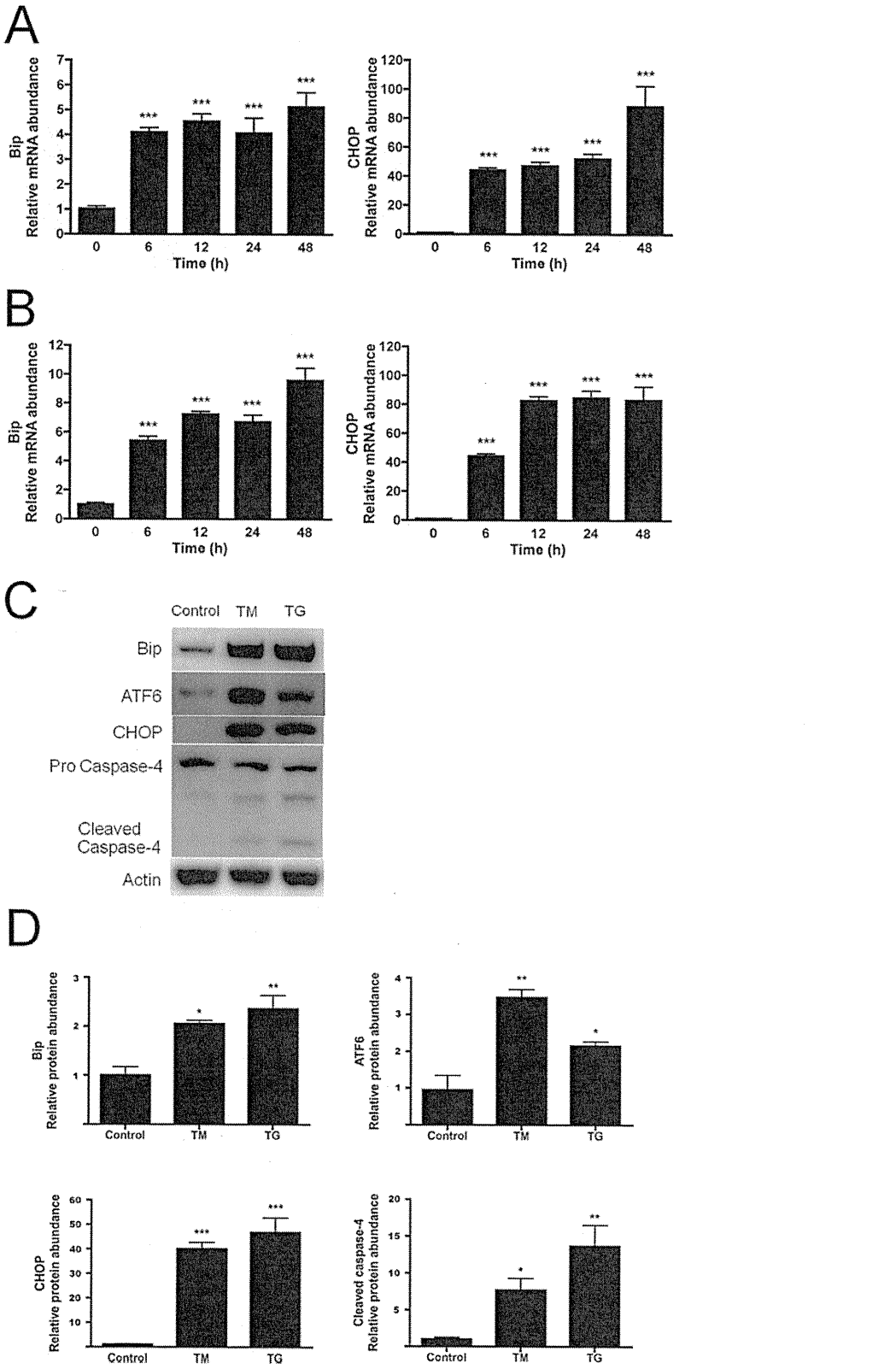

FIGURE 1 Induction of ER stress markers, Bip, ATF6, CHOP and caspase-4. (A) Expressions of Bip mRNA and CHOP mRNA are significantly up-regulated 6–48 hours after the TM exposure compared to that of the initial levels. (B) Expressions of Bip and CHOP mRNA are significantly up-regulated 6–48 hours after the TG exposure compared to that of the initial levels. (C) Results of Western blot and (D) densitometric analysis of Western blot. The levels of Bip, ATF6, CHOP, and caspase-4 proteins are increased by TM and TG. Data are presented as the means ± SEMs. ***$P < 0.001$.

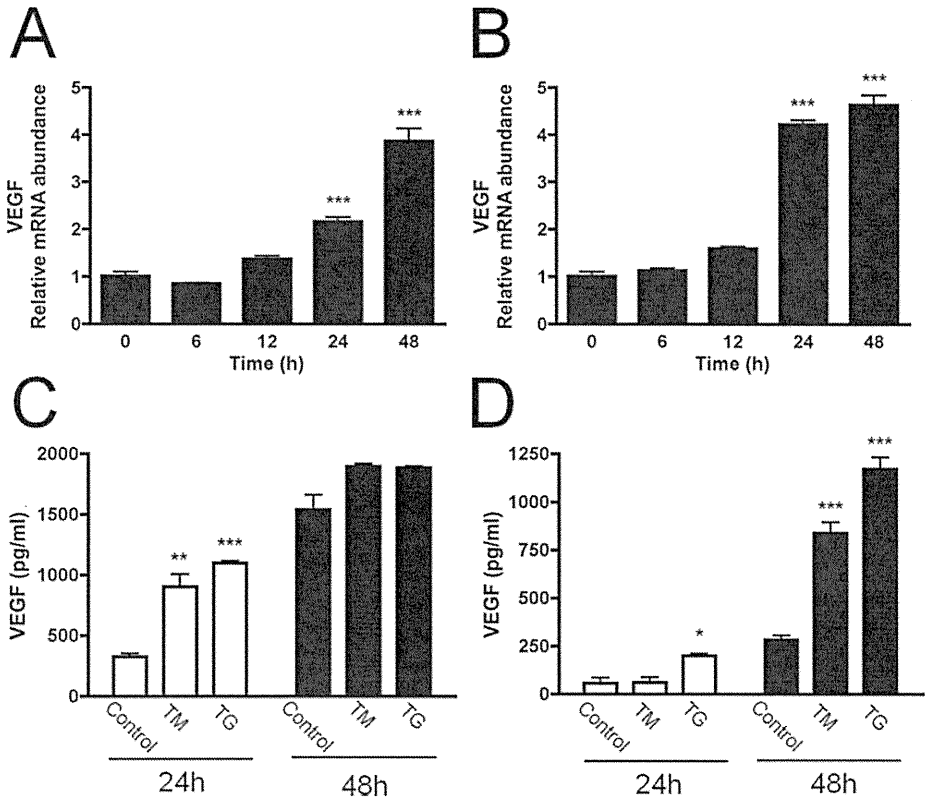

FIGURE 2 VEGF induction under ER stress-induced by TM or TG. (A) (B) After 6, 12, 24 and 48 hours TM (A) and TG (B) exposure, the expression of VEGF mRNA is up-regulated in a time dependent way, and it is significantly increased at 24–48 hours. (C) In apical chamber medium, the concentration of VEGF165 was significantly increased after 24 hours but the increase was not significant after 48 hours. (D) In the basolateral chamber medium, the concentration of VEGF165 was significantly increased after 48 hours. Data are presented as the means ± SEMs. *=$P < 0.05$, **=$P < 0.01$, ***=$P < 0.001$.

increased to 13.6-fold by TM ($P<0.05$) and to 10.9-fold by TG ($P<0.05$, Figure 3C and 3D).

Immunohistochemistry for Tight Junction Components Under ER Stress

Immunohistochemistry was also performed in monolayered cells to detect the alterations of ZO-1, occludin, and claudin-1 expressions under ER stress for 48 hours (Figure 4). The staining of the junctions for each peptide was weak in the controls. ER stress induced by TM or TG increased the intensity of staining for ZO-1, occludin, and claudin-1 to higher levels than that of the control (Figure 4). Throughout the experiments, cell death was not detected.

Measurement of TER

The cells grown under for 48 hours of ER stress induced by TM or TG had higher TER values than that of the control (TM, 2.2-fold, $P<0.001$; TG, 1.7-fold, $P<0.001$, Figure 5). This significant increase in the TER values indicated that the permeability through the monolayer of RPE cells was reduced under ER stress.

DISCUSSION

ER stress has been suggested to be the cause of a broad range of diseases involving the accumulation of unfolded or misfolded proteins in the ER.[7-10] Several studies have shown that ER stress up-regulates the expression of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, in different cultured cell lines.[5,24] Thus, ER stress would possibly be a cause of inflammation.[6,24]

The integrity of the intercellular junctions of RPE cells can be affected by several pro-inflammatory cytokines, e.g., TNF-α, interleukin-1β, hepatocyte growth factor, and placental growth factor-1.[25–28] These cytokines decrease the TER, increase the permeability, and alter the expression or content of the tight junction molecules.[25–28] The expression of VEGF mRNA in ARPE-19 cells up-regulated by ER stress has been reported.[12] VEGF has been reported to decrease the expression and the function of tight junctions.[29] The tight junction proteins occludin and claudin-1 could not be detected, and ZO-1 was weakly affected in the microvascular retinal endothelial cells exposure to VEGF165.[29] In addition, exposure of ARPE-19 cells to VEGF induced a significant drop in the TER.[30]