

FIGURE 1. Bevacizumab concentration in the aqueous humor, iris/ciliary body, vitreous humor, and retina/choroid after topical administration of bevacizumab (1.25 mg/0.05 mL; six times daily for the first 7 days) in the treated (A) and fellow (B) eyes.

start of topical drug administration. In the vitreous, a very low level of bevacizumab was detected (0.4 ± 0.2 ng/mL, both 1 and 2 weeks after the start of topical drug administration) and bevacizumab was not detected in the aqueous.

Subconjunctival Injection. The concentrations of bevacizumab in the aqueous humor, iris/ciliary body, vitreous humor, and retina/choroid after subconjunctival administration are shown in Table 1 and Figure 2. In the treated eyes (Fig. 2A), the C_{max} in the iris/ciliary body and retina/choroid was 1418.7 ± 359.2 and 295.8 ± 48.1 ng/g at 1 week after administration, respectively. The $T_{1/2}$ in the iris/ciliary body, and retina/choroid was 1.80 and 2.85 weeks, respectively. A low level of bevacizumab was detected in the aqueous (31.0 ± 5.2 ng/mL at 1 week after administration) and the vitreous (11.1 ± 1.0 ng/mL at 1 week) humors.

On the other hand, in the fellow eyes (Fig. 2B), the C_{max} of bevacizumab in the iris/ciliary body and retina/choroid were 1192.9 ± 80.8 and 187.0 ± 26.7 ng/g at 1 week after administration, respectively. A low level of bevacizumab was detected in the aqueous (11.9 ± 2.3 ng/mL at 1 week after administration) and the vitreous (9.3 ± 0.6 ng/mL at 2 weeks) humors.

Intravitreal Injection. The concentrations of bevacizumab in the aqueous humor, iris/ciliary body, vitreous humor, and retina/choroid after intravitreal administration are shown in Table 1 and Figure 3. In the treated eyes (Fig. 3A), the C_{max} of bevacizumab in the aqueous humor, iris/ciliary body, vitreous humor, and retina/choroid were 373.6 ± 150.6 ng/mL,

$109,192.6 \pm 13,273.1$ ng/g, $59,730.8 \pm 10,552.7$ ng/mL, and $93,990.0 \pm 38,271.6$ ng/g, at 1 week after administration, respectively. The $T_{1/2}$ in the iris/ciliary body, and retina/choroid were 0.82 and 0.89 weeks, respectively.

In the fellow eyes (Fig. 3B), a very low level of bevacizumab was detected in the aqueous (5.4 ± 5.4 ng/mL at 1 week) and vitreous (6.7 ± 6.7 ng/mL at 2 weeks) humors. The C_{max} of bevacizumab in iris/ciliary body and retina/choroid were 753.6 ± 221.9 ng/g at 2 weeks and 224.2 ± 34.3 ng/g at 1 week after administration, respectively.

Systemic Pharmacokinetics

The concentrations of bevacizumab in the plasma from each of the three types of administration routes are shown in Table 1 and Figure 4.

In the case of eye drops, the C_{max} in the plasma was 14.3 ± 6.4 ng/mL at 1 week after administration. For subconjunctival injection, the C_{max} was 3733.1 ± 174.9 ng/mL at 1 week after injection and $T_{1/2}$ was 1.75 weeks. For intravitreal injection, the C_{max} was 2087.2 ± 200.8 ng/mL at 2 weeks after injection and $T_{1/2}$ was 1.85 weeks.

Contribution of Topical and Systemic Absorption in the Iris/Ciliary Body and Retina/Choroid of the Treated Eye

Figure 5 shows the percentage of bevacizumab concentration in the iris/ciliary body and retina/choroid delivered by topical

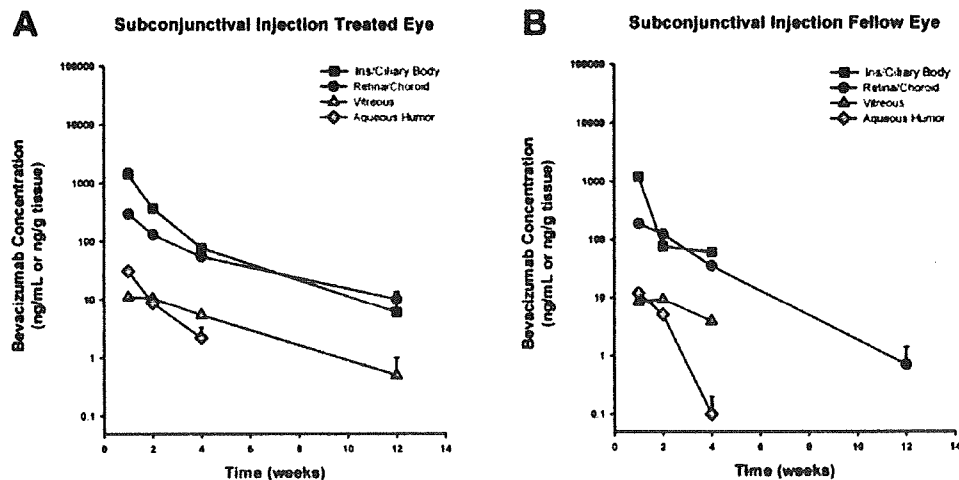


FIGURE 2. Bevacizumab concentration in the aqueous humor, iris/ciliary body, vitreous humor, and retina/choroid after subconjunctival injection (1.25 mg/0.05 mL) into the treated (A) and fellow (B) eyes.

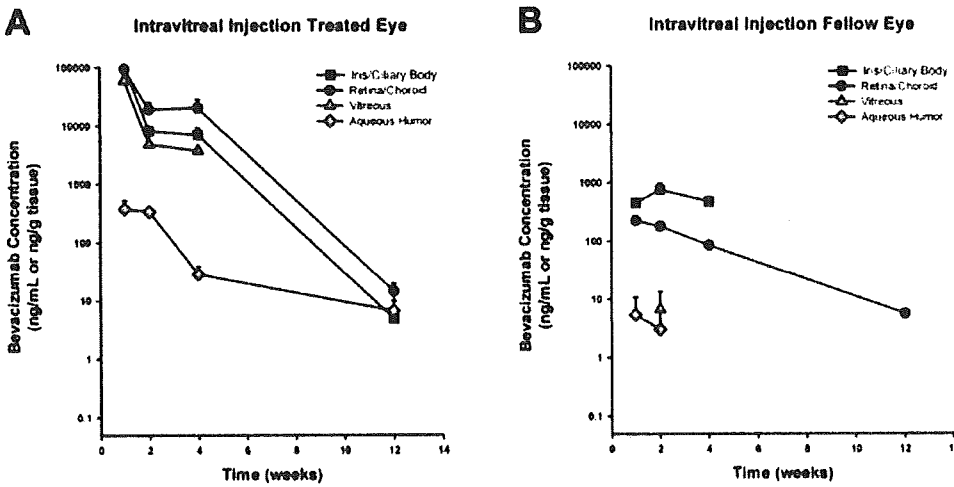


FIGURE 3. Bevacizumab concentration in the aqueous humor, iris/ciliary body, vitreous humor, and retina/choroid after intravitreal injection (1.25 mg/0.05 mL) into the treated (A) and fellow (B) eyes.

and systemic absorption when administered by the three different routes. In the case of intravitreal injection, most of the bevacizumab detected in the retina/choroid and iris/ciliary body was a result of the direct injection of bevacizumab, with less than 1% obtained from the systemic circulation. For subconjunctival injection, 20% and 40% of the total absorbed bevacizumab in the iris/ciliary body and the retina/choroid, respectively, penetrated through the sclera. For the eye drops, 30% and 50% of the total absorbed bevacizumab in the iris/ciliary body and the retina/choroid, respectively, were absorbed via the transcorneal and/or transscleral route. Therefore, after subconjunctival injection or eye drops, most of the bevacizumab in the iris/ciliary body and retina/choroid was absorbed systemically.

DISCUSSION

Other studies have described the pharmacokinetics of bevacizumab after intravitreal injection of bevacizumab in rabbits¹⁹ and humans (Csaky KG, et al. *IOVS*. 2007;48:ARVO E-Abstract 4936).^{33,34} It is important to understand the pharmacokinetics of drugs administered via two additional routes because there have been reports of increased bioavailability of bevacizumab

by subconjunctival injection²⁴⁻²⁷ and topical solution.²⁸⁻³⁰ In this study, we compared bevacizumab pharmacokinetics in ocular tissue and plasma after administration by repeated doses in eye drops, a single subconjunctival injection, or a single intravitreal injection. It should be noted that our rabbit study model is somewhat different from the human eye, as the rabbit vitreous volume is smaller, the lens is larger, and the retina is thinner, is avascular, and has no fovea. Therefore, the behavior of bevacizumab in human eyes may differ from that in rabbit eyes. Moreover, the actual therapeutic concentration of bevacizumab for the treatment of iris, retinal, and choroidal neovascularization is still unknown. Results in *in vitro* studies, however, have suggested that a molar ratio of 2.6 to 1 of bevacizumab to VEGF₁₆₅ is necessary for maximum inhibition of endothelial proliferation. The half-maximum inhibitory concentration of bevacizumab (IC₅₀) is 22 ng/mL, and the minimum concentration that completely blocks VEGF activity, including VEGF-induced endothelial cell growth, migration, and hyperpermeability, is 500 ng/mL.³⁵

Our study showed that the most effective administration route to the retina/choroid and iris/ciliary body was intravitreal injection. Csaky et al. showed that bevacizumab elimination

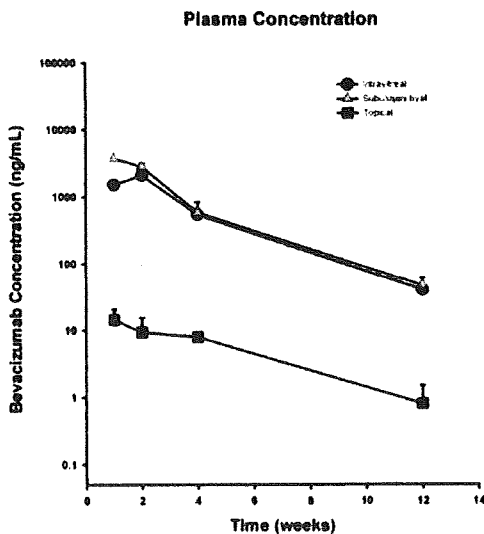


FIGURE 4. Bevacizumab concentration in the plasma after topical, subconjunctival, and intravitreal injection (1.25 mg/0.05 mL).

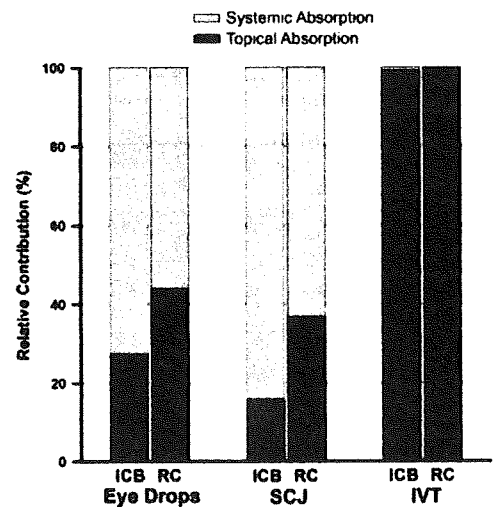


FIGURE 5. Relative contribution of topical and systemic absorption in the retina/choroid and iris/ciliary body of the treated eyes when administered by topical, subconjunctival, and intravitreal injection. ICB, iris/ciliary body; RC, retina/choroid; SCJ, subconjunctival injection; IVT, intravitreal injection.

followed first-order kinetics with $T_{1/2}$ in the human vitreous of approximately 10 days (Csaky KG, et al. *IOVS*. 2007;48:ARVO E-Abstract 4936). In contrast, Zhu et al.³³ reported that the pharmacokinetics of intravitreal bevacizumab follow a two-compartment model with initial and terminal $T_{1/2}$ of 0.5 and 6.7 days, respectively. Our half-life results in rabbits were slightly shorter ($T_{1/2} \sim 6$ days) compared with the results of Csaky et al. and Zhu et al. in humans.

In addition, Bakri et al.¹⁹ showed in rabbits that after a 1.25-mg intravitreal injection of bevacizumab, in the treated eye, the $T_{1/2}$ in the aqueous and vitreous humors was 4.88 and 4.32 days, respectively. In contrast, in our rabbit study, $T_{1/2}$ in the vitreous after direct injection was approximately 6 days.

The differences in our results and those of these previous studies could be explained by a difference in ocular anatomy as well as detection methods for bevacizumab. Csaky et al. (*IOVS*. 2007;48:ARVO E-Abstract 4936) and Zhu et al.³³ performed their studies in humans, whereas we used rabbits. The vitreous volume of rabbits is approximately 1.5 mL, which is only one third the volume in humans. A larger vitreous volume may need a longer duration for equal distribution and therefore possibly a longer $T_{1/2}$ in the human vitreous. This notion would be in accordance with the comparison of the pharmacokinetics of triamcinolone acetonide in rabbits³⁶ and humans.³⁷

Furthermore, although we and Bakri et al.¹⁹ both used rabbits, the pharmacokinetic parameters in Bakri et al. differed from those in our study in that their method detected only free bevacizumab. In this study, we used the rabbit anti-human IgG (H+L) (AffiniPure; Jackson ImmunoResearch, Inc.) which has the ability to detect both the heavy and light chains of bevacizumab. Therefore, our assay had the ability to detect three possible variants of bevacizumab in vitro: fragments of the bevacizumab molecules, the entire VEGF-bevacizumab complex, and free bevacizumab.

In the retina/choroid and iris/ciliary body after intravitreal injection, bevacizumab concentration above IC_{50} was maintained for approximately 11.7 and 10.3 weeks, respectively, whereas that above 500 ng/mL was maintained for 7.7 and 6.6 weeks. A recent report regarding long-term follow-up results for intravitreal bevacizumab treatment of neovascular age-related macular degeneration has described the mean number of injections during 1 year as 3.4.¹ Thus, a single intravitreal injection of bevacizumab is active for approximately 3 months. Our results in rabbits showed bevacizumab concentration in the retina/choroid after intravitreal injection were maintained for approximately 3 months (11.7 weeks) in the effective range and were consistent with reinjection intervals in clinical situations.

Subconjunctival injection is also an effective mode of administration for intraocular neovascular diseases. In general, drug injected into subconjunctival space has two fates: direct transscleral delivery into intraocular tissues or clearance via conjunctival blood and lymphatic flow.³⁸⁻⁴⁰ Since IgG has a relatively high scleral permeability and has the same molecular weight as bevacizumab, some of the bevacizumab injected into the subconjunctival space may penetrate intraocular tissues including the retina/choroid, iris/ciliary body, and vitreous via the sclera.^{41,42} The bevacizumab level in the retina/choroid and iris/ciliary body was maintained above IC_{50} for 8.6 and 8.4 weeks, respectively, whereas it was maintained above 500 ng/mL for 0.3 weeks in the iris/ciliary body. The sclera consists of collagen and elastin chains that create a fiber matrix in which the pore diameter and intracellular space may determine the permeability of drugs. Negatively charged drugs have been found to have higher permeability than those with positive charges in bovine and porcine sclera.^{43,44} Proteoglycans in the sclera are negatively charged,⁴⁵ which may contribute to the binding of positively charged molecules. An isoelectric

point of bevacizumab has been found to be approximately 8.4.⁴⁶ Therefore, longer $T_{1/2}$ in the iris/ciliary body and retina/choroid after subconjunctival injection compared with those after intravitreal injection may sustain bevacizumab delivery into intraocular tissues due to scleral depot binding of bevacizumab to the scleral matrix.

Since the conjunctival blood vessels do not form a tight junction barrier,⁴⁷ bevacizumab can enter into the blood circulation by pinocytosis and/or convective transport through paracellular pores in the vascular endothelial layer. The Fc receptor, which binds to both albumin and the Fc portion of IgG,^{48,49} was detected in the lymphatic vessels but not in the blood vessels of the conjunctiva.⁵⁰ It may be that the function of the Fc receptor in the conjunctival lymphatic vessels is to act as an efflux receptor for the efficient elimination from the conjunctival space. Residual bevacizumab, other than the bevacizumab that directly permeated the sclera and was introduced into the blood circulation, may be eliminated from the conjunctival tissue into the lymphatic vessels via convective transport with lymphatic fluid.⁵¹

Systemic exposure of bevacizumab when administered by intravitreal and subconjunctival injection was very similar. Bevacizumab was detected in the fellow eyes treated by intravitreal or subconjunctival injection. Bevacizumab may have been transported through the systemic circulation into the fellow eyes. In the fellow eyes treated by intravitreal injection, bevacizumab concentration in the retina/choroid was maintained above IC_{50} for 8.0 weeks.

In the case of subconjunctival injection, our data showed that most of the bevacizumab in the treated eyes was derived from the systemic circulation, and so the retinal and choroidal pharmacokinetics profile in the fellow eyes was similar to that in the treated eyes. In the fellow eyes, bevacizumab concentration in the retina/choroid was maintained above IC_{50} for 5.2 weeks. In the iris/ciliary body, a similar profile was observed, but in the fellow eye, bevacizumab was not detected at 12 weeks after injection. Of interest, corneal deposition of bevacizumab for 4 weeks after subconjunctival injection was observed by immunohistochemistry (data not shown). Therefore, subconjunctival bevacizumab may be a promising treatment, not only for neovascular diseases of the iris, retina, and choroid but also for corneal neovascularization.

In this study, we concluded that topical administration of bevacizumab by eye drops was not effective for the treatment of intraocular neovascular diseases because of poor distribution in the target tissues. These results are in accordance with data that showed that a monoclonal antibody cannot penetrate the cornea from an in vitro chamber system.⁵² Since previous reports showed that topically applied bevacizumab was effective for corneal neovascularization,²⁸⁻³⁰ further studies are needed regarding the pharmacokinetics of drugs administered to the cornea in eye drops.

There are several reports that have noted the systemic adverse effects of bevacizumab after intravitreal injection.⁵³⁻⁵⁵ These adverse effects are similar to the ones reported for intravenous administration of bevacizumab for cancer treatment, such as systemic hypertension, thromboembolic diseases and death.^{56,57} In addition, Shima et al.⁵⁵ found irregular vaginal bleeding as a complication in young women receiving intravitreal injection of bevacizumab. On the other hand, there have been no complications reported in previous studies of bevacizumab injected subconjunctivally at a concentration of 2.5 mg/0.1 mL,²⁴⁻²⁷ which is twice the dose that we used in the current study. It should be noted, however, that these were small studies with a small amount of participants. Our results suggest that systemic adverse effects of bevacizumab may occur after subconjunctival injection, as well as after intravitreal or intravenous injection. Therefore, intravitreal or subconjunc-

tival bevacizumab should be used in elderly patients with choroidal or iris neovascularization. However, efficacy in the fellow eye should be expected to derive from the systemic circulation.

In conclusion, in this study we investigated the pharmacokinetics of bevacizumab in intraocular tissues and plasma by three different routes of administration. First, we found that intravitreal injection of bevacizumab was the most effective route of administration for intraocular tissue. Second, we found that both intravitreal and subconjunctival injections of bevacizumab resulted in a high concentration in plasma, and bevacizumab was distributed into the intraocular tissues in fellow eyes via systemic circulation at a level that may be effective in blocking VEGF activity. In addition, when administered by subconjunctival injection, bevacizumab was transported systemically into intraocular tissues of treated eyes at an effective level. More research is needed to define the ideal dose of bevacizumab, administration route, and intervals that will achieve the best clinical outcome possible without systemic side effects.

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Neuroprotective Effects of Angiotensin II Type 1 Receptor Blocker in a Rat Model of Chronic Glaucoma

Hongwei Yang,^{1,2} Kazuyuki Hirooka,¹ Kouki Fukuda,¹ and Fumio Shiraga¹

PURPOSE. To investigate the neuroprotective effect of candesartan, an angiotensin II type 1 receptor (AT1-R) blocker, against the neurotoxicity of the retinal ganglion cells (RGCs) in an animal model of glaucoma.

METHODS. Cauterization of three episcleral vessels in rats was used to create chronically elevated intraocular pressure (IOP) in one eye. Rats were then treated orally with candesartan (1 mg/kg/d). At 10 weeks, immunohistochemistry was used for quantification of RGC survival and examination of retinal localization of AT1-R.

RESULTS. Compared with the contralateral control eyes, there was a consistently elevated IOP of approximately 2.5-fold during the experimental period. At the end of the 10-week candesartan treatment, there were no changes noted for the blood pressure. Compared with the contralateral control eyes that had normal IOP, the RGC survival rate in the central retina of eyes with the chronic, elevated IOP was $46.5\% \pm 19.4\%$ (mean \pm SD) in the untreated animals and $84.2\% \pm 4.9\%$ in the candesartan-treated animals ($P < 0.05$; unpaired *t*-test). In the retina of the normal IOP rat eyes, retinal vessels were positive for AT1-R. After 10 weeks of IOP elevation, immunohistochemical analysis of the retina indicated there were many AT1-R-positive RGCs in the candesartan-treated rat, whereas there was an apparent AT1-R decrease in the vehicle-treated rats.

CONCLUSIONS. In the rat chronic glaucoma model, continuous pharmacologic treatment using candesartan results in significant neuroprotection against RGC loss. (*Invest Ophthalmol Vis Sci.* 2009;50:5800-5804) DOI:10.1167/iovs.09-3678

Primary open-angle glaucoma, which is one of the leading causes of vision loss in the world, is an optic neuropathy that is associated with elevated intraocular pressure (IOP). Current glaucoma treatments are based on attempts to lower elevated IOP to slow or stop the progressive loss of the visual field that occurs as a result of optic nerve degeneration and the subsequent loss of retinal ganglion cells (RGCs). However, glaucomatous damage progresses in many of these cases despite adequate control of the IOP.¹ Consequently, many investigators are trying to find a therapeutic modality that not only

stabilizes the IOP at a lower level but that can prevent the death of RGCs or even lead to RGC regeneration.^{2,3}

The renin-angiotensin system (RAS) plays an important part in the control of blood pressure and electrolyte homeostasis. Recent evidence suggests that in addition to the circulating RAS, there is tissue or local RAS in the vasculature, adrenal gland, kidney, brain, testis, and ovary that also may have a role in the overall control.⁴⁻⁶ Many of the known RAS components have been identified in the human eye.^{4,7-9} Angiotensinogen is an obligatory component for the eventual production of angiotensin II. Angiotensin I and angiotensin II are generated within the circulation by a sequential cleavage of the liver-derived angiotensinogen. Renin, which is synthesized in the kidney, cleaves this substrate, leading to the formation of angiotensin I. Angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II. Angiotensin II is the effector molecule of the system and has two cognate receptors that are designated as angiotensin II type 1 and type 2 receptors (AT1-R and AT2-R, respectively).^{10,11} Because AT1-R mediates the major functions of angiotensin II, its antagonists are widely used to treat patients with hypertension and cardiovascular diseases. Emerging evidence obtained when using the experimental cerebral ischemia model has suggested that peripherally administered AT1-R blockers (ARBs) can cross the blood-brain barrier and interact with AT1-R, thereby reducing the infarct volume.¹²⁻¹⁴ Recently, ARBs have also been proven to attenuate inflammation and oxidative stress in the brain^{15,16} and retina.^{17,18}

The long-term use of ACE inhibitors, which are widely used as antihypertensive drugs, are believed to have a favorable effect on the visual fields in patients with normal-tension glaucoma.¹⁹ Angiotensin II receptor gene polymorphisms have been found in humans, and these may be associated with the risk for glaucoma.²⁰ The purpose of the present study was to investigate the effects of candesartan along with the role of ARBs in RGC degeneration in an animal model of glaucoma.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Charles River Japan, Yokohama, Japan), weighing 180 to 210 g each, were housed in a standard animal room under a 12-hour light/12-hour dark cycle with free access to food and water. All surgical procedures were performed under general anesthesia that used intraperitoneal injections of pentobarbital (40-50 mg/kg Nembutal; Abbott, Abbott Park, IL). Animal care and all experiments were conducted under the approved standard guidelines for animal experimentation of the Kagawa University Faculty of Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two groups of rats that had unilateral elevated IOP were followed up for 10 weeks. The rats in the first group did not receive any treatments for their elevated IOP, whereas rats in the second group underwent 10 weeks of candesartan treatment (1 mg/kg/d; a gift from Takeda Pharmaceutical Co., Ltd., Osaka, Japan), with the initial dose administered on the first day of IOP elevation. Candesartan or water was orally administered to animals on a daily basis via the use of feeding needles.

From the ¹Department of Ophthalmology, Kagawa University Faculty of Medicine, Kagawa, Japan; and the ²Department of Ophthalmology, Shengjing Affiliated Hospital, China Medical University, Shenyang, China.

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Corresponding author: Kazuyuki Hirooka, Department of Ophthalmology, Kagawa University Faculty of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan; kazuyk@med.kagawa-u.ac.jp.

Chronic Elevated IOP Rat Model

We performed cauterization of three episcleral vessels in six rats to unilaterally create the chronic elevated IOP conditions. The contralateral eye served as the comparative control. Incision of the conjunctiva at the equator exposed 3 of the 4 to 5 major trunks that are formed by the limbal-derived veins. Each vessel was then lifted with a small muscle hook and cauterized by direct application of ophthalmic cautery (Tagawa, Tokyo, Japan) against the muscle hook. Successful cauterization was defined as an immediate retraction and subsequent absence of bleeding at the cauterized end of the vessels. The eyes were dressed with 0.3% ofloxacin ophthalmic ointment (Santen, Osaka, Japan).

IOP and BP Measurements

IOP was measured three times in the anesthetized rat using a handheld electronic tonometer (TonoPen XL; Bio-Rad, Glendale, CA), with the mean value used for the analysis. Bilateral IOP measurements were performed within 3 hours and 1 week of the surgery to confirm the unilateral, moderately elevated IOP. Thereafter, IOP was measured weekly.

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured weekly in unanesthetized rats using the tail-cuff method (BP-98A; Softron Co., Tokyo, Japan).

Retrograde Labeling of Retinal Ganglion Cells

At 7 days before kill, fluorescent dye (Fast Blue; Polysciences Inc., Warrington, PA) 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 the left hemispheres. The windows were drilled to a depth of 3.6 mm from the surface of the skull, located 6.8 mm behind the bregma on the anteroposterior axis and 1.5 mm lateral to the midline. Using a Hamilton syringe, 1.5 μ L of 3% fluorescent dye (Fast Blue; Polysciences Inc.) was slowly injected into the bilateral superior colliculi. After suturing the skin over the wound, antibiotic ointment was applied.

Tissue Preparation and Assessment of RGC Survival

Animals were killed by an overdose of pentobarbital (Nembutal) at 1 week after the fluorescent dye (Fast Blue; Polysciences 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 hours 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 the 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 RGCs were visualized under a fluorescence microscope (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 two regions in each quadrant at two different eccentricities, 1 mm (central) and 4 mm (peripheral) away from the optic disc.^{21,22} Imaging software (Image-Pro Plus, version 4.0; Media Cybernetics, Bethesda, MD) was used to count approximately 15% to 20% of the total number of RGCs in each eye. Cell counts were conducted by the same person in a masked fashion, with the identity of the original retinas unknown to the investigator until completion of the cell counts from all the different groups. Changes in the densities of the RGCs were expressed as the RGC survival percentage, which was based on the comparison between the surgical and contralateral control eyes. The specimens that were compared came from different retinal regions of the same animal.

Immunohistochemistry

At 3 or 10 weeks after the initial elevation of the IOP, rats were anesthetized by intraperitoneal injection of pentobarbital and then perfused intracardially with phosphate-buffered saline (PBS). Animals were subsequently perfused with 4% paraformaldehyde in PBS. Retinal sections were rinsed in 100% ethanol twice for 5 minutes each, followed by a separate 95% ethanol and 90% ethanol rinse for 3 minutes. The sections were then washed using PBS, pH 7.4, three times for 10 minutes each, followed by treatment with 0.3% Triton-X-100 in PBS, at pH 7.4, for 1 hour. After further washing three times for 10 minutes each with PBS at pH 7.4, sections were blocked in 3% normal horse serum and 1% bovine serum albumin (BSA) in PBS for 1 hour to reduce the nonspecific labeling. Tissues were incubated overnight at 4°C with primary antibodies in PBS containing 0.5% Triton X-100, 5% normal horse serum, and 1% BSA. Primary antibody was diluted as follows: rabbit polyclonal antibody against human AT1-R (Santa Cruz Biotechnology, Santa Cruz, CA) 1:100. Control sections were prepared by omitting both the primary antibody and the rabbit IgG (1:1000; Vector Laboratories Inc., Burlingame, CA) in PBS containing 0.5% Triton X-100, 5% normal horse serum, and 1% BSA overnight at 4°C. After washing in PBS for 50 minutes, tissues were immersed in the alkaline phosphatase (AP; Vectastain ABC-AP Kit; Vector Laboratories Inc.) for 30 minutes at room temperature, washed in PBS for 15 minutes, and processed using the avidin-biotin complex reagent (ABC-Reagent; Vectastain ABC-AP Kit; Vector Laboratories Inc.) for 1 hour at room temperature. Images were acquired using 40 \times objective lenses (DXM 1200; Nikon, Tokyo, Japan). Graphics editing software (PhotoShop version 5.0; Adobe, San Jose, CA) was used to adjust the brightness and contrast of the images.

Statistical Analysis

Statistical analysis was performed (SPSS for Windows; SPSS Inc., Chicago, IL). A paired *t*-test was used to determine the statistical significance of the BP measurements. An independent Student's *t*-test was used to compare the RGC survival rate and IOP measurements. *P* < 0.05 was considered statistically significant. All statistical values are presented as the mean \pm SD.

RESULTS

Intraocular Pressure and Blood Pressure

There was no significant difference noted between the baseline IOP values of vehicle- and candesartan-treated rats. Elevated IOP was observed over the 10-week experimental period in all eyes that underwent the three-vessel cauterization (Fig. 1). Three hours after the surgery, IOP was elevated to approximately 26 mm Hg (range, 23.7–30.3 mm Hg), and it was consistently elevated approximately 2.5-fold compared with the contralateral control eyes. In both groups, the elevated IOP was significantly higher than in contralateral control eyes. The elevation in the IOP was essentially the same between the candesartan-treated and the nontreated animals. Our success rate was 6 of 8 animals. Animals in which elevated IOP were not maintained for more than 2 weeks were excluded from the subjects.

The changes in the SBP and DBP are shown in Figure 2. Treatment with vehicle did not affect either the SBP or the DBP at week 10 in the rats (118.8 \pm 6.9 mm Hg and 93.1 \pm 6.4 mm Hg, respectively). Furthermore, there were no changes seen in the SBP and DBP in the rats at 10 weeks after the candesartan treatment (113.1 \pm 6.1 mm Hg and 87.9 \pm 5.9 mm Hg, respectively).

Effect of Candesartan on RGC Survival

Figure 3A shows representative results of the RGC labeling in both the vehicle- and the candesartan-treated rat. Compared

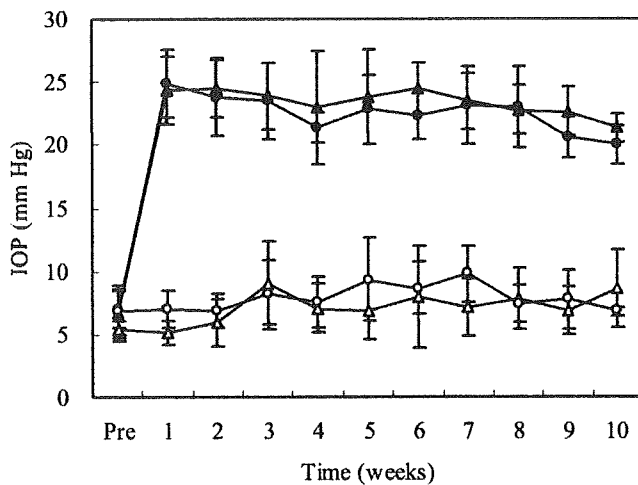


FIGURE 1. Elevated IOP in rat eyes that underwent unilateral cauterization of three episcleral vessels (Operated) compared with the opposite eye (Control). *Filled triangles*: operated, vehicle. *Filled circles*: operated, candesartan. *Open triangles*: control, vehicle. *Open circles*: control, candesartan. During the 10-week experimental period, one group of animals was not treated pharmacologically (*triangles*), whereas the other group was treated with candesartan (*circles*). All values are mean \pm SD ($n = 6$).

with the vehicle-treated rat, RGC death seemed to be mild in the candesartan-treated rat. RGC survival rates in the central retinas of the eyes with elevated IOP were $46.5\% \pm 19.4\%$ (mean \pm SD) in the vehicle-treated group and $84.2\% \pm 5.0\%$ in the candesartan-treated group ($P = 0.02$; Fig. 3B). In the peripheral retina, RGC survival rates in the eyes with elevated IOP were $52.3\% \pm 21.8\%$ in the vehicle-treated group and $82.5\% \pm 2.4\%$ in the candesartan-treated group ($P = 0.004$; Fig. 3B).

Immunohistochemistry

In the retinas of rat eyes with normal IOP, AT1-R immunoreactivity was detected on the endothelial cells of the inner retinal vessels (Fig. 4A). In the retinas of rat eyes with chronic elevated IOP, immunohistochemical analysis indicated there were many AT1-R-positive cells within the ganglion cell layer in the candesartan-treated rat, though this number was apparently decreased in the vehicle-treated rat (Figs. 4B, C). However, immunohistochemical analysis at 3 weeks after the initial elevation of IOP showed there were many AT1-R-positive cells within the retinal ganglion cell layer in the vehicle-treated rat (Fig. 4D).

DISCUSSION

The present study demonstrates for the first time that AT1-R upregulation is associated with chronic elevated IOP in the rat model of glaucoma and that the AT1-R signaling blockade of candesartan effectively prevents retinal neuronal death. Candesartan (1 mg/kg/d) has neuroprotective effects against cerebral ischemia in spontaneously hypertensive rats.²³ In animals pretreated with 0.1 or 1 mg/kg candesartan, only 1 mg/kg candesartan reduced ischemic damage to the retina against retinal ischemia-reperfusion injury in the rat retina (KF, KH, et al., unpublished data, 2009). Therefore, the 1-mg/kg/d dose used in this study was selected.

Oral administration of the ARB losartan reduced the IOP in human subjects with both normal tension and glaucoma.²⁴ Hasizume et al.²⁰ have recently shown that candesartan causes a decrease in IOP, with an IOP reduction noted for 5 hours

after drug administration in healthy subjects. The total outflow facility increased significantly in all subjects, with a decrease in the SBP noted only in patients with hypertension.²⁴ These results suggest that the mechanism is not mediated by a decrease in blood pressure but, rather, is more specific. These findings confirm the role of the RAS in the regulation of IOP.²⁴ Because candesartan treatment was performed before elevation of the IOP, we were unable to compare the IOP before and after its oral administration in this study. However, given that all the animals had cautery-induced IOP elevation, we compared the animals that were not treated pharmacologically with those that did receive candesartan and determined there was no significant difference between the two groups. Thus, we concluded that candesartan treatment provides a neuroprotective effect that is independent of IOP reduction.

IOP in the control eyes was lower than has been previously reported by other investigators (11.6–13.2 mm Hg).^{21,25} This might have been due to differences associated with the anesthetic used in the present study. Anesthetics can affect IOP, and this effect can change over time, depending on the level of anesthesia present.^{26,27} The most commonly used device for measuring IOP in rats is the TonoPen electronic tonometer (Reichert, Depew, NY).^{21,25} Recent reports have shown the TonoLab device (Colonial Medical Supply, Franconia, NH) to be more effective than the TonoPen when measuring IOP in rats.^{28,29} Moore et al.³⁰ have shown that there is a high correlation between the results obtained when using a TonoPen and those obtained by directly measuring the rat eye ($r = 0.94$ – 0.98). They also have reported the repeatability and consistency of IOP measurements when using the TonoPen.³⁰ In our study, the coefficient of variation in measuring IOP with TonoPen was 8.5%.

In the present study, we found that the 1 mg/kg/d candesartan treatment in normal rats did not affect either the SBP or the DBP. Although our results are similar to those of another study that found there was no SBP decrease from baseline after the same dose of candesartan was administered in adult and old rats, a second study has shown that a higher dose (10 mg/kg/d) of candesartan caused a significant decrease in the SBP.³¹ However, since a 1-mg/kg/d candesartan dose has been shown to reduce BP in spontaneously hypertensive rats,²³ it may be that the 1-mg/kg/d candesartan dose level affects only hypertensive rats.

Kurihara et al.³² recently reported that intense inflammation caused local upregulation of angiotensin II expression and disturbed visual function by electroretinography. The RAS polymorphisms that exist for the angiotensin II receptor gene may be a major genetic risk factor for the development or progression of glaucoma in the Japanese population.²⁰ Angiotensin II activates the NADPH-dependent oxidase complex, which serves as a major source of superoxide and is upregulated in several pathologic conditions associated with oxidative stress.^{33,34} Given that oxidative stress induces apoptosis in neurons,³⁴ hydrostatic pressure-induced oxidative stress could

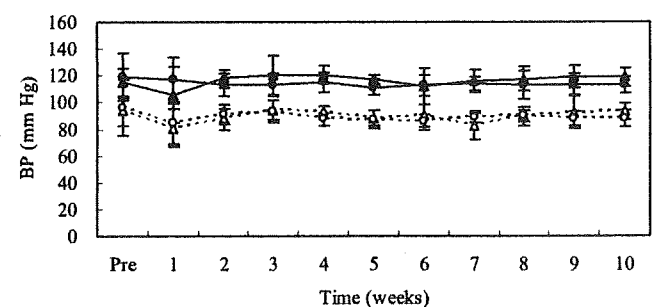


FIGURE 2. Systolic (*solid line*) and diastolic (*dotted line*) blood pressure (BP) measurements after oral administration of candesartan (*circles*) or vehicle (*triangles*). All values are mean \pm SD ($n = 6$).

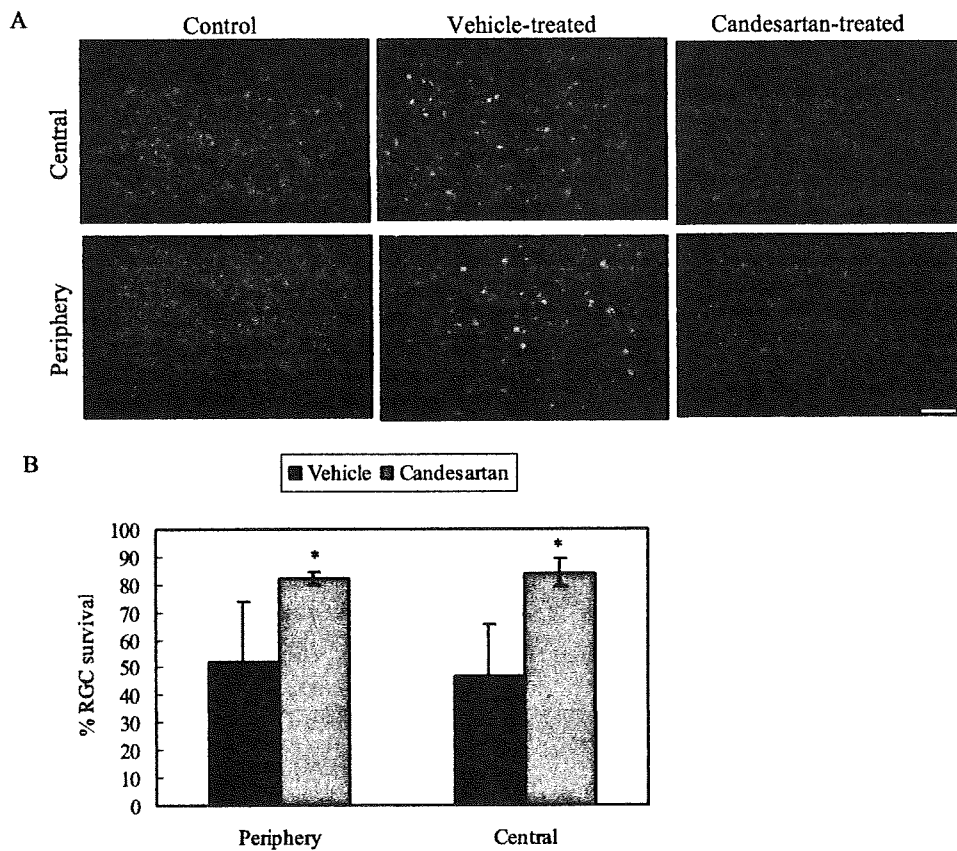


FIGURE 3. Survival of RGCs at 10 weeks in rat eyes with chronic elevated IOP. (A) Retrograde labeling of the RGCs in eyes with normal IOP and in eyes with elevated IOP at 10 weeks after administration of candesartan or vehicle. Micrographs in the central and peripheral areas were taken approximately 1 and 4 mm from the optic nerve head, respectively. Scale bar, 20 μ m. (B) RGCs were counted in the central and peripheral areas at approximately 1 and 4 mm from the optic nerve head, respectively. The graph depicts the mean \pm SD of six animals treated with candesartan and six animals treated with vehicle. A significant difference in the RGC survival rate in eyes with elevated IOP that were treated with candesartan and vehicle was evident between the central ($P = 0.02$) and peripheral ($P = 0.004$) areas. * $P < 0.05$

very well be the mechanism responsible for the similar pressure-induced apoptosis seen in animal models^{36,37} and in glaucoma patients with high IOP.³⁸ In the present study, we demonstrated that the AT1-R level increased in the ganglion cell

layer in candesartan-treated rats. In vehicle-treated rats, however, there was a decrease in the AT1-R-positive RGCs in the retina 10 weeks after the elevation of IOP compared with that seen in the candesartan-treated rat. Vehicle-treated rats were

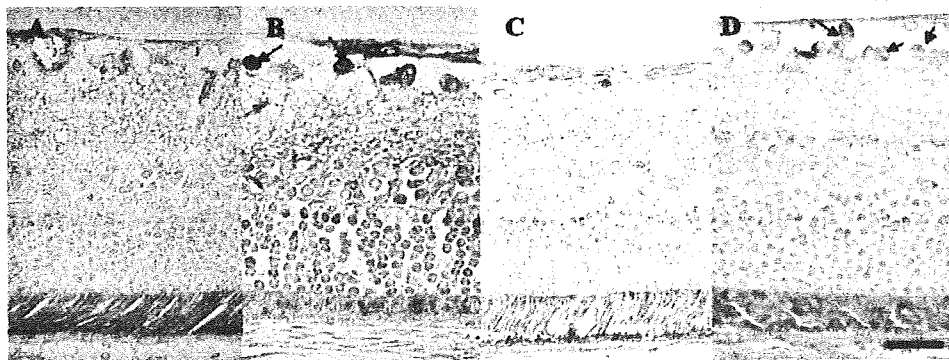


FIGURE 4. Immunohistochemical staining of the AT1R expression in the retina. Retinal sections from normal animals (A) and from animals with chronic elevated IOP that were administered candesartan over a 10-week period (B) or vehicle over a 10-week (C) or a 3-week period (D). An increase in the AT1-R-positive RGCs (arrow) was noted for the candesartan-treated rats at 10 weeks and vehicle-treated rats at 3 weeks, compared with the normal and vehicle-treated rats at 10 weeks. Scale bar, 25 μ m.

also found to have many AT1-R-positive RGCs in the retina 3 weeks after IOP elevation (i.e., a few weeks before the beginning of cell death). Thus, increases in the AT1-R-positive RGCs occur when IOP is chronically elevated. Treatment with candesartan effectively prevented cell death.

In conclusion, the current data suggest that candesartan does indeed have a therapeutic effect in this animal model of glaucoma. Because ARBs are safely and widely used to treat hypertension, clinical administration of candesartan for the purpose of pharmacologic neuroprotection may be a new and beneficial therapy that can be used in patients with glaucoma.

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Unintentional Displacement of the Retina after Standard Vitrectomy for Rhegmatogenous Retinal Detachment

Chieko Shiragami, MD, Fumio Shiraga, MD, Hidetaka Yamaji, MD, Kouki Fukuda, MD, Mai Takagishi, MD, Misako Morita, Co, Takehiro Kishikami, Co

Objective: To study unintentional displacement of the retina after standard vitrectomy for rhegmatogenous retinal detachment (RRD).

Design: Prospective interventional case series.

Participants: Forty-three eyes of 43 consecutive patients with cystic RRD involving 1 or more quadrants underwent successful standard vitrectomy with 20% sulfur hexafluoride gas injection. Neither scleral buckling nor retinotomy was performed.

Methods: Fundus autofluorescence (FAF) imaging was subsequently recorded to detect displacement of the retina using the Topcon TRC-50DX (Topcon, Tokyo, Japan) at 10 days and 1, 3, and 6 months postoperatively. Fluorescein angiography was also recorded using standard techniques for patients with abnormal FAF findings. Cyclotorsion and vertical deviation were measured postoperatively.

Main Outcome Measures: The proportion of eyes with postoperative retinal displacement detected by FAF imaging.

Results: The mean age of these 43 patients was 60 years with a range of 39 to 77 years. Of the 43 eyes, retinal detachment involved 1 quadrant in 2 eyes, 2 quadrants in 31 eyes, 3 quadrants in 8 eyes, and 4 quadrants in 2 eyes. After complete reattachment of the retina, FAF photography demonstrated hyperfluorescent lines superiorly parallel to retinal vessels within the vascular arcade in 27 of the 43 eyes (62.8%). Fluorescein angiography did not demonstrate any abnormalities corresponding to the linear autofluorescence. This autofluorescence was hypothesized to originate from increased metabolic activity of the retinal pigment epithelium that had been preoperatively located under the major retinal vessels and was postoperatively exposed to light because of downward displacement of the retina. Of the 27 eyes with retinal displacement, 1 to 5 degrees of extorsion were seen in 16 eyes (59.3%), and 1 to 4 degrees of vertical deviation were seen in 13 eyes (48.1%). None of the 27 patients had diplopia or slant. The extent of retinal detachment ($P = 0.019$) and the macular status (on or off) ($P = 0.016$) were significantly associated with postoperative displacement of the retina.

Conclusions: In eyes with RRD treated with standard vitrectomy and gas injection, the retina may move downward after the surgery. If the extent of retinal detachment is large, or macular detachment is present, unintentional postoperative retinal translocation may easily occur.

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The commonly used treatments for rhegmatogenous retinal detachment (RRD) are scleral buckling surgery and primary pars plana vitrectomy (PPV).^{1,2} Pneumatic retinopexy or scleral buckling is usually performed for localized cases. At the other end of the spectrum, complicated cases with proliferative vitreoretinopathy, giant tears, bullous RRD, and retinal breaks with marked vitreous traction are commonly treated with primary PPV. Pars plana vitrectomy surgery for retinal detachments has become safer and more effective using perfluorocarbon liquids (PFCL).³

Inferior limited macular translocation with scleral imbrication was performed to move the posterior retina downward and translocate the fovea outside a choroidal neovascular membrane.⁴⁻⁶ However, as de Juan and Vander reported in 1999,⁷ creation of retinal detachment and air

tamponade without scleral imbrication could move the retina downward. Even if the retina is totally reattached during PPV, in patients with RRD, subtle subretinal fluid remains at the end of surgery. After the surgery, the patients usually take a sitting position for a while before they maintain an appropriate position. At that time, force of gravity may slightly move the retina downward. Thus, we aimed to study unintentional displacement of the retina after standard vitrectomy for RRD by using fundus autofluorescence (FAF).

Materials and Methods

Patients

Forty-three eyes of 43 consecutive patients with RRD involving 1 or more quadrants underwent successful standard PPV with 20%

sulfur hexafluoride gas injection at the Kagawa University Hospital between November 2006 and June 2008. The mean age of all patients (26 male, 17 female) was 60.0 years with a range of 39 to 77 years. No patients had binocular diplopia in their preoperative history. The eyes with reoperations, silicon oil tamponade, or proliferative vitreoretinopathy were excluded from this study. The study was conducted in accordance with the recommendations of the Declaration of Helsinki and was approved by the institutional review board at the Kagawa University Hospital. After an explanation of the purpose of the study and the procedures to be used, a signed informed consent was obtained from all patients before surgery and examinations.

Surgical Technique

Local anesthesia was induced by retrobulbar nerve block. A 3-port PPV was performed in all cases using the Accurus vitrectomy system (Alcon Labs, Fort Worth, TX). The patients aged more than 50 years underwent combined cataract surgery. The posterior hyaloid was separated and removed using the vitreous cutter. After that, all vitreous traction on retinal tears was removed and the peripheral vitreous was subtotally shaved. In 30 eyes, PFCL was injected over the posterior pole and reached the posterior border of retinal breaks through a 20- or 23-gauge blunt cannula. Fluid-air exchange was performed to drain the subretinal fluid through original retinal breaks in all 43 eyes. After endolaser photocoagulation around retinal breaks was performed and PFCL was completely removed, 20% sulfur hexafluoride gas was used for internal tamponade in all cases. Neither scleral buckling nor retinotomy was performed. The patients did not take a face-down positioning but performed a sitting position for several minutes before they went back to the recovery room after the operation. After that, face-down or appropriate positioning was maintained for 7 to 10 days after the surgery.

Ophthalmic Examinations and Fundus Photography

All patients underwent a regular ophthalmic examination, including measurement of the best-corrected visual acuity and fundus examination. Color fundus photography and fluorescein angiography using the Topcon TRC-50DX fundus camera (Topcon, Tokyo, Japan) were carried out preoperatively and postoperatively in all eyes.

Color fundus photography was performed using 50-degree fields of the panoramic image preoperatively and postoperatively (10 days and 1, 3, and 6 months after PPV in all patients, and 12 months after PPV in 4 patients). The FAF imaging was recorded to detect displacement of the retina using the Topcon TRC-50DX fundus camera with 2 bandpass filters (based on the modification by Spaide with an excitation bandwidth of 500–610 nm and an emission bandwidth of 675–715 nm)⁸ at 10 days, 1 month, 3 months, and 6 months after the surgery. Fluorescein angiography was also recorded using standard techniques for patients with abnormal FAF findings.

Orthoptic Examination

All patients underwent an orthoptic examination 3 months after surgery. A synoptophore (Clement and Clark, Edinburgh, UK) was used to measure the objective and subjective angles of deviation and fusion ability.⁹ The elevation-dependent torsional disparity during various degrees of convergence was measured. With a synoptophore, the subject views 2 images at optical infinity through 2 eyepieces. These images fill the visual field so that

nothing in the surrounding laboratory is seen. The images can be rotated vertically and torsionally about a Fick system of gimbals, independently for each eye.

Statistical Methods

To determine whether there was a significant association among several factors (independent variables), including age, gender, PFCL use, macular status (on or off) and extent of retinal detachment, and unintentional displacement of the retina (dependent variable), multiple logistic regression analysis was performed. A *P* value of less than 0.05 was considered significant. This statistical analysis was carried out using SPSS 11.5 statistical software (SPSS Inc., Chicago, IL).

Results

On preoperative fundus examinations, 43 eyes had bullous RRD involving 1 or more quadrants. Of the 43 eyes, RRD involved 1

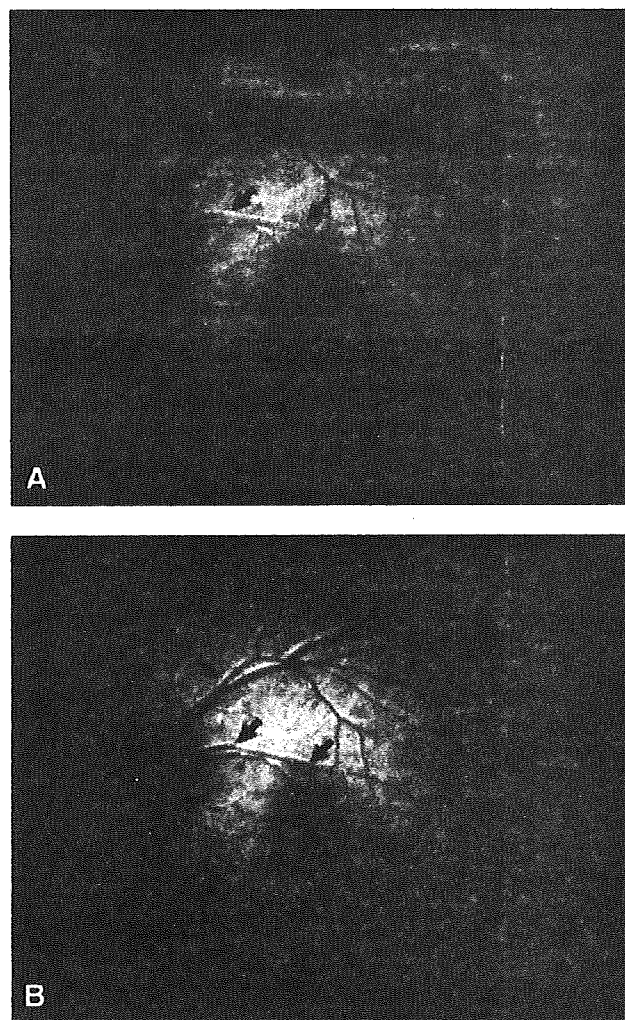


Figure 1. A, The fundus autofluorescence (FAF) image taken 10 days postoperatively demonstrates hyperfluorescent lines (arrows) parallel to retinal vessels. B, The FAF image taken 3 months postoperatively demonstrates that the hyperfluorescent lines are morphologically identical to those observed 10 days postoperatively in their form, range, and brightness (arrows).

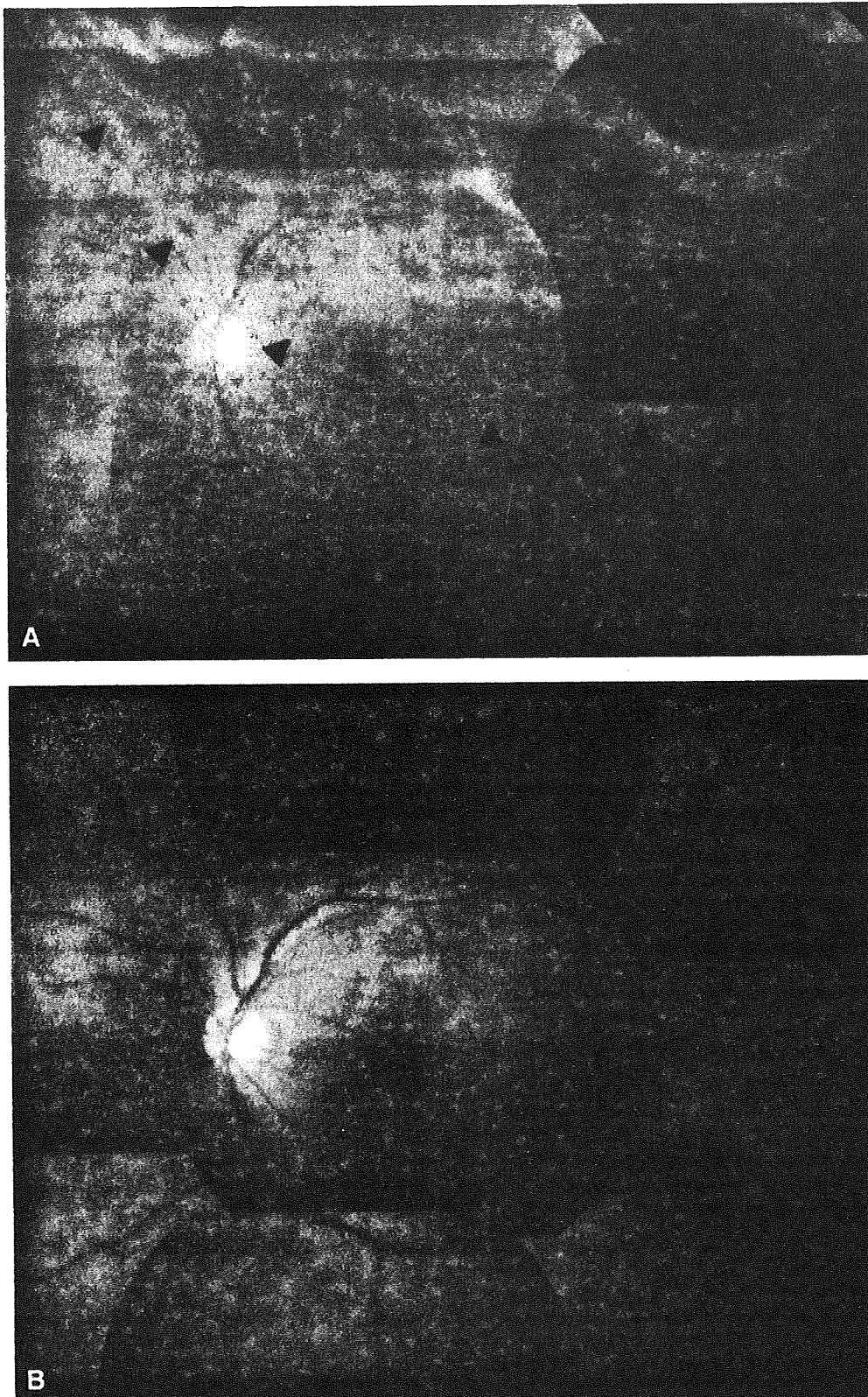


Figure 2. A, Preoperative color photograph of the eye with cystic rhegmatogenous retinal detachment (RRD). The retinal tear is present in the tempoor superior region. The RRD demarcation line (*arrowheads*). B, Color photograph taken 1 month postoperatively. The retina is attached completely. C, The fundus autofluorescence (FAF) image taken 1 month postoperatively demonstrates hyperfluorescent lines also in the region where there was no retinal detachment before surgery (*arrowheads*).



Figure 2. (Continued.)

quadrant in 2 eyes, 2 quadrants in 31 eyes, 3 quadrants in 8 eyes, and 4 quadrants in 2 eyes on fundus examination. Retinal breaks were located in the superior region (19 eyes), the temporal region (13 eyes), the nasal region (5 eyes), the inferior region (1 eye), and multiple regions (5 eyes).

After complete reattachment of the retina, autofluorescence photography demonstrated hyperfluorescent lines superiorly parallel to the retinal vessels in 27 of the 43 eyes (62.8%). This autofluorescence was hypothesized to originate from increased metabolic activity of the retinal pigment epithelium (RPE) that had been preoperatively located under the major retinal vessels and was postoperatively exposed to light because of downward displacement of the retina. The clinical findings of 27 patients whose retina moved downward postoperatively are shown in Table 1 (available at <http://aaojournal.org>). These hyperfluorescent lines on FAF images were observed 10 days after PPV (Fig 1A), and these lines were morphologically identical to those taken 3 months postoperatively in their form, range, and brightness (Fig 1B). In 3 of 27 eyes, hyperfluorescent lines parallel to retinal vessels were also observed in the region where there had been no retinal detachment before surgery (Fig 2). However, in 4 eyes of 4 patients who were followed over 12 months, the hyperfluorescent lines moved slightly closer to the original retinal vessels (Fig 3). In the 27 eyes, fluorescein angiography did not demonstrate any abnormalities corresponding to the linear autofluorescence (Fig 4). On the other hand, there were no significant FAF abnormalities in the remaining 16 eyes (37.2%).

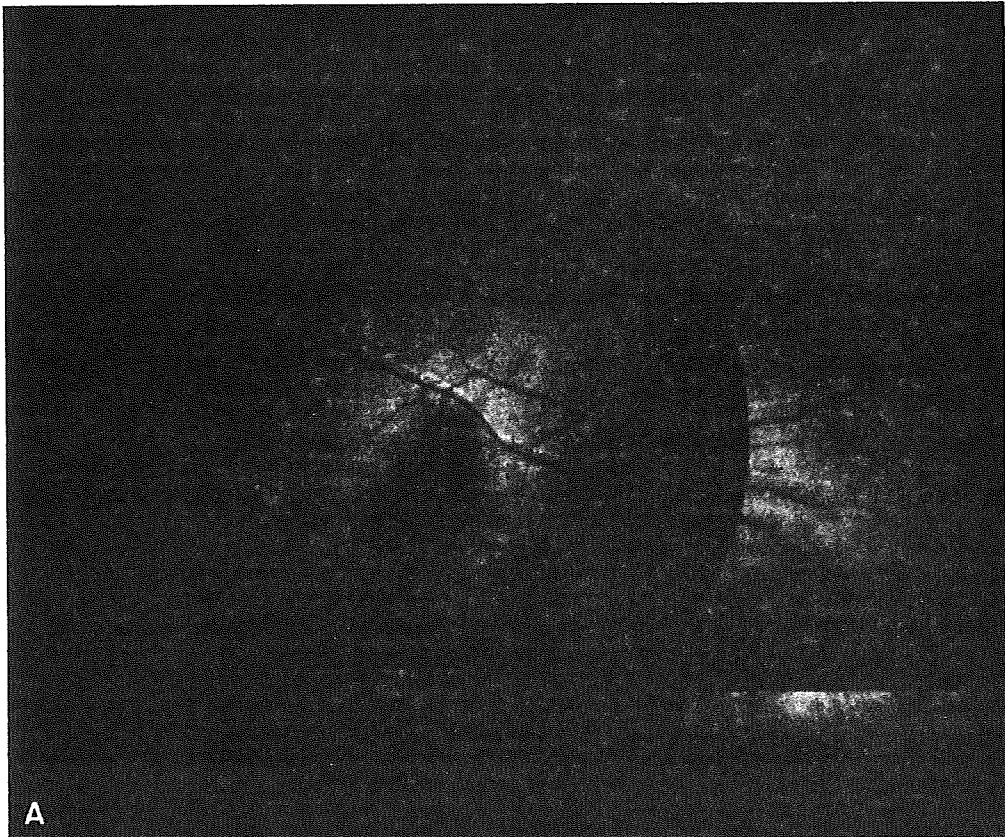
Multiple logistic regression analysis revealed that the extent of retinal detachment ($P = 0.019$) and the macular status (on or off) ($P = 0.016$) were significantly associated with postoperative retinal displacement (Table 2).

In the 27 patients whose eyes showed unintentional displacement of the retina with macular rotation around the disc, postoperative examination with a synoptophore revealed 1 to 5 degrees of extorsion in 16 patients (59.3%) and 1 to 4 degrees of vertical deviation in 13 patients (48.1%) (Table 1, available at <http://aaojournal.org>). However, none of the 27 patients had cyclovertical diplopia or slant. The remaining 16 patients with no displacement of the retina did not have obvious strabismic deviation by examination with a synoptophore.

Discussion

Fundus autofluorescence is a noninvasive test that provides discrete funduscopy images based on stimulated emission of light from lipofuscin. Lipofuscin is a cellular waste product containing lipid, protein, and fluorophores such as A2E. Visualization of the signal depends on the distribution pattern of the fluorophore-containing lipofuscin.¹⁰⁻¹² FAF imaging allows topographic mapping of lipofuscin distribution in the RPE cell monolayer, as well as of other fluorophores that may occur with disease in the outer

Figure 3. A, The fundus autofluorescence (FAF) image taken 10 days postoperatively demonstrates the hyperfluorescent lines. B, The FAF image taken 12 months postoperatively demonstrates the hyperfluorescent lines. These hyperfluorescent lines appear to be closer to the original retinal vessels than those observed 10 days postoperatively.



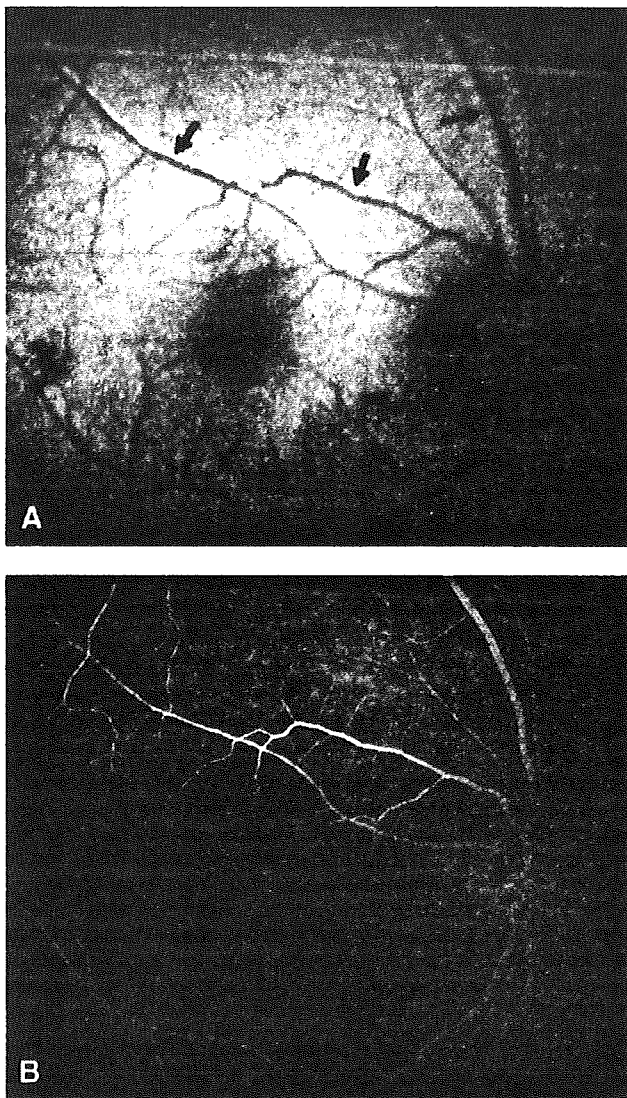


Figure 4. A, The fundus autofluorescence (FAF) image taken 3 months postoperatively (the same eye as Figure 3). Hyperfluorescent lines (arrows). B, The fluorescein angiography does not demonstrate any abnormalities corresponding to the linear autofluorescence.

retina and the subneurosensory space.⁸ Fundus autofluorescence in the RPE is dependent on outer segment renewal and potentially affected by a balance between accumulation and clearance. Therefore, the autofluorescence can be interpreted as a clinical sign for the metabolic activity of the RPE. Increased phagocytosis of photoreceptor outer segments gives rise to hyperautofluorescence.^{8,12} FAF imaging, therefore, is a valuable technique for the assessment of unintentional displacement of the retina after standard vitrectomy for patients with RRD. The reason why increased autofluorescence indicates the preoperative site of retinal vessels is unclear. For a long time, the RPE cells beneath the retinal vessels were prevented from light irradiation, in other words, they maintained dark adaptation because they were shaded by the retinal vessels. After that, acute exposure to the excitation light, which was caused by translocat-

tion of the retinal vessels, may have induced increased FAF signals.¹² Because fluorescein angiography did not demonstrate any abnormalities corresponding to the linear autofluorescence, it is considered that these hyperautofluorescent lines might be due to metabolic changes in RPE cells, but not morphologic changes. The hyperfluorescent lines on FAF images observed 10 days after PPV were morphologically identical to those taken 3 months postoperatively in their form, range, and brightness. However, in 4 eyes of 4 patients who were followed over 12 months, the hyperfluorescent lines moved slightly closer to the original retinal vessels. The retina might return to the preoperative, original position with times.

Immediately after the end of PPV surgery, a little subretinal fluid usually remains, despite intraocular gas tamponade. The fact that the retina moved downward suggests that intraocular gas could move the retina downward because of the shift of residual subretinal fluid during the sitting position immediately after PPV surgery. Large retinal detachment including macular detachment, which was independently associated with postoperative displacement of the retina, may easily shift residual subretinal fluid downward. The patients did not take a face-down position but took a sitting position for several minutes before they went back to the recovery room after the operation. If patients take the strict prone position as soon as the operation is completed, retinal displacement may be prevented.

If the macula is translocated vertically around the disk but not the fovea, cyclotorsional diplopia or awareness of a tilted image is likely to occur.^{13,14} However, the cyclodeviation could be easily compensated for by the sensory fusion ability, which is driven by the central nervous system, without a clinically visible motor adaptation.¹⁵ Limited macular translocation or full macular translocation with 360-degree retinotomy surgery has been performed for age-related macular degeneration.^{4-6,16-18} The retina in the macula is relocated to a new location away from the subretinal pathology, overlying healthier RPE. In the majority of cases undergoing full macular translocation, the macula is rotated upward, creating intorsion in the operated eye. The rotation of the retina leads to horizontal and vertical strabismus. This torsion also greatly exceeds the maximum total amplitude of cyclofusion, which can be as large as 15 degrees.^{11,19,20} Extraocular muscle surgery can reduce cyclotorsion after macular translocation surgery, alleviating symptoms of tilted vision and

Table 2. Multiple Logistic Regression Model of Variables Associated with Displacement of Retina

| | Standard | | |
|-------------------------------|----------|--------------|---------|
| | OR | 95% CI | P Value |
| Age | 1.064 | 0.971-1.167 | 0.184 |
| Gender | 3.022 | 0.595-15.347 | 0.182 |
| Quadrants of RD | 7.743 | 1.409-42.569 | 0.019 |
| Macula status (0, on; 1, off) | 10.909 | 1.560-76.290 | 0.016 |
| PFCL use (0, not use; 1, use) | 0.697 | 0.135-3.599 | 0.667 |

CI = confidence interval; OR = odds ratio; PFCL = perfluorocarbon liquids; RD = retinal detachment.

facilitating use of the vision in the translocated eye.^{13,14} In the present study, 1 to 5 degrees of extorsion were seen in 16 of the 27 eyes (59.3%) and 1 to 4 degrees of vertical deviation were seen in 13 of 27 eyes (48.1%) that had unintentional displacement of the retina after PPV for RRD. Because normal subjects have no vertical deviation and little cyclodeviation, cyclotorsion and vertical deviation were thought to be caused by the displacement of the retina.^{21,22} Cyclotorsion and vertical deviation can be compensated for by the sensory and motor fusion ability. In fact, none of the 27 patients had binocular diplopia or slant. However, there is a possibility that larger-angle extorsion or vertical deviation may occur after PPV in patients with RRD, resulting in unexpected binocular diplopia. Therefore, patients with RRD must be followed carefully after PPV, especially with respect to their binocular vision, except for the patients with poor vision at the affected eyes.

In conclusion, the current study suggests that the retina may move downward postoperatively in eyes with cystic RRD treated with standard vitrectomy and gas injection. If there is large retinal detachment, or macular detachment is present, unintentional postoperative retinal translocation may easily occur. Despite the fact that approximately half of the patients demonstrated excyclotorsion or vertical deviation on orthoptic examination, no patients had binocular diplopia. A long-term FAF study after PPV for RRD may help to identify the position shift trend of the retina, and the disappearance of the line may help to better understand the RPE metabolism.

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Footnotes and Financial Disclosures

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Department of Ophthalmology, Kagawa University Faculty of Medicine, Kagawa, Japan.

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Correspondence:

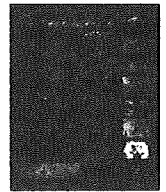
Chieko Shiragami, MD, Department of Ophthalmology, Kagawa University School of Medicine, 1750-1 Ikenobe Miki-cho, Kagawa 761-0793, Japan. E-mail: chappi@kms.ac.jp.

Shiragami *et al* · Displacement of the Retina after Vitrectomy

Table 1. Clinical Characteristics of 27 Patients Whose Retina Moved Downward Postoperatively

| Case No. | Gender | Affected Eye | Age (yrs) | Preoperative VA | Postoperative VA | Quadrants of RD | Macula Status | PFCL Use | Postoperative Cyclodeviation | Postoperative Vertical Deviation |
|----------|--------|--------------|-----------|-----------------|------------------|-----------------|---------------|----------|------------------------------|----------------------------------|
| 1 | M | L | 76 | 0.2 | 1 | 3 | off | + | Ex3° | L/R2° |
| 2 | M | L | 47 | 0.5 | 0.9 | 3 | off | + | Ex5° | L/R1° |
| 3 | M | R | 53 | 0.4 | 0.9 | 2 | off | + | 0° | 0° |
| 4 | M | R | 59 | 0.3 | 0.6 | 2 | off | + | Ex1° | R/L2° |
| 5 | F | R | 58 | 0.01 | 0.5 | 4 | off | + | Ex3° | R/L2° |
| 6 | M | L | 61 | 1.2 | 1.2 | 2 | on | - | 0° | 0° |
| 7 | F | L | 64 | 1.2 | 0.7 | 2 | on | + | 0° | 0° |
| 8 | F | R | 58 | 0.04 | 0.5 | 3 | off | - | 0° | 0° |
| 9 | M | L | 65 | 0.9 | 1.2 | 2 | on | + | 0° | 0° |
| 10 | M | R | 63 | 0.8 | 1 | 2 | off | + | 0° | R/L4° |
| 11 | F | L | 65 | 0.1 | 0.6 | 2 | off | + | 0° | 0° |
| 12 | F | R | 56 | 1.2 | 1.5 | 2 | on | + | Ex3° | R/L2° |
| 13 | F | L | 75 | 0.5 | 0.8 | 3 | on | - | Ex3° | L/R2° |
| 14 | F | L | 64 | 0.09 | 1.2 | 3 | off | + | 0° | 0° |
| 15 | F | L | 60 | 0.9 | 1.2 | 1 | on | + | 0° | 0° |
| 16 | M | L | 72 | 0.03 | 0.4 | 2 | off | - | Ex5° | L/R4° |
| 17 | M | R | 77 | L.S. | 0.4 | 3 | off | - | Ex3° | R/L2° |
| 18 | F | L | 67 | 0.04 | 0.4 | 4 | off | + | Ex5° | L/R3° |
| 19 | F | R | 56 | 1.2 | 1.5 | 2 | on | - | Ex3° | R/L2° |
| 20 | M | R | 39 | 1 | 0.5 | 2 | on | - | 0° | 0° |
| 21 | M | L | 53 | 1.2 | 1.2 | 2 | on | + | 0° | 0° |
| 22 | M | L | 61 | 1.5 | 1.5 | 2 | on | + | Ex2° | 0° |
| 23 | M | R | 58 | 0.04 | 1.2 | 2 | off | + | Ex3° | R/L2° |
| 24 | M | R | 58 | 0.01 | 0.5 | 3 | off | + | Ex3° | 0° |
| 25 | M | L | 64 | 0.7 | 1 | 2 | on | + | Ex5° | L/R1° |
| 26 | M | L | 57 | 0.04 | 0.7 | 3 | off | + | Ex5° | 0° |
| 27 | M | L | 67 | 2 | 1 | 1 | on | - | Ex2° | 0° |

Ex = extorsion; L = left; PFCL = perfluorocarbon liquids; R = right; RD = retinal detachment; VA = visual acuity.



A novel missense mutation (Leu46Val) of PAX6 found in an autistic patient

Motoko Maekawa^{a,*}, Yoshimi Iwayama^a, Kazuhiko Nakamura^b, Miho Sato^c, Tomoko Toyota^a, Tetsuo Ohnishi^a, Kazuo Yamada^a, Taishi Miyachi^d, Masatsugu Tsujii^{d,e}, Eiji Hattori^a, Nobuo Maekawa^f, Noriko Osumi^{g,h}, Norio Mori^b, Takeo Yoshikawa^{a,h}

^a Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

^b Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Shizuoka, Japan

^c Department of Ophthalmology, Hamamatsu University School of Medicine, Shizuoka, Japan

^d The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Hamamatsu University School of Medicine, Shizuoka, Japan

^e Faculty of Sociology, Chukyo University, Toyota, Japan

^f Maekawa Eye Clinic, Miyagi, Japan

^g Department of Developmental Neurobiology, Tohoku University Graduate School of Medicine, Miyagi, Japan

^h CREST, Japanese Science and Technology Agency, Tokyo, Japan

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ABSTRACT

The paired box 6 (PAX6) is a transcription factor expressed early in development, predominantly in the eye, brain and pancreas. Mutations in PAX6 are responsible for eye abnormalities including aniridia, and it is also known that some PAX6 mutations result in autism with incomplete penetrance. We resequenced all the exons and flanking introns of PAX6 in 285 autistic patients in the Japanese, with the possibility that novel mutations may underlie autism. Fifteen different polymorphisms were identified: 13 are novel, and 2 were previously reported (rs667773 and rs3026393). Among the novel ones, there is one missense mutation that was found in a patient: 136C > G (Leu46Val) (single nucleotide polymorphism ID "ss130452457" is temporarily assigned). Leu46 is extremely conserved from fly to human, and we did not detect Val46 in 2120 nonautistic subjects. The autistic patient carrying this heterozygous mutation showed reduced vision, photophobia and eyelid ptosis, but no other ocular abnormality such as aniridia. Our findings suggest the necessity of further studies on the causal relationship between PAX6 and autism.

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The human paired box 6 (PAX6) gene is located on chromosome 11p13, and its orthologues have been found in diverse species. This gene encodes a transcription factor that is involved in multiple developmental pathways and is expressed early in the development of the eye, numerous regions of the brain, and the pancreas [25]. The PAX6 gene of 22 kb size contains 14 exons, including an alternatively spliced exon 5a that encodes 14 amino acids, and encodes a protein of 422 amino acid (Fig. 1). The PAX6 protein contains two DNA-binding domains (one paired domain and one homeodomain), and one proline/serine/threonine-rich (PST) transactivation domain. PAX6 affects the development of the nervous system and brain by regulating proneural genes such as neurogenin 2 (*Ngn2*) and achaete-scute complex homolog-like 1 (*Mash-1*) [28,36]. The *Pax6* heterozygous mutant mice and rats, i.e., "small eye", show both ocular and neuronal phenotypes including an absent olfactory bulb, a decrease in the number of cortical neurons and cortical plate thickness, as well as altered dorsoventral patterning of the forebrain,

and a decrease in hippocampal neurogenesis at the postnatal stage [18].

In human, in addition to the reports of associations of various PAX6 mutations with eye abnormalities including syndromic ones like Peters anomaly [8,26], there is emerging evidence of the roles of PAX6 mutations in behavioral and neurodevelopmental disease phenotypes such as autism and mental retardation. For instance, recent studies have identified individuals with PAX6 mutations who manifest mental retardation and aniridia [12,20,32]. In addition, magnetic resonance imaging (MRI) studies of patients with aniridia have shown subtle brain abnormalities including a lack of the anterior commissure and pineal gland [23]. These accumulating lines of evidence suggest that concomitant phenotypes are determined by the nature of PAX6 mutations. In this study, we carried out resequencing analysis of the gene in autistic patients with, with the aim of searching for additional mutations of PAX6, which might be associated with the disease.

Two hundred and eighty-five autistic patients of Japanese descent (236 men, 49 women; age, from 3 to 32 years old) were analyzed. The diagnosis of autism was made on the basis of the Autism Diagnostic Interview-Revised (ADI-R) criteria [17] with consensus from at least two experienced psychiatrists. All available medical

* Corresponding author. Tel.: +81 48 467 5968; fax: +81 48 467 7462.
E-mail address: mmaekawa@brain.riken.jp (M. Maekawa).

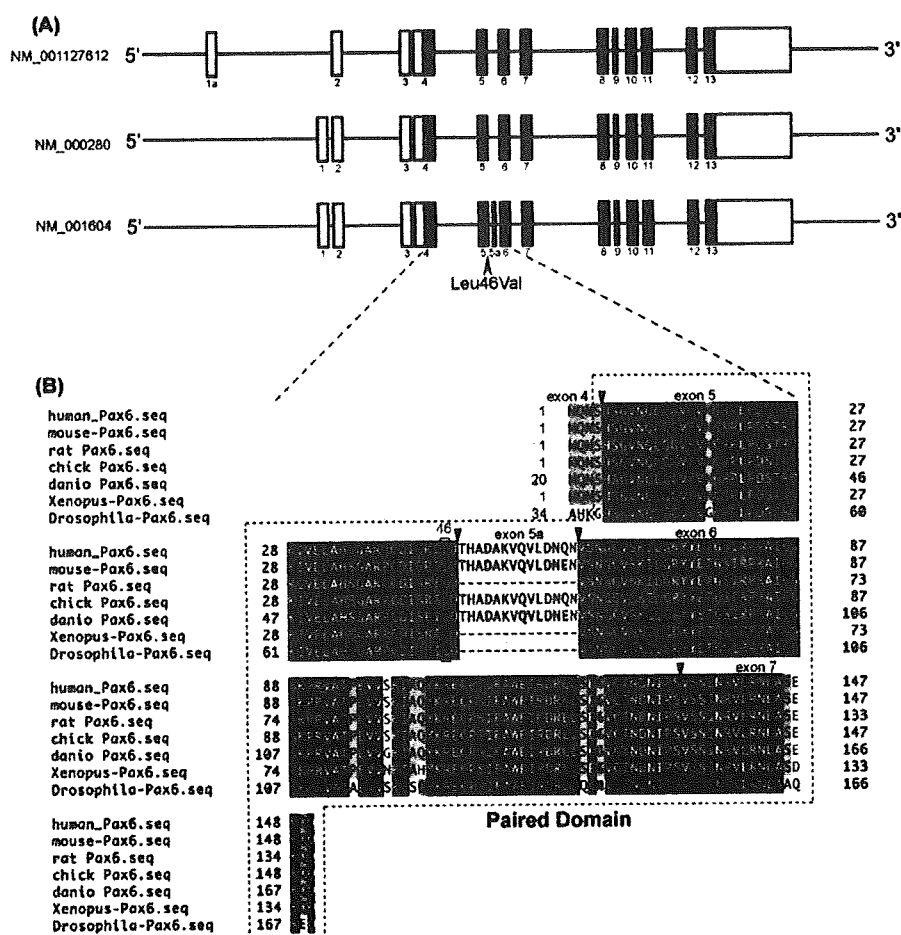


Fig. 1. Genomic structure of human *PAX6* and amino acid sequences of *PAX6/Pax6*. (A) Genomic structure and location of 136G (Val46) missense mutation for human *PAX6*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. A black arrowhead indicates the location of the missense mutation. Note that the three isoforms are shown (<http://genome.ucsc.edu/cgi-bin/hgGateway?org=human>). (B) Amino acid sequence alignments of *PAX6/Pax6* among species. The black boxes indicate the conserved amino acids from *Drosophila* to human. The grey boxes show the partially conserved ones. The conserved 46Leu is indicated by a red square. The red-broken-line box shows the paired domain that contains the conventional 128 residues [34] and five subsequent residues (SEKQQ) [38]. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

records were also taken into consideration. Regarding a detected missense mutation in patient samples, we analyzed 2120 nonautistic subjects, who are 1060 patients with schizophrenia (503 men, 557 women; mean age 48.0 ± 13.8 years) and 1060 controls who are free of mental disorders (503 men, 557 women; mean age 47.7 ± 13.6 years) [16]. All the subjects were from central Japan. The study was approved by the ethics committees of RIKEN and Hamamatsu University and was conducted in accordance with the Declaration of Helsinki (<http://www.wma.net>). All the participants provided written informed consent (for patients under 16 years old, we explained the aim of this study to the patients' parents and as long as possible to the patients in easy terms, and obtained written informed consent from at least their parents).

Genomic DNA was isolated from blood samples by standard methods. All the exons and exon/intron boundaries of *PAX6* were screened for polymorphisms by direct sequencing of polymerase chain reaction (PCR) products. The primers used for amplification are listed in Supplementary Table 1. PCR was performed with an initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, $60\text{--}63^\circ\text{C}$ (optimized for each primer pair) for 15 s, 72°C for 30 s, and a final extension at 72°C for 10 min, with AmpliTaqGold (Applied Biosystems, Foster City, CA) or it was performed with an initial denaturation at 98°C for 5 min, followed by 35 cycles at 98°C for 45 s, $58\text{--}61^\circ\text{C}$ for 30 s, 68°C for 30 s, and a final extension at 68°C

for 10 min, with Pwo SuperYield DNA Polymerase (Roche, Basel, Switzerland). Direct sequencing of PCR products was performed with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems). Polymorphisms were detected with the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI). The genomic structure of *PAX6* is based on the UCSC March 2006 draft assembly of the human genome database (<http://www.genome.ucsc.edu>), and the NCBI (<http://www.ncbi.nlm.nih.gov/>) database was searched for known single nucleotide polymorphisms (SNPs). Custom TaqMan SNP genotyping assays (Applied Biosystems) were used to score the identified missense SNP by the TaqMan assay method [27], and using ABI PRISM 7900 Sequence Detection System (SDS) and SDS v2.3 software (Applied Biosystems).

Our polymorphism screening detected a total of 15 different variants in the *PAX6*: 13 are novel ($-11,521\text{C} > \text{T}$, $\text{IVS4-85T} > \text{C}$, $\text{IVS4-70} \sim -72\text{Ins/DelTCT}$, $\text{IVS4-42C} > \text{T}$, $117\text{G} > \text{A}$, $136\text{C} > \text{G}$, $\text{IVS5-163C} > \text{T}$, $319\text{C} > \text{T}$, $\text{IVS6} + 28\text{C} > \text{T}$, $\text{IVS8} + 14\text{Ins/DelT}$, $867\text{T} > \text{C}$, $\text{IVS12} + 11\text{G} > \text{A}$ and $1194\text{C} > \text{T}$), and 2 were previously reported (rs667773 and rs3026393) (Table 1). With respect to novel variants, the $136\text{C} > \text{G}$ was a missense mutation (Leu46Val; ss130452457) located in the paired domain, which was found in one patient as a heterozygote (Table 1 and Fig. 1). We then examined 2120 nonautistic subjects for this missense mutation. However, the mutation was not detected