

OCT Evaluation of Neuroprotective Effects of Tafluprost on Retinal Injury After Intravitreal Injection of Endothelin-1 in the Rat Eye

Atsushi Nagata,^{1,2} Katsumi Omachi,³ Tomomi Higashide,¹ Satoshi Shirae,³ Atsushi Shimazaki,³ Masatsugu Nakamura,³ Naruhiro Ishida,³ and Kazuhisa Sugiyama¹

¹Department of Ophthalmology and Visual Science, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

²Department of Ophthalmology, National Hospital Organization Kanazawa Medical Center, Kanazawa, Japan

³Research & Development Center, Santen Pharmaceutical Co., Ltd., Osaka, Japan

Correspondence: Tomomi Higashide, Department of Ophthalmology and Visual Science, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa 920-8641, Japan; eyetomo@med.kanazawa-u.ac.jp.

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PURPOSE. To determine whether optical coherence tomography (OCT) is a useful technique to monitor retinal damage and to evaluate the neuroprotective effect of topical tafluprost in a rat model of intravitreal endothelin-1 (ET-1) injection.

METHODS. A single intravitreal injection of ET-1 (0.2–200 pmol/eye) was performed in one eye. Optical coherence tomography imaging was performed until 2 weeks after ET-1 injection. Subsequently, an intravitreal injection of ET-1 (20 pmol/eye) was performed in one eye of each rat, which was followed by topical instillation of tafluprost or saline once daily for 4 weeks. Optical coherence tomography imaging was performed until 4 weeks after ET-1 injection. After the last OCT session, retinal ganglion cells (RGCs) were retrogradely labeled with Fluorogold.

RESULTS. Endothelin-1 at doses of 20 to 200 pmol/eye caused a significant decrease in inner retinal thickness, whereas ET-1 at doses of 0.2 to 5 pmol/eye did not. The inner retinal thickness at 2 weeks postinjection was strongly correlated with Fluorogold-labeled RGC counts in the central retina ($r = 0.92$, $P < 0.001$). The inner retina of eyes treated with tafluprost was significantly thicker than eyes treated with saline at 1 and 2 weeks ($P = 0.038$ and $P = 0.045$, respectively). Fluorogold-labeled RGC counts in the central retina of eyes treated with tafluprost were significantly greater than in eyes treated with saline ($P = 0.03$).

CONCLUSIONS. Optical coherence tomography is useful for monitoring inner retinal damage in a rat model of intravitreal ET-1 injection. Daily topical administration of tafluprost may be protective against ET-1-induced retinal injury in the rat.

Keywords: optical coherence tomography, retinal ganglion cell, endothelin-1, rat, tafluprost

Glaucoma, which is characterized by the progressive degeneration and loss of retinal ganglion cells (RGCs) and their axons, is one of the most common causes of visual loss.^{1,2} Although elevated intraocular pressure is known to be the major risk factor for progression of glaucoma, many reports suggest that other factors are associated with the pathogenesis of this disease.^{3–5} There is evidence to suggest that a disturbance of ocular blood flow may be one such factor.^{6–8} Endothelin-1 (ET-1) is a potent vasoactive peptide that causes vasoconstriction of retinal vessels. Endothelin-1 and its receptors have been found in ocular tissues where it appears to have a regulatory function.^{9,10} Endothelin-1 is found in both the aqueous and vitreous humors and its concentration is elevated in glaucoma patients^{11–14} and in animal models of glaucoma.^{15,16} These findings suggest that ET-1 may be an important contributing factor in the development of glaucomatous optic neuropathy.

Optical coherence tomography (OCT) is a technology capable of producing high-resolution optical cross sections of the retina and providing quantitative measurements of retinal nerve fiber layer (RNFL) thickness, macular retinal thickness,

and optic disc morphology in human eyes. Optical coherence tomography has been also used for imaging the rodent retina.^{17–20} Recently, we reported the *in vivo* evaluation of changes in the RNFL with an experimental OCT system in a rat model of optic nerve injury, and showed that RNFL thickness determined by OCT scans had a good correlation with that determined in retinal sections.¹⁸ A literature search did not find any reports on OCT evaluation of retinal changes in rodent eyes with ET-1-induced retinal injuries.

Prostaglandin F_{2α} (PGF_{2α}) analogs are widely used for the treatment of glaucoma because they produce powerful reductions of intraocular pressure.²¹ Tafluprost is a novel PGF_{2α} analog with high affinity for the prostaglandin F receptor. Some studies have reported the effect of tafluprost on ocular blood flow^{22–26} and neuroprotection.^{27,28}

In this study, we examined the relationship between retinal injury after an intravitreal injection of ET-1 in the rat eye and the dose of ET-1. Subsequently, using the lowest dose of ET-1 that caused surely retinal injury, we examined the neuroprotective effect of tafluprost on ET-1-induced retinal injury in the rat.

METHODS

Animal Handling and ET-1–Induced Retinal Injury

Male Brown-Norway rats, aged 10 weeks and weighing 200 to 250 g, were used in the study. The rats had free access to food and water and were maintained in cages in an environmentally controlled room with a 12-hour light-dark cycle. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experimental procedures were approved by the Committee on Animal Experimentation of Kanazawa University and Santen Pharmaceutical Co., Ltd. A total of 69 rats were used in the study: 45 were used for the examination of a dose-response relationship and the other 24 for the examination of the neuroprotective effect of tafluprost. All experiments were conducted on rats anesthetized by an intraperitoneal injection (65 mg/kg) of sodium pentobarbital (Somnopenil; Schering-Plough Animal Health, Omaha, NE). An intravitreal injection of ET-1 was achieved by inserting a 29-gauge needle attached to a syringe (TERUMO, Tokyo, Japan) through a site 0.5 mm posterior to the superior limbus. For the examination of a dose-response relationship, 5 μ L of ET-1 (0.2, 2.0, 5.0, 20, 60, or 200 pmol/eye; Peptide Institute, Osaka, Japan) was injected into the vitreous body of the right eye ($n = 7, 8, 6, 6, 5, 6$, respectively). Contralateral eyes with no injection served as a control ($n = 14$). Intravitreal injection of PBS did not cause significant changes in retinal thickness in OCT images (Nagata A, Higashide T, Sugiyama K, unpublished observations, 2009). For the examination of the tafluprost effect, an intravitreal injection of ET-1 (20 pmol/eye) was performed in one eye of each rat. Observation of retinal vessels was performed at 1 hour after ET-1 injection by indirect ophthalmoscopy using a handheld retinal camera (GENESIS-D; Kowa Co., Ltd., Nagoya, Japan). Constriction of retinal vessels was photographed and graded into three groups: grade 0, few vessels constricted; grade 1, nearly one half of major vessels constricted; and grade 2, almost all major vessels constricted (Supplementary Fig. S1). Thereafter, these rats received topical administration of tafluprost (Santen Pharmaceuticals, Osaka, Japan) eye drops ($n = 12$) or saline ($n = 12$) once daily for 4 weeks in a double-blind manner.

OCT Imaging of Rat Retinal Layers

An experimental OCT system was developed with a time-domain OCT (EG SCANNER, MTEX MATSUMURA CORP., Tendo, Japan). Optical components in the experimental OCT system were optimized to acquire retinal images of the rat fundus.¹⁸ The scan rate of the OCT was 133 A-scans per second, with an axial resolution of 4.3 μ m. To improve the signal-to-noise ratio, the rat was placed on a platform with a fixation device for the rat's head, which successfully maintained stability of the rat fundus for several minutes. Ten-scan averaging was employed. Auto segmentation of retinal layers was performed for an objective quantification of thickness measurements except for areas of the RNFL that contain major retinal vessels. In these cases, segmentation was manually corrected as follows: In areas where a major blood vessel interrupted the RNFL tissue, RNFL thickness was determined as the average of RNFL thickness measurements from two adjacent points.¹⁸ An example of RNFL segmentation is illustrated in Figure 1B. The eyes were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceuticals, Osaka, Japan). To preserve corneal clarity throughout the experiment, a custom-made contact lens with a radius of curvature of 2.75 mm, a diameter of 5.0 mm, and 0 diopters (Unicon Corp., Osaka, Japan) was placed on the cornea after topical anesthesia with 0.4%

oxybuprocaine hydrochloride eye drops (Santen Pharmaceuticals). Cross-sectional OCT imaging of the retina was performed in a circumpapillary manner, with a circle diameter of 1000 μ m centered on the optic disc under general anesthesia with intraperitoneal pentobarbital. Measurements were recorded for total retinal thickness, RNFL thickness, and inner retinal thickness (from the RNFL to the inner nuclear layer).

For examination of the dose-response relationship, OCT imaging was performed before (baseline) and at 1 and 2 weeks after intravitreal injection for ET-1–treated eyes, while it was performed at 2 weeks after injection for control eyes. For examination of the tafluprost effect, OCT imaging was performed before (baseline), and at 1, 2, and 4 weeks after an intravitreal injection of ET-1.

Quantification of RGCs in Rat Retinal Flatmounts

Retrograde staining of RGCs of both eyes was achieved by injecting a fluorescent dye into the superior colliculus bilaterally after the last OCT imaging session. Rats were placed in a stereotaxic apparatus (Narishige Co. Ltd., Tokyo, Japan), and the skin of the skull was incised. The brain surface was exposed by perforating the parietal bone with a dental drill to facilitate dye injection. Fluorogold 5% (FG; Wako Pure Chemical Industries, Ltd., 2.1 μ L each) was injected at a point 6 mm caudal to the bregma and 1.2 mm lateral to the midline on both sides to a depth of 4.5 mm from the surface of the skull.

Seven days after FG was injected into the superior colliculus, the eyes were enucleated after an overdose administration of intraperitoneal pentobarbital. The eyes were fixed in 4% paraformaldehyde in PBS for 1 hour in the dark at 4°C. The anterior segments were removed and the eye cups were fixed in 4% paraformaldehyde/PBS for 30 minutes in the same conditions. Six radial cuts were made in the periphery of the eye cup and the retina was carefully separated from the retinal pigment epithelium. A small cut was placed in the peripheral corner of the superior retinal portion in order to correctly identify retinal orientation.

The retina was then flatmounted on a glass slide, covered with an anti-fade mounting solution (VECTASHIELD; Vector Laboratories, Inc., Burlingame, CA) and a glass coverslip, and kept in the dark at 4°C until microscopic observation. The retinal flatmounts were viewed with a universal microscope (Axioplan 2; Carl Zeiss Co., Ltd., Tokyo, Japan). Fluorogold-labeled RGCs were manually counted in a masked fashion by the same investigator in areas 1500 μ m from the center of the optic disc and 500 μ m from the end of the retina in each sextant (12 areas per eye, 0.345 mm² each, Fig. 1F). Morphological criteria of RGC identification was the same as our previous study.²⁹ Cells with irregular shape, intense dye staining, and smaller or larger size than typical RGCs were considered to be non-RGC cells such as microglia.

Statistical Analysis

For the examination of a dose-response relationship, differences in retinal thicknesses were analyzed by repeated-measures ANOVA for comparisons between different time points and by one-way ANOVA for comparisons between different ET-1 doses. For the examination of the tafluprost effect, differences in thickness of each retinal layer were analyzed by two-way repeated-measures ANOVA for comparisons between saline-treated eyes and tafluprost-treated eyes, or between different time points. The difference in average FG-labeled RGC counts in retinal flatmounts was compared with one-way ANOVA between control and ET-1–treated eyes, or two sample *t*-tests between saline-treated and tafluprost-treated eyes. The difference in grading scale for vasoconstrict-

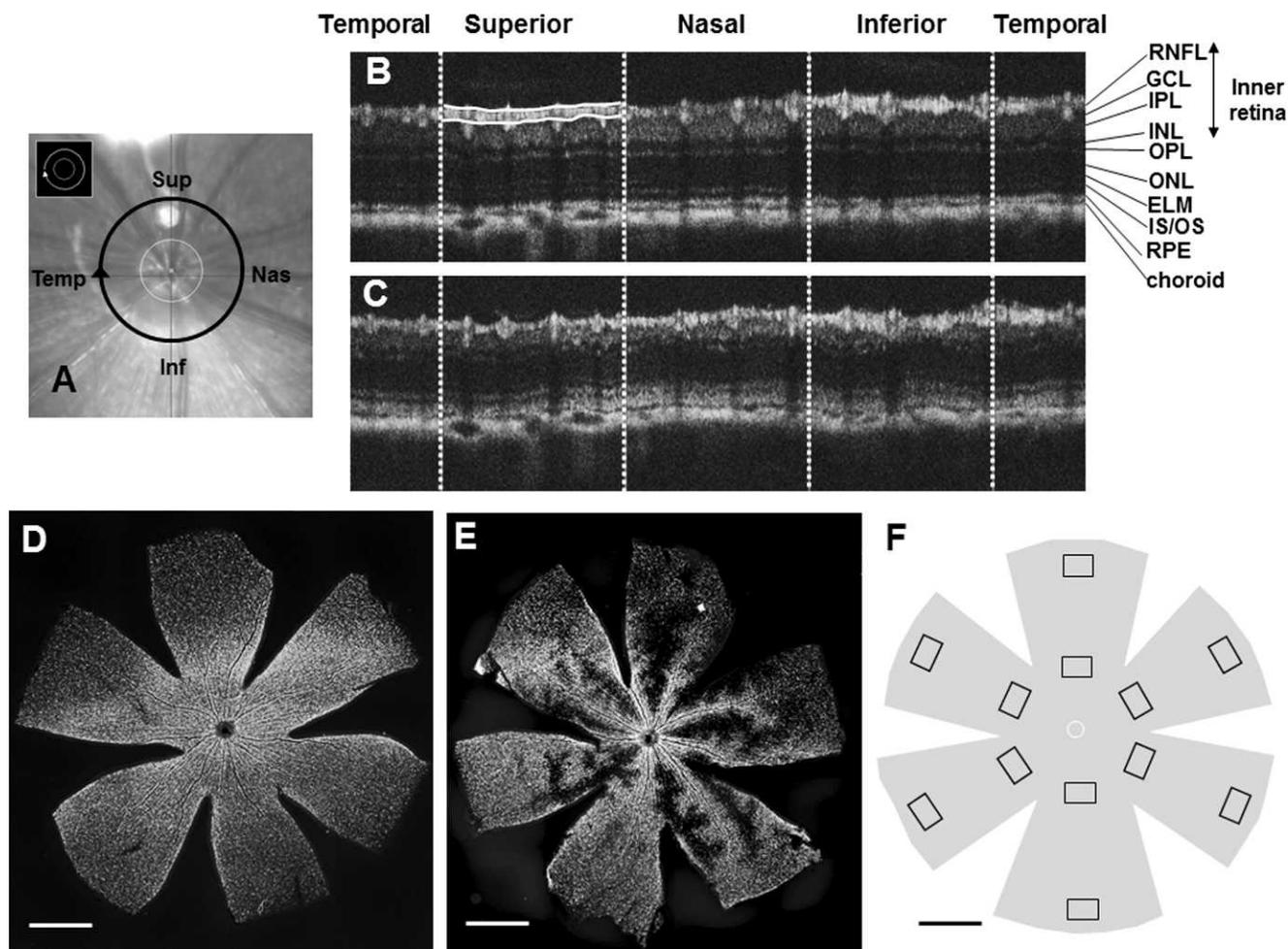


FIGURE 1. Representative fundus and OCT images and photographs of retinal flatmounts. (A) Fundus image by a CCD camera with a scan circle (1000- μ m diameter) centered on the optic disc. *Circle with an arrow*: scan position and direction. (B, C) Cross-sectional OCT image of the same rat retina along the circle before (B) and 2 weeks after (C) ET-1 injection (20 pmol/eye). Cross-sectional OCT images were segmented to identify the RNFL and the inner retina. A part of the RNFL segmentation is shown in (B). (D, E) Representative photographs of retinal flatmounts showing FG-labeled retinal ganglion cells in a control eye (D) and an ET-1-treated eye (20 pmol/eye, [E]), each at 3 weeks after intravitreal injection. (F) Schema showing the locations (*rectangles*) of RGC counting. ELM, external limiting membrane; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS/OS, inner and outer segment junction of the photoreceptor cell; ONL, outer nuclear layer; OPL, outer plexiform layer. *Scale bars* in (D-F): 1500 μ m.

tion was compared with Mann-Whitney test. Pearson's correlation coefficient was used to test the correlation between mean thickness of each retinal layer and mean FG-labeled RGC density in retinal flatmounts.

In order to examine regional differences in retinal thickness by OCT and FG-labeled RGCs in retinal flatmounts, the retinal thickness was compared among four quadrants: superior (S), nasal (N), inferior (I), and temporal (T). Retinal ganglion cell densities were analyzed in six regions: S, NS, NI, I, TI, and TS regions. For the examination of a dose-response relationship, repeated-measures ANOVA was used for comparison between different regions. For the examination of the tafluprost effect, two-way repeated-measures ANOVA was used for comparison between saline-treated and tafluprost-treated eyes, and between different time points. Bonferroni post hoc test was used to detect pairs with a significant difference by one-way ANOVA and repeated-measures ANOVA and two-way repeated measures ANOVA. $P < 0.05$ was considered statistically significant. Data are expressed as the mean \pm SD.

RESULTS

Thickness Changes of Retinal Layers Over Time and a Decrease in FG-Labeled RGCs After an Intravitreal Injection of ET-1

Our OCT system successfully imaged thickness changes of retinal layers over time after an intravitreal injection of ET-1 (Figs. 1B, 1C). Thickness of total retina, RNFL, and inner retina at baseline were 205.7 ± 4.8 μ m, 27.2 ± 2.3 μ m, and 93.7 ± 3.1 μ m (mean \pm SD), respectively. No significant changes in thickness of total retina, inner retina, or RNFL were observed in eyes injected with 0.2 to 5 pmol/eye of ET-1 during the experimental period. Endothelin-1 at doses of 20, 60, or 200 pmol/eye caused a significant and progressive decrease of total retinal thickness and inner retinal thickness (Figs. 2A, 2C). In eyes injected with 20 and 60 pmol/eye of ET-1, RNFL thickness was unchanged 1 week after ET-1 injection, but then decreased significantly at 2 weeks (Fig. 2B). In eyes injected with 200 pmol/eye of ET-1, RNFL thickness was increased 1 week after

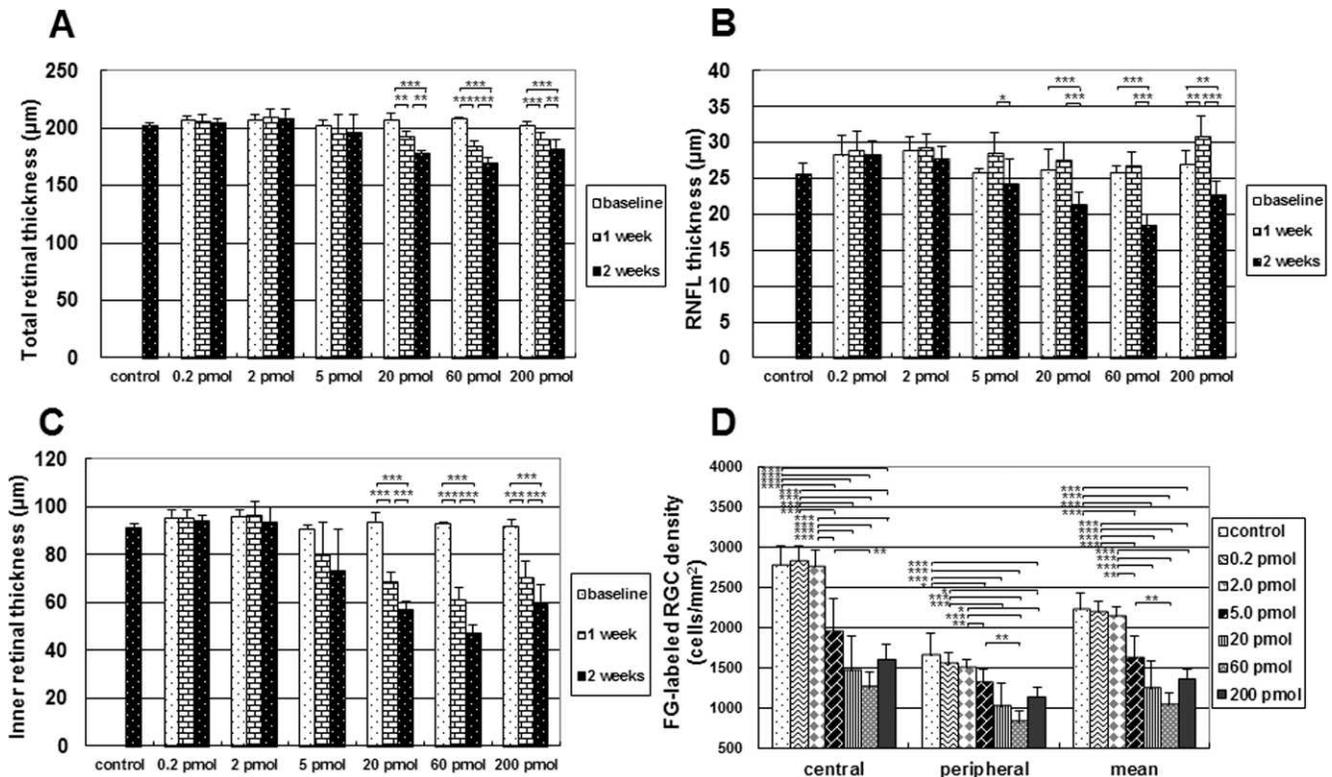


FIGURE 2. Changes in mean retinal thickness in circumpapillary OCT scans and FG-labeled RGC density in retinal flatmounts caused by intravitreal injection of ET-1. Changes in the total retinal thickness (A), RNFL thickness (B), and the inner retinal thickness (C). Retinal ganglion cell density in rat retinal flatmounts (D). Mean, mean of RGC counts in all retinal areas examined. Data represent the mean \pm SD. (A–C) Between two time points, repeated-measures ANOVA. (D) One-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ET-1 injection ($P < 0.01$), but then decreased at 2 weeks (Fig. 2B). As for comparisons between different ET-1 doses, lower doses of Endothelin-1 (0.2, 2, 5 pmol) caused a smaller decrease in retinal thickness than higher doses (20, 60, and 200 pmol) except for the RNFL at 1 week after ET-1 injection, which showed no differences between any pairs of ET-1 doses (Supplementary Fig. S2). When comparing higher doses, there were no statistically significant differences in retinal thickness among the three groups except for total retina, which was significantly thicker in eyes with 200 pmol than in eyes with 60 pmol (Supplementary Fig. S2).

Fluorogold-labeled RGC counts decreased at a dose ≥ 5 pmol/eye in the flatmount retinas harvested 3 weeks after intravitreal injection of ET-1 (Figs. 1D, 1E, 2D, Supplementary Fig. S3). The average number of RGCs of control eyes was 2773.9 ± 234.3 , 1654.6 ± 268.0 , and 2214.3 ± 208.9 cells/mm² in the central retina, peripheral retina, and all retinal areas examined, respectively. Fluorogold-labeled RGC counts in eyes injected with 0.2 and 2 pmol/eye of ET-1 were not significantly different from those of control eyes. When comparing the 20, 60, and 200 pmol groups, there were no statistically significant differences in RGC densities among the three groups (Fig. 2D).

Thickness of total retina, inner retina, and RNFL in OCT images correlated significantly with the number of FG-labeled RGCs in the central retina, peripheral retina, and all retinal areas examined ($P < 0.001$, Table). The strongest association was found between the inner retinal thickness and the average number of FG-labeled RGCs in the central retina ($r = 0.92$, $P < 0.001$, Table, Fig. 3). Because ET-1 at a dose of 20 pmol/eye or greater caused a significant decrease of inner retinal thickness, a dose of 20 pmol/eye was chosen to examine the neuroprotective effect of tafluprost in the following study.

Reduced Retinal Injury Following Application of Tafluprost Eye Drops

During the experimental period to examine the effects of tafluprost on ET-1-induced retinal injury, two rats treated with saline died and were excluded. Constriction of retinal vessels was observed 1 hour after ET-1 injection in all rats. Grade 1 was observed in two tafluprost-treated eyes and one saline-treated eye. Grade 2 was observed in 10 tafluprost-treated eyes and nine saline-treated eyes. There was no significant difference in the grade of vessel constriction between the two groups. Figures 4A through 4C illustrate changes in retinal thickness over time after ET-1 injection. When comparing tafluprost-treated and control eyes, the thickness of total retina, RNFL, and inner retina in eyes treated with tafluprost was significantly thicker than in eyes treated with saline after 1 and 2 weeks (total retina, $P < 0.01$ and $P = 0.019$; RNFL, $P = 0.012$ and $P < 0.01$; inner retina, $P = 0.038$ and $P = 0.045$, at 1 and 2 weeks, respectively).

TABLE. Pearson's Correlation Coefficient for the Relationship Between FG-Labeled RGC Counts in Retinal Flatmounts and Retinal Thickness in OCT Images

Retinal Flatmounts OCT	RGC Counts in Central Retina	RGC Counts in Peripheral Retina	Mean*
Total retinal thickness	0.84	0.71	0.82
RNFL thickness	0.79	0.72	0.79
Inner retinal thickness	0.92	0.80	0.90

* Mean of RGC counts in all retinal areas examined.

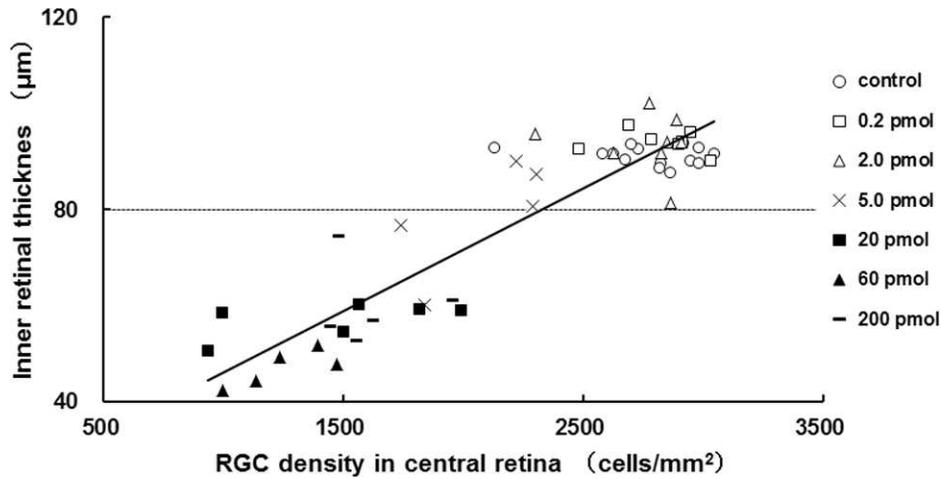


FIGURE 3. Correlation between inner retinal thickness determined by OCT and FG-labeled RGC density in the central retinal areas. Pearson's correlation coefficient analysis shows a significantly positive relationship between inner retinal thickness determined by OCT and FG-labeled RGC density in the central retinal areas ($r = 0.92$, $P < 0.001$).

Although FG-labeled RGC counts in the peripheral retina and the mean retina were not different between the two groups (1406 ± 127 vs. 1380 ± 176 cells/mm², $P = 0.69$; 1908 ± 151 vs. 1740 ± 258 cells/mm², $P = 0.09$; in the peripheral

retina and in all retinal areas examined, respectively), Fluorogold-labeled RGCs in the central retina were higher in tafluprost-treated eyes than in control eyes (2411 ± 213 vs. 2101 ± 397 cells/mm², $P = 0.03$, Fig. 4D).

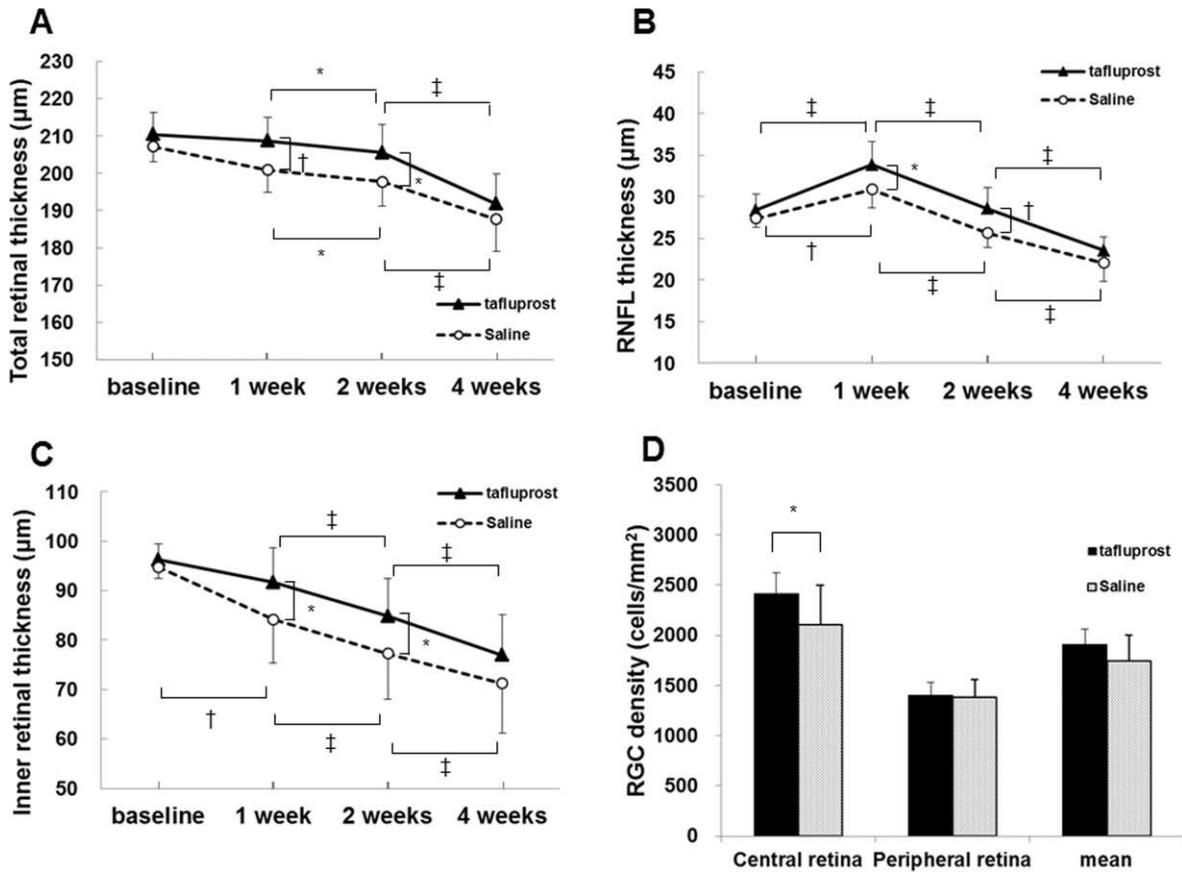


FIGURE 4. Changes in mean retinal thickness in circumpapillary OCT scans and FG-labeled RGC density after intravitreal injection of ET-1 with topical treatment of tafluprost or saline. Changes in the total retinal thickness (A), RNFL thickness (B), and the inner retinal thickness (C). Retinal ganglion cell density in rat retinal flatmounts (D). Mean, mean of RGC counts in all retinal areas examined. Data represent the mean \pm SD. (A-C) Between groups or between different time points, two-way repeated-measures ANOVA. (D) Two sample *t*-test. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

Regional Differences in Retinal Thickness and FG-Labeled RGCs

Normal Eyes. The mean retinal thickness was as follows ($n = 38$): total retina, 202.0 ± 5.2 , 206.7 ± 5.0 , 207.5 ± 5.1 , and $206.5 \pm 5.8 \mu\text{m}$; inner retina, 91.5 ± 3.7 , 94.6 ± 3.5 , 93.2 ± 3.8 , and $95.6 \pm 3.5 \mu\text{m}$; RNFL, 27.0 ± 2.8 , 26.4 ± 2.9 , 27.8 ± 3.0 , and $27.5 \pm 3.2 \mu\text{m}$ in the S, N, I, and T quadrants, respectively (Supplementary Fig. S4A). The thickness of the total retina and the inner retina in the S quadrant was significantly thinner than in the other quadrants. Inner retinal thickness in the N and T quadrants was significantly thicker than in the I quadrant. On the contrary, RNFL thickness in the I quadrant was significantly thicker than in the N quadrant. Retinal ganglion cell density in the I region was significantly lower than in the TS region in the peripheral and the mean retina (Supplementary Fig. S5A).

The Effects of ET-1. Relative thickness compared with baseline was evaluated. In eyes with 2 pmol/eye of ET-1, total retinal thickness in the T quadrant was significantly greater than in the S quadrant 2 weeks after ET-1 injection ($P = 0.021$), although the difference was less than 2%. In eyes injected with other doses of ET-1, there were no significant regional differences in thickness changes of the total retina. The thickness changes in the inner retina did not show regional differences at any ET-1 dose. In contrast, the RNFL thickness changes were larger in the S quadrant with some statistically significant differences in eyes injected with 2, 5, and 200 pmol of ET-1 (Supplementary Fig. S4B). Regional differences in relative RGC density were most prominent in eyes injected with 5 pmol of ET-1 compared with normal eyes (Supplementary Figs. S5B-D, S6A-C).

Effects of Tafluprost. Total retinal thickness in eyes treated with tafluprost was significantly thicker than in eyes treated with saline in all quadrants except the S quadrant after 1 and 2 weeks (Supplementary Fig. S7). Inner retinal thickness in eyes treated with tafluprost was significantly thicker than in eyes treated with saline in the N and I quadrants after ET-1 injection (Supplementary Fig. S8). The N quadrant in particular showed significant differences at 1 to 4 weeks. RNFL thickness in eyes treated with tafluprost was significantly thicker than in eyes treated with saline in the S, N, and I quadrants at 1 or 2 weeks after ET-1 injection (Supplementary Fig. S9). Although there was a significant difference in RNFL thickness in the T quadrant between the two groups at baseline, the difference was not significant after ET-1 injection. Relative RGC density compared with normal eyes was greater in the N regions of the central, peripheral, and mean retina although the differences were not statistically significant (Supplementary Fig. S10).

DISCUSSION

In previous studies, intravitreal injection of ET-1 into rat eyes led to prolonged retinal circulation time,³⁰ impairment of anterograde axonal transport,³¹ RGC loss,^{32,33} increased glial fibrillary acidic protein expression,³² reduction in the number of axons,³² change in pupillary light reflex,³² impairment of retrograde axonal transport,³³ and histological optic nerve damage.³³ Retrobulbar delivery of ET-1 to the rat optic nerve also induced loss of RGCs and their axons in a chronic model and a transient impairment of retrograde axonal transport in an acute model.^{34,35} Similarly, loss of optic nerve axons was shown in a primate model of chronic retrobulbar administration of ET-1.³⁶ Furthermore, astrogliosis coincided with ET-B receptor upregulation was reported in human glaucomatous optic nerves.³⁷ The present study showed thinning of the inner

retina and a decrease in FG-labeled RGCs caused by an intravitreal injection of ET-1 in rat eyes.

Previous studies proposed mechanisms of RGC damage caused by ET-1. One mechanism is the induction of nitric oxide (NO) synthetase by ET-1 receptor binding. When ET-1 binds to ET-B receptors,^{38,39} it promotes the activation of endothelial nitric oxide synthase and causes release of NO, which has been linked to apoptotic signaling cascades.⁴⁰ Other theories involve blockage of target-derived growth factors via an alteration in axonal transport^{33,35} and the ability of ET-1 to induce ischemia via vasoconstriction.⁴¹ Two studies reported retinal circulation disturbance after intravitreal injection of ET-1 into the rat eye. Intravitreal injection of ET-1 (1–10 pmol/eye) terminated retinal circulation and caused a significant prolongation of retinal circulation time.³⁰ Taniguchi et al.³³ reported that intravitreal injection of 5 pmol/eye of ET-1 caused a significant constriction of retinal vessels and a statistically significant decrease in the number of retrogradely labeled RGCs. Because the inner retina receives blood supply from the central retinal artery, the retinal ischemia-reperfusion model in rats also showed a marked thinning of the inner retina.⁴² In the present study, intravitreal injection of ET-1 at doses of 20 pmol/eye or greater caused a significant thinning first in the inner retina at 1 week and then in RNFL at 2 weeks, and a decrease in the number of RGCs. The degree of FG-labeled RGC decrease was most comparable with inner retinal thickness at 2 weeks after ET-1 injection. Combining these changes in the inner retina with the retinal vessel constriction observed 1 hour after intravitreal injection of ET-1 at a dose of 20 pmol/eye, the mechanism of ET-1-induced inner retinal damage in our model is likely to be retinal ischemia.

Regarding the dose-response relationship, a decrease in retinal thickness and FG-labeled RGCs was not significantly different at a dose of 20 pmol/eye or greater, and even showed a trend of less damage at 200 pmol/eye. These results indicate that higher doses of intravitreal ET-1 may be an overdose for the receptor activation responsible for retinal damage, or might even indicate a possible neuroprotective mechanism in ischemic insults. In ET-B receptor-deficient rats, cerebral neural damage by hypoxia-ischemia was pronounced,⁴³ although RGC loss by ocular hypertension was attenuated.⁴⁴

In previous studies with an intravitreal ET-1 injection, a similar decrease in FG-labeled RGCs was observed at 1 week postinjection with 5 pmol/eye of ET-1 injection followed by FG labeling (23.1% decrease),³⁵ and with 2.5 nmol/eye of ET-1 injection after FG labeling (24.6% decrease).³² In the present study, an injection of 20, 60, and 200 pmol/eye caused a 43.1%, 53.4%, and 38.2% decrease, respectively, at 3 weeks post injection. In a study where RGCs were FG-labeled before ET-1 injection,³² lower ET-1 doses (25 and 250 pmol/eye), which are similar levels to those used in our study, did not cause a decrease in FG-labeled RGCs. This apparent discrepancy may be explained as follows: Since ET-1 was reported to impair axonal transport of RGCs,^{31,33,35} RGCs that were not labeled by FG after ET-1 injection may include dead RGCs as well as RGCs with functional impairment of axonal transport. Therefore, the number of FG-labeled RGCs may be less than that of live RGCs in the protocol of postlabeling with FG after ET-1 injection, whereas prelabeling before ET-1 injection will delineate all live RGCs.

In the present intravitreal ET-1 injection model, the number of FG-labeled RGCs was most correlated with inner retinal thickness in OCT images. Therefore, inner retinal thickness measured by OCT may be a useful parameter of RGC injury in the present model. On the other hand, RNFL thickness decreased at 2 weeks' post injection of ET-1, although previous experiments revealed that RGCs had decreased at 1 week after injection of ET-1.³² We previously reported that RNFL thinning

was not observed until 2 weeks after optic nerve crush in rats,¹⁸ although our group demonstrated 50% RGC loss even 1 week after the insult in the same model.²⁹ Thus, there is a discrepancy between the onset of RGC loss and RNFL thinning after optic nerve crush; RGC loss was followed by RNFL thinning. Similarly, there may be a time lag between the decrease in FG-labeled RGCs and RNFL thinning after ET-1 injection. Furthermore, it may be more difficult for RNFL thickness to reflect RGC damage compared with inner retinal thickness because RNFL thickness in rats is thinner than that in humans and inner retinal thickness in rats. Accordingly, inner retinal thickness may be more suitable than RNFL thickness for monitoring RGC loss and for evaluating the neuroprotective effects of tafluprost in this model.

The present data suggested that tafluprost instillation reduced ET-1-induced retinal injury. Previous experiments have revealed that topical administration of tafluprost increases retinal blood flow in cat²² and optic nerve head blood flow in rabbits²³ and monkeys.²⁶ Kurashima et al.²⁵ reported that tafluprost instillation could inhibit the ET-1-induced impairment of ocular blood flow. Another study identified a direct anti-apoptotic effect of tafluprost on cultured RGCs and rat RGCs after optic nerve crush.²⁷ Our results warrant further experiments to explore the potential neuroprotective mechanism of tafluprost in this model.

Regarding the regional differences in retinal thickness and FG-labeled RGCs, the retinal thickness was significantly different in several comparisons between different regions by OCT. However, retinal thickness along the circular scan can be considered relatively uniform given that the differences did not exceed the coefficient of repeatability and reproducibility of total retinal thickness ($\leq 9.2 \mu\text{m}$, 4.5%; Nagata A, Higashide T, Sugiyama K, unpublished observations, 2008), inner retinal thickness ($\leq 4.0 \mu\text{m}$, 4.2%; Nagata A, Higashide T, Sugiyama K, unpublished observations, 2008), and RNFL thickness ($\leq 2.5 \mu\text{m}$, 9.0%).¹⁸ The relatively smaller RGC densities in the I region was consistent with a previous study.⁴⁵ The preferential damage by ET-1 injection was observed in the S region both in the changes of RNFL thickness and FG-labeled RGCs at relatively smaller ET-1 doses. The proximity of the S quadrant to the ET-1 injection site may be one reason for the regional differences. Inner retinal thickness showed the most prominent differences between tafluprost-treated and saline-treated eyes when compared with the magnitude of the variability (repeatability and reproducibility) of thickness measurements by OCT. The protective effects of tafluprost on the inner retinal thickness were relatively large in the N region, and FG-labeled RGCs showed a similar trend. The reason for the possible regional differences in the effects of tafluprost is currently unknown.

There are several limitations of our study. Since we postlabeled RGCs in order to see the whole effects on the retina by intravitreal injection of ET-1, the loss of FG labeling by axonal damage or cell death cannot be differentiated. There is also a time lag between retinal thickness measurements by OCT and FG-labeled RGC counts in retinal flatmounts, and this may have affected the correlation between retinal thickness and RGC counts. Further studies using both pre- and post-FG labeling of RGCs should clarify these issues. A further limitation was that retinal thickness was evaluated only with a single circle scan. Although a significant correlation between retinal thickness and FG-labeled RGCs was observed, a circular scan with multiple scan diameters will give us more information about retinal changes than a single circular scan.

In conclusion, inner retinal thickness measured by OCT was strongly correlated with FG-labeled RGCs in a rat model of intravitreal ET-1 injection. Topical tafluprost applied daily after ET-1 injection partly inhibited the process of inner retinal

damage. Given that OCT could successfully monitor the inhibitory effects of tafluprost in our rat model, OCT may be a useful tool that can offer a valuable in vivo marker to evaluate potential neuroprotective drugs in rat models of RGC damage.

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