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Neuroprotective Effects of R(−)-1-(benzo[b]thiophen-5-yl)-2-[2-(N,N-diethylamino)ethoxy]Ethanol Hydrochloride (T-588) Against Retinal Ganglion Cell Death Induced by Elevated Intraocular Pressure in Rat

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Purpose: We investigated whether T-588 can attenuate retinal ganglion cell (RGC) death induced by elevated intraocular pressure (IOP).

Methods: IOP elevation was induced unilaterally by argon laser irradiation of the rat trabecular meshwork 4 days after an intracameral injection of India ink. We orally administered either the vehicle, or 10, 30, or 100 mg/kg body weight (BW) of T-588 24 hours before the laser application. Five days after the laser application, 1.5 μL of 3% Fast Blue was injected into the superior colliculi bilaterally. Three days after the Fast Blue injection, the eye was enucleated and the retinal whole flatmount was prepared. Labeled ganglion cells were counted by fluorescence microscope with an ultraviolet filter.

Results: Laser treatment significantly increased the IOP. The percentages of labeled RGCs in the lasered eyes as compared with the nonlasered contralateral eyes were 78.0 ± 11.6% in the control group, 78.7 ± 12.9% in the 10 mg/kg BW group, 79.1 ± 13.0% in the 30 mg/kg BW group, and 91.0 ± 9.0% in the 100 mg/kg BW T-588–treated group. The survival rate of RGCs was significantly higher in the 100 mg/kg BW T-588–treated group than in the control group.

Conclusion: T-588 appears to have a neuroprotective effect on retinal ganglion cells in this ocular hypertensive model. **Jpn J Ophthalmol 2002;46:621–626** © 2002 Japanese Ophthalmological Society

Key Words: Elevated intraocular pressure model, neuroprotection, rats, T-588.

Introduction

A common feature of all glaucomas is the progressive death of retinal ganglion cells,[1,2] and elevated intraocular pressure (IOP) is a major risk factor for glaucomatous optic nerve damage.[3,4] Although other factors are postulated to play a role in glaucoma, IOP remains the best documented criterion[5,6] and nearly all of our current glaucoma therapy is directed towards lowering IOP. Nonetheless, normalization of the IOP cannot completely stop glaucomatous visual loss.[7,8] While IOP clearly plays a critical role in the management of glaucoma, it may be helpful to look beyond pressure control in the treatment of this disease. This premise underlies the concept of direct neuroprotection in glaucoma, whereby agents are sought that may retard glaucomatous loss without necessarily affecting the IOP.

T-588 is a potential therapeutic agent for reversing the dementia associated with Alzheimer's disease and cerebrovascular disease. This compound has an antihypoxic effect in mice[9] and ameliorates memory and learning impairment in animal models, including

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cerebral embolization, basal forebrain lesion, and transient forebrain ischemia.[10,18] The pharmacological effects of T-588 are considered to be mediated at least partly by cholinergic and noradrenergic systems in the brain,[10–12] but the molecular mechanisms underlying the effect are not known. No information is available on the function of T-588 in the retina of the rat or in that of higher vertebrates. The present study was designed to investigate the capability of T-588 to retard the death of retinal ganglion cells (RGCs) induced by elevated IOP in the rat.

Materials and Methods

Animals

Male albino Wistar rats were handled in accordance with the ARVO principles for the use of animals in ophthalmic and vision research. We used 40 rats 9 weeks after birth, weighing 280–300 g. The rats were housed in plastic cages and allowed free access to food and water. All operations were carried out under general anesthesia with ketamine hydrochloride, 40 mg/kg body weight (BW) and xylazine, 4 mg/kg BW. During all procedures under anesthesia, the rats were kept on a heating pad until they began to recover from the anesthesia.

IOP-Elevated Model and Drug Administration

All surgical procedures were performed unilaterally, in the right eye. The left eye served as a control. IOP elevation in the rat was induced by laser photocoagulation in the trabecular meshwork according to the method reported by Ueda et al.[13] Approximately 30 μL of the aqueous humor from the right eye was aspirated using a 30-gauge needle, then the same volume of 35% India ink was injected into the anterior chamber. Three days after the injection of India ink, T-588 was dissolved in distilled water (0.2%, 10 mg/kg BW; 0.6%, 30 mg/kg BW; and 2.0%, 100 mg/kg BW), and 1.45 mL of this drug solution or distilled water was randomly administered to one of the following oral treatment groups by gastric tube: 1.45 mL of distilled water (n = 10), 10 mg/kg BW of T-588 (n = 11), 30 mg/kg BW of T-588 (n = 10), and 100 mg/kg BW of T-588 (n = 9). One day after drug administration, laser irradiation of the trabecular meshwork was performed in the right eye. The laser pulses were delivered around the perilimbal tissues stained black with India ink, at a spot-size setting of 150–200 μm, a power setting of 150–250 mW, and for a duration of 0.2 seconds.

IOP Measurements

IOP in the anesthetized rat was measured three times using a pneumatonometer (Model Classic 30; Mentor, Norwell, MA, USA) and the mean value was used for the analyses. Measurements were taken before the injection of India ink, and 5 and 8 days after the laser photocoagulation of the trabecular meshwork.

Visualization of RGCs

To assess RGC survival, the rats were kept alive for 8 days. Five days after laser irradiation, for retrograde labeling of the RGCs, 1.5 μL of a 3% Fast Blue solution (Sigma Chemical, Steinheim, Germany) was injected over a period of 15 minutes into the bilateral superior colliculi of rats fixed in a stereotaxic apparatus with the following coordinates: 5.8–6.0 mm posterior to the bregma, 1.2–1.5 mm lateral to the midline, and 3.8–4.0 mm from the surface of the skull.

Tissue Processing

Three days after the tracer injection, the rats were deeply anesthetized and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Immediately thereafter, both eyes were enucleated, and the cornea and lens were removed. The remaining eyecup was postfixed for 12 hours in 4% paraformaldehyde in PBS. The eyecup was cut and the retina was flatmounted.

Cell Count

Retrograde-labeled RGCs in the flatmounts were visualized using a fluorescence microscope (Axioskop H, Carl Zeiss, Jena, Germany) with an ultraviolet filter (Blue-Violet: 395-440 nm). We counted in four predetermined areas, ie, superior, inferior, nasal, and temporal, from each flatmounted retina. All areas chosen were within a radial distance of approximately 1 mm from the optic disc. RGC numbers were determined by computer scanning for particle analysis using image analysis software (Optimas Image, Version 6.5). Cell counts were performed following a double-blind protocol.

Statistical Analysis

All data are given as mean ± SD. Analysis of variance (ANOVA) was used to determine statistical significance of the IOP measurements. Additionally, a one-way ANOVA and post-hoc comparisons based

Table 1. Intraocular Pressure in the Lasered Eyes*

Days	Control (n=10)	10 mg/kg BW (n=11)	30 mg/kg BW (n=10)	100 mg/kg BW (n=9)	$P^{\dagger}$
−4	11.±0.5 (10.3–11.7)	11.4 ±0.5 (10.7–12.5)	11.1±0.4 (10.7–11.8)	11.2 ±0.4 (10.7–11.7)	NS
+5	18.3±0.6‡ (17.7–19.7)	18.3±0.5‡ (17.5–19.2)	18.0±0.5‡ (17.5–19.0)	18.4±0.5‡ 17.5–18.8)	NS
+8	16.9±0.4‡ (16.3–17.3)	17.4±0.4‡ (17.0–18.2)	17.2±0.4‡ (16.7–17.8)	16.9±0.4‡ (16.5–17.7)	NS

*mm Hg, mean ± SD (range). BW: body weight.
†analysis of variance. NS: not significant.
‡P<.0001(compared with day −4; paired t-test).

on the Fisher protected least significant difference approach were used to determine statistical significance in RGC density determinations. A difference of $P <$ .05 was considered significant.

Result

IOP in the Lasered Eye

The IOP was measured before laser irradiation, and on the 5th and 8th day following laser therapy. Laser treatment significantly increased the IOP. The range of IOPs and their mean ± SD for lasered eyes and nonlasered eyes have been summarized in Table 1. Statistical analyses showed a highly significant difference ($P <$ .0001, ANOVA). The IOPs on the 5th and 8th days were essentially similar between the control rats and the T-588–treated rats. The mean values of the IOPs did not show any difference between the 5th and 8th days following T-588 administration, regardless of doses ($P >$ .05, ANOVA). Similarly, the baseline IOP values were not significantly different between the control and the T-588–treated groups.

Effects of T-588 on RGCs Survival

After preparing retinal flatmounts, we found RGCs were focused 1 mm from the optic disc. RGCs were counted in the superior, inferior, nasal, and temporal quadrants. Laser-induced elevated IOP severely reduced the RGC number in the right eyes (Figure 1A) while the RGC number remained high in the nonlasered left eyes (Figure 1B). This indicates that the time provided was sufficient to induce cell death following laser therapy. This fact is consistent with the results of previous experiments conducted in our laboratory.[14] On the other hand, eyes treated with 100 mg/kg BW of T-588 exhibited a substantially larger number of RGCs (Figure 1C), suggesting that T-588 averted RGC death due to increased IOP-induced neurodegeneration. However, a lower dose of T-588, either 10 mg/kg BW or 30 mg/kg BW, failed to prevent RGC death. Table 2 shows the density of RGCs labeled by Fast Blue in the lasered and nonlasered eyes and the survival rate, which is the percentage of the labeled RGCs in the lasered eyes as compared with the nonlasered contralateral eyes 8 days after laser irradiation. The mean value of survival rates of RGCs in control rats was 78.0 ± 11.6. The mean survival rate in 10 mg/kg BW T-588–treated rats was 78.7 ± 12.9. A dose of 30 mg/kg BW of T-588 also expressed a similar mean survival rate of 79.1 ± 13.0. By contrast, an increment of the survival rate was observed after 100 mg/kg BW T-588 treatment.

We constructed a bar graph (Figure 2) to show the mean ± SD of survival rates of RGCs after T-588 treatment and the level of significance of the drug effects. An ANOVA test revealed that the increment of survival rate after 100 mg/kg BW of T-588 pretreatment was statistically significant ($P <$ .05). The survival rate of RGCs in the 100 mg/kg BW T-588–treated group was significantly higher than that in the control group and in the rats treated with 10 mg/kg BW T-588 and 30 mg/kg BW T-588.

Discussion

In the present study, we demonstrated that T-588 has neuroprotective effects against RGC death induced by increased IOP in the rat. This is the first study to report that T-588 can attenuate the RGC death induced by elevated IOP.

Ikeda et al[15] used an animal model of motoneuron disease (MND) to assess the neuroprotective effect of T-588 in the neuromuscular junction. They observed that T-588 treatment attenuated forelimb contracture and muscle weakness, increased the weight of bicep muscles, and delayed progression of neuromuscular dysfunction in MND wobbler mice. A pharmacokinetic study showed that T-588 was transported efficiently into the cerebrum and spinal cord following oral administration. T-588 potentiates

Jpn J Ophthalmol
Vol 46: 621–626, 2002

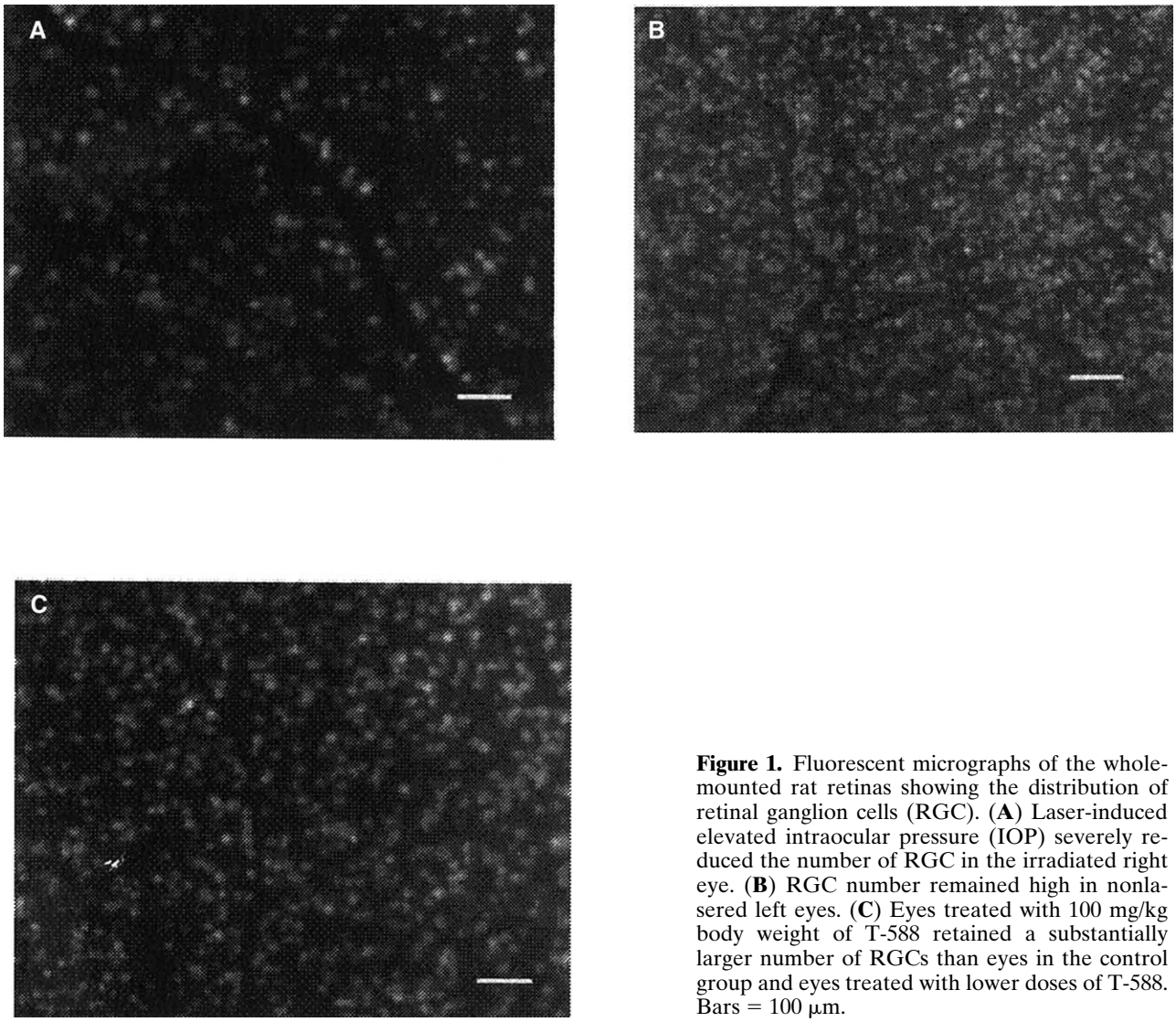

Figure 1. Fluorescent micrographs of the whole-mounted rat retinas showing the distribution of retinal ganglion cells (RGC). (**A**) Laser-induced elevated intraocular pressure (IOP) severely reduced the number of RGC in the irradiated right eye. (**B**) RGC number remained high in nonlasered left eyes. (**C**) Eyes treated with 100 mg/kg body weight of T-588 retained a substantially larger number of RGCs than eyes in the control group and eyes treated with lower doses of T-588. Bars = 100 μm.

the neurotrophic effects of nerve growth factor in PC12 cell culture,[16] enhances acetylcholine release from the frontal cortex and the hippocampus of rats,[17] and can ameliorate cognitive dysfunction in the various amnesia models of rodents.[18,19] T-588 also rescues rat cerebellar granule cells from glutamate neurotoxicity in vitro.[20] This agent also inhibits synaptic facilitation in both crustacean and mammalian neuromuscular junction.[21]

Table 2. Retinal Ganglion Cell (RGC)Density in the Lasered and Nonlasered Eyes on Day 8*

	Control (n=10)	10 mg/kg BW (n=11)	30 mg/kg BW (n=10)	100 mg/kg BW (n=9)
Lasered eyes (RGCs/mm^2)	1493.6±132.1	1518.2±233.6	1560.4±212.0	1800.8±346.4
	(1248.4–1702.9)	(1195.6–1762.2)	(1143.0–1796.8)	(1366.9–2580.7)
Nonlasered eyes (RGCs/mm^2)	1962.0±157.0	1980±199.2	2048.3±325.9	2000.8±307.3)
	(1665.0–2221.7)	(1562.9–2300.7)	(1648.6–2672.9)	(1762.2–2789.9)
Survival rate (%)	78.0±11.6	78.7±12.9	79.1±13.0	91.0±9.0
	(61.5–103.7)	(57.0–97.6)	(64.1–100.1)	(76.0–103.5)

*Mean ± SD (range). BW: body weight.

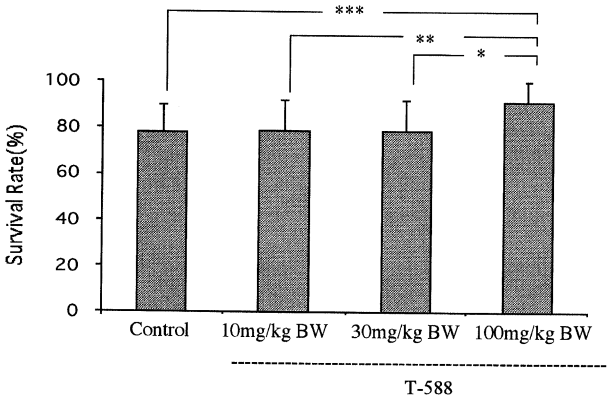

Figure 2. The percentage of Fast Blue-labeled retinal ganglion cells in the lasered eyes as compared with the nonlasered contralateral eyes 8 days after laser irradiation (*P = .0347, **P = .0258, ***P = .0219, Fisher protected least significant difference). Values indicate mean ± SD.

The mechanism of action of T-588 in the glaucoma rat model is not fully understood. It efficiently penetrates the spinal cord without delaying the loss of large motor neurons in MND; yet it also increases the choline acetyltransferase and cyclic adenosine monophosphate levels of the cervical cord. In wobbler mice, when the initial symptoms of MND occur at the age of 3–4 weeks, astrocytosis takes place in the gray matter of the spinal cord.[22] T-588 is known to promote mitogen-activated protein kinase and can prevent rat astrocytes from reperfusion-induced apoptosis in culture.[23] Therefore, one possibility is that T-588 acts directly on spinal motor neurons or glia. Several neuropeptides are altered in the spinal motor neurons of the wobbler mouse.[24] The activities of Cu/Zn superoxide dismutase decrease while nitric oxide synthase levels increase in the spinal cord of this animal.[25] At least in the wobbler mouse, T-588 treatment may adjust the abnormal levels of free radicals and neurotransmitters.

Because the mechanism related to the pharmacological effects of T-588 is still unknown, it is difficult, at present, to point out exactly which mechanisms play the pivotal role in exerting the neuroprotective effect of T-588 on RGCs against ocular hypertension. Nevertheless, it is convincing that T-588 could provide spatial buffering of free radicals to stabilize cellular integrity, thereby preventing cell death.[25] A recent report suggests that T-588 reduced vesicular endocytosis of Ca^{2+}[21] essential for Ca^{2+}-dependent neurotransmitter release. Therefore, it is likely that T-588 could suppress Ca^{2+} influx to block the release of glutamate, an excitatory neurotransmitter responsible for excitotoxicity. Excitotoxicity is, indeed, a major factor associated with RGC degeneration in glaucoma. Electrophysiological studies also support the notion that T-588 reduced the facilitated transmitter release.[21] However, a different mechanism involving γ-aminobutyric acid (GABA), an inhibitory neurotransmitter, could not be ruled out. Apart from glutamate and GABA, interactions with cholinergic, adrenergic, or dopaminergic systems could account for such neuroprotective effects. However, these remain to be elucidated.

In the present study, we failed to observe any significant IOP reduction by oral administration of T-588, suggesting that the neuroprotective action of T-588 is not the result of its ocular hypotensive effect. After laser treatment, the IOP significantly increased and the IOPs on the 5th and 8th days were quite similar among the treatment and control groups. The survival rate of RGCs in the 100 mg/kg BW T-588–treated group was significantly higher than that in the control group, although there was no statistically significant difference among the control group and the groups treated with 10 mg/kg BW and 30 mg/kg BW T-588. Thus, T-588 appears to induce a neuroprotective effect on the RGCs in the ocular hypertensive model. However, further investigation will be required to address many issues with respect to the mechanism by which T-588 promotes RGC survival, or the question of the clinical application, such as optimum dosing.

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Jpn J Ophthalmol
Vol 46: 621–626, 2002

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