ment of RS in the right eye, which resulted in an improvement of VA from 0.2 to 0.6.

Fourteen of the 16 eyes had a final VA of 0.1 or better, and six eyes had a BCVA of better than 0.5.

Complications

Intraoperative or postoperative complications were observed in seven eyes. The retina redetached in three eyes [patients 5 (L), 8, and 9], as described above.

A full-thickness macular hole was observed after the initial vitrectomy in 5 of the 16 eyes. In four of these five eyes, the full-thickness macular hole associated with the retinal detachment appeared before (patients 13 and 14) or after the vitrectomy (patients 8 and 9), as described above. In the fifth eye (patient 4), a macular hole was found in the reattached retina after vitrectomy with ILM peeling and C_3F_8 gas tamponade. All five of these eyes had severe preoperative FD associated with RS and a poor VA.

Patient 5 developed a peripheral retinal break in the right eye intraoperatively during PVD induction that was treated successfully with laser photocoagulation.

Discussion

We confirmed that OCT is a very important tool for observing the pathological appearance of highly myopic eyes, as concluded in previous reports.[2–9] These previous reports also noted that RS and FD were not uncommon. Baba and associates[8] reported that none of seven patients with foveal retinal detachment complained of recent, progressive visual impairment. In addition, one case report described the spontaneous resolution of a myopic foveal retinoschisis.[13] Benhamou and associates[9] reported that this condition is fairly stable, in terms of visual acuity and retinal thickness, and changes slowly over time. In the present study, we studied eyes with RS and/or FD and progressive visual impairment. Fourteen of the 16 eyes exhibited a symptomatic visual impairment at the time of the initial patient visit, and the other two eyes were noted to have asymptomatic RS during routine follow-ups for the other eye, with subsequent visual impairment in the following few years. Two of the 11 eyes associated with FD developed macular hole retinal detachment during the preoperative follow-up period. The patients noticed an acute decrease in BCVA after the development of macular hole retinal detachment. In one eye, OCT revealed changes in the macular appearance during a follow-up examination, with no accompanying visual disturbance. This change detected by OCT and the symptomatic visual impairment associated with RS or FD may reflect the high risk of developing a macular hole.

A high incidence of macular hole retinal detachment in the opposite eye (in 3 out of 14 patients) and the preoperative progression leading to the development of macular hole retinal detachment in 2 out of the 16 eyes support the hypothesis of Takano et al.,[2] who suggested that RS and FD in highly myopic eyes may precede macular hole retinal detachment.

In this study, OCT examination revealed various macular profiles of myopic RS and FD, similar to the description by Benhamou et al.[9] RS involving the entire posterior pole connected to the conus of the optic disc was observed in all 16 cases; thus, we would like to propose that the term "posterior retinoschisis" is more appropriate than "macular retinoschisis" or "foveal retinoschisis." Although the BCVA in eyes with FD seemed to be poorer than that in eyes without FD, six eyes had a BCVA of better than 0.2, and the presence of FD could not be determined based only on visual acuity.

Figure 5A–D. Fundus photograph and OCT images of the left eye of patient 10. A A preoperative fundus photograph shows a shallow macular detachment in an eye with a BCVA of 0.3. B A preoperative OCT image shows a marked elevation of the posterior retina. A posterior retinoschisis is visible from the edge of the conus of the optic disc to the edge of the posterior staphyloma. The outer layer detachment is remarkable. A partial separation of the posterior hyaloid is visible between the fovea and the conus of the optic disc (scan length, 9.0 mm). C Two months after the vitrectomy, the posterior retina elevation was remarkably reduced (scan length, 10.0 mm). D Fifteen months postoperatively, the retina had completely reattached (scan length, 10.0 mm).

Figure 6A–E. Fundus photograph and OCT images of the left eye of patient 13. A, B A 66-year-old man with a history of macular hole retinal detachment surgery in the right eye was noted to have posterior retinoschisis of the asymptomatic left eye during a routine follow-up examination. The BCVA of the left eye was 0.5 (scan length, 9.0 mm). C After observation for 20 months, an OCT examination revealed the development of a foveal detachment associated with the posterior retinoschisis. The BCVA had decreased to 0.3, but the patient had not noticed the development of any visual disturbance (scan length, 9.0 mm). D After observation for 30 months, he complained of reduced vision and scored a BCVA of 0.1. An OCT examination revealed a posterior retinal detachment associated with a macular hole, and vitrectomy was performed (scan length, 10.0 mm). E Ten months postoperatively, the retina had completely reattached, but the macular hole persisted (scan length, 5.0 mm).

Figure 7A–D. Fundus photograph and OCT images of the right eye of patient 14. A A fundus photograph taken at presentation shows a shallow posterior detachment over a posterior staphyloma in an eye with a BCVA of 0.5. B An OCT image taken at presentation shows a foveal detachment with posterior retinoschisis (scan length, 9.0 mm). C Three months after presentation, the BCVA had decreased to 0.06; an OCT image shows macular hole retinal detachment. A vitrectomy was performed (scan length, 10.0 mm). D Three months after the operation, an OCT image shows complete retinal reattachment, but the macular hole remains visible (scan length, 5.0 mm).

Figure 8A–E. Fundus photograph and OCT images of the left eye of patient 9. A A preoperative fundus photograph shows a shallow posterior detachment over a posterior staphyloma in an eye with a BCVA of 0.2. B A preoperative OCT image shows posterior retinoschisis and foveal detachment (scan length, 2.8 mm). C Two weeks after the vitrectomy, the posterior retinoschisis elevation has decreased, but foveal detachment has increased (scan length, 5.0 mm). D One month after the vitrectomy, the patient noticed a reduced BCVA. An OCT image shows the development of macular hole retinal detachment (scan length, 5.0 mm). E About 1.5 years postoperatively, the retina had completely reattached, but the macular hole remained visible (scan length, 5.0 mm).

In 7 of the 16 eyes, OCT revealed the presence of a detached posterior hyaloid surrounding the macula, which may have widely stretched the posterior pole. The appearance of posterior vitreous adhesions over the macula was consistent with our experience of performing vitrectomies for retinal detachment associated with a macular hole[14] and a previous clinicopathological report,[15] which suggested that the posterior hyaloid might remain tightly attached to the macula, despite the presence of PVD in highly myopic eyes. Although PVD was not observed in the other nine eyes preoperatively, OCT examinations may be limited in detecting preretinal structures in highly myopic cases. In the present study, final reattachment was obtained in all 16 eyes, and no recurrences were observed in any eyes for over 6 months (the mean follow-up period was 23.3 months) after final surgery. This result suggests that the release of vitreous traction at the posterior pole may have an important role in the treatment of myopic RS and FS.

We performed vitrectomy, including vitreous cortex removal, in all eyes and internal limiting membrane (ILM) peeling in six eyes. All five eyes with RS and without FD achieved retinal reattachment after the initial vitrectomy. However, 3 of the 11 eyes with RS and FD required reoperation after the initial vitrectomy. In two of these three eyes, a full-thickness macular hole associated with posterior retinal detachment occurred about 1 month after vitrectomy with or without ILM peeling. The incidence of the development of macular hole retinal detachment seems to be higher than in previous reports,[3–7] in which most cases received ILM peeling. Kuhn[6] suggested that ILM may be responsible for macular detachment in highly myopic eyes. We also observed the proliferation of glial cells, which cause an abnormal ILM figure in highly myopic eyes.[14] ILM peeling may be highly beneficial for reducing the traction on the detached retina. However, the side effects of ILM peeling remain unknown. In addition, ILM peeling is technically difficult in highly myopic eyes, and ICG may be toxic to the neural retina as well as to the RPE.[16] The number of surgical reports remains insufficient, and we were able to obtain retinal reattachment in most of the eyes without ILM peeling. Thus, we were unable to conclude whether ILM peeling leads to a better anatomical prognosis. However, current techniques, such as TA-assisted vitrectomy or viscodissection, may be useful to avoid ILM peeling.

The role of gas tamponade in treatment is also uncertain. Gas tamponade can induce pneumatic displacement of outer-layer detachments and improve vision in retinoschisis associated with optic disc pits.[17] We expected intravitreal tamponade to have a similar effect in myopic eyes with RS and FD. However, intravitreal tamponade may push the subretinal fluid inside the limited area of the posterior staphyloma toward the weak point of the fovea, causing the formation of a macular hole. In two eyes with RS but without FD, retinal reattachment was obtained without using gas tamponade. Further study is needed to optimize surgical techniques.

Progression to a macular hole occurred preoperatively in 2 out of the 16 eyes and postoperatively in 3 out of 14 eyes. These eyes had relatively severe FD with posterior staphyloma prior to the development of a macular hole, and the BCVA of these eyes was worse than that of the others. These cases might have been at an advanced stage following the early development of a macular hole, similar to the two cases reported by Ikuno and Tano.[18] Patients and surgeons should regard this probable advanced stage with severe FD and poor vision as being equivalent to the early stage of macular hole retinal detachment when treating this condition.

Visual acuity improved or remained stable in all the eyes in the present study. Considering the poor prognosis of macular hole retinal detachment, vitrectomy using modern surgical techniques to induce PVD or to perform ILM peeling may be effective for the treatment of RS and/or FD in highly myopic eyes. However, further study is needed to select surgical indications and optimize surgical techniques to avoid complications, including macular hole.

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Lattice Corneal Dystrophy Type III in Patients with a Homozygous L527R Mutation in the *TGFBI* Gene

Late-onset lattice corneal dystrophy (LCD) is associated with decreasing vision, minor recurrent epithelial erosions or no erosions at all, and lattice lines much thicker than those usually observed in LCD types I and II. A patient with this type of LCD is classified as LCD type III.[1] Most LDC type III cases have been reported in Japanese patients, and the inheritance pattern is proposed to be autosomal recessive. However, Stock et al.[2] reported that although LCD type IIIA resembles type III clinically, it differs in that type IIIA has an autosomal dominant inheritance pattern. A later study reported that LCD type IIIA is caused by mutations in the transforming growth factor beta-induced (*TGFBI*) gene.

More recently, a heterozygous L527R mutation in the *TGFBI* gene has been reported to be the cause of late-onset LCD in six Japanese patients.[3] Interestingly, only two of these had a family history of LCD. Hirano et al.[4] reported that two Japanese patients with late-onset LCD also had a heterozygous L527R mutation and no family history. They clinically diagnosed LCD type III in these two patients.

We present the characteristics of two patients with late-onset LCD who were homozygous for the L527R mutation.

Case Reports

Patient 1 was a 78-year-old man who presented with decreased vision in both eyes. His corrected visual acuity was 0.3 OD and 0.1 OS. He is the younger brother of patients 2 and 3 in the family with LCD type III reported by Hida et al.[1] (their Fig. 2). His corneal opacities were bilateral and observed as grayish nodular deposits in the midstroma with relatively thick lattice lines that extended from limbus to limbus (Figs. 1A, B). He had no history of corneal erosions. Penetrating keratoplasty was performed on his left eye in March 1996 and on his right eye in December 2001. Histologic findings of the excised corneas showed large deposits of amyloid in the stroma, predominantly midway between the epithelium and the endothelium.

Patient 2 was a 78-year-old man who presented with decreased vision in both eyes. His corrected visual acuity was 0.3 OD and 0.01 OS. The corneal opacities consisted of grayish nodular deposits in the central midstroma and relatively thick lattice lines that extended from limbus to limbus in both eyes (Fig. 1C). The appearance of the corneal opacities was similar to those of patient 1. His sister had undergone keratoplasty at the age of 60 years, but the details of the condition of her cornea could not be obtained. There was no history of corneal erosions in patient 2. Penetrating keratoplasty was performed on the left eye in January 1993. Histologic findings of the excised cornea showed large stromal deposits of amyloid (Fig. 1D).

A genetic investigation was performed according to the guidelines of the Declaration of Helsinki. Written informed consent was obtained. Direct sequencing[5] of the *TGFBI* gene revealed homozygous L527R mutations (Fig. 2).

Comments

Recently, we reported on five Japanese patients with late-onset LCD who also carried a heterozygous L527R mutation.[5] One (case 3 in reference 5) of the five patients had been previously reported as having LCD type III by Hida et al.[1] (case 5 in reference 1), and only one (case 4 in reference 5) with the L527R mutation had a family history of LCD, an affected sibling. Thus, most patients with late-onset LCD reported in Japan have relatively thick lattice lines and/or tiny, discrete nodular deposits, but no family history or affected siblings. They are heterozygous[3–5] or homozygous for the L527R mutation. These results indicate that the heterozygous L527R mutation in the *TGFBI* gene for LCD has low penetrance in the Japanese population, but the homozygous L527R mutation might have increased penetrance. Interestingly, the corneas in those patients with a homozygous L527*R* mutation appeared very similar to the corneas of the heterozygous patients. The reason for this is unknown.

Mutations in the *TGFBI* gene can cause LCD type IIIA as well as LCD type III, both late-onset LCD. Because the corneal appearance in patients with type III is very similar to that in patients with type IIIA, the two types are diagnosed by the inheritance pattern. However, the difference most likely results from the degree of penetrance of the *TGFBI* gene mutation.

Transcorneal Electrical Stimulation Promotes the Survival of Photoreceptors and Preserves Retinal Function in Royal College of Surgeons Rats

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Abstract

Purpose: To determine whether transcorneal electrical stimulation (TES) has neuroprotective effects on photoreceptors and retinal function in Royal College of Surgeons (RCS) rats.

Methods: Three-week-old RCS rats received TES through a contact lens electrode on the left eyes weekly for 2 to 6 weeks. The right eyes received sham stimulation on the same days. Another group of RCS rats were not treated and served as controls. Electroretinograms (ERGs) were recorded from the rats at 3-weeks (before TES), 5-, 7-, and 9--weeks-of-age. After the ERG recordings, the rats were killed for morphological analyses of the retina.

Results: Morphological analyses showed that the mean thickness of the outer nuclear layer (ONL) was significantly thicker in eyes treated with TES than in eyes with sham stimulation (P <0.05). At 5-weeks-of-age, the threshold for eliciting an ERG was 1.23-log units lower in eyes treated with TES than in sham-stimulated rats (P <0.001). At 7-weeks-of-age, the threshold was approximate 0.88 log units lower in the TES than sham-stimulated RCS rats (P = 0.027).

Conclusions: TES prolongs the survival of photoreceptors and delays the decrease of retinal function in RCS rats. These findings indicate that TES is a potential therapeutic treatment for diseases of the RPE and photoreceptors such as retinitis pigmentosa.

Introduction

Electrical activity is important for both the development and survival of neurons. For example, depolarization of neurons exerts some trophic influence on their development,[1,2] and depolarization by high KCl concentrations inhibits death of mature retinal ganglion cells (RGCs) in culture.[3-5] In the auditory system, chronic electrical stimulation promoted the survival of spiral ganglion cells which otherwise would have degenerated from the administration of an ototoxic drug *in vivo*.[6-8] In motor neurons, electrical stimulation activated the cell body, and accelerated axonal regeneration and increased the expression of the mRNAs of BDNF and trkB.[9,10]

In the visual system, we have demonstrated that direct electrical stimulation of the transected optic nerve (ON) stump promotes the survival of axotomized RGCs in adult Wistar rats.[11] In addition, we have demonstrated that transcorneal electrical stimulation (TES) which is less invasive than electrical stimulation of the transected (ON), also promoted the survival of axotomized RGCs *in vivo*.[12] We concluded that electrical stimulation may have a potential therapeutic effect on injured RGCs in patients with optic nerve diseases such as optic neuropathy. In fact, we have applied TES on patients with traumatic optic neuropathy (TON) or with nonarteritic ischemic optic neuropathy (NAION), and have found an improvement of visual function.[13]

These findings led us to hypothesize that TES will also have a neuroprotective effect on photoreceptors in eyes with photoreceptor degeneration such as in patients with retinitis pigmentosa (RP). This is important because RP is one of the leading causes of blindness worldwide and no established treatment is available clinically, although many experimental approaches have been tried to save photoreceptors in various animal models of RP.[14-19]

The purpose of this study was to determine whether TES would have a neuroprotective effect on the photoreceptors and preserve retinal function in RCS rats. RCS rats have been extensively used as an animal with photoreceptor degeneration and serve as an animal model of RP.[20] We shall show from morphological and electrophysiological analyses that TES had a neuroprotective effect on of the photoreceptors and delayed the decrease of visual function in RCS rats.

MATERIALS AND METHODS

Animals

All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the procedures were approved by the Animal Research Committee, Osaka University Medical School. Tan-hooded, pink-eyed RCS rats (*rdy/rdy*) were purchased from Clea Japan Inc, Tokyo, and inbred at the animal facilities of Osaka University. They were raised on a 12-hour dark:12-hour light cycle with an ambient light intensity of 100 lux.

Transcorneal Electrical Stimulation

Rats were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg). Only the left eye was electrically stimulated as described in detail.[12] For the stimulation, the cornea was anesthetized with a drop of 0.4% oxyburocaine HCl, and a contact lens electrode with internal and external concentric electrodes (Kyoto contact lens, Kyoto, Japan) was placed on the cornea with a drop of 2.5 % methylcellulose to maintain good electrical contact and prevent corneal dehydration.

Biphasic, rectangular (1 ms/phase duration) current pulses were delivered at a frequency of 20 Hz from an electrical stimulation system (Stimulator: SEN-7320, Nihonkohden, Japan; Isolator: WPI, Sarasots, FL) through the contact lens electrode. The current intensities used were 0 μA (sham stimulation), 50 μA, and 100 μA, and the duration of stimulation was 1 hour.

TES was applied initially on 3-weeks-old RCS rats (postnatal 20-23 days) and was applied once a week for an hour. The TES-treated RCS rats were divided into three groups. TES was applied between 3- and 5-weeks of age in Group 1 (n = 6), was applied between 3- and 7-weeks of age in Group 2 (n = 6), and was applied between 3- and 9-weeks of age in group 3 (n = 6).

For the TES, the left eyes received TES (50 or 100 μA), and the right eyes received either sham electrical stimulation or no treatment as control.

Electroretinography

Electroretinograms (ERGs) were recorded from RCS rats after the end of the TES-treatment, i.e., at 5-weeks of age in Group 1, at 7-weeks of age in Group 2, and at 9-weeks of age in Group 3. For the ERGs, animals were kept in total darkness for at least 12 hours and were prepared for the recordings under dim red light. They were anesthetized intramuscularly with a loading dose of xylazine (13 mg/kg) and ketamine (86 mg/kg). The pupils were dilated with 0.1% atropine, 0.5% tropicamide, and 0.5% phenylephrine HCl. The animals were held steady with a bite bar and nose clamp in a stereotaxic frame. A heating pad maintained the body temperature at approximately 37°C.

ERGs were recorded from both eyes simultaneously with contact lens electrodes (Kyoto

contact lens, Kyoto, Japan), and the ground electrode was inserted subcutaneously near the tail. Responses were amplified 10,000 X and bandpass filtered from 0.08 Hz to 1000 Hz with a 60 Hz notch filter. The recordings were digitized at 5 kHz. Ten to twenty responses were averaged with interstimulus intervals from 3 seconds to 30 seconds depending on the intensity of stimulus.

ERGs were elicited by white light of 50 ms duration and a maximum luminance of +2.1 log candela (cd) /m^2 . The luminance was attenuated with neural density filters in 0.5 or 1.0 log unit steps. The threshold amplitude was set at 10 μV for the b-wave and 5 μV for the scotopic threshold response.

Histological Analyses

Immediately after the ERG recording, the rats were killed with an overdose of sodium pentobarbital. The eyes were removed and kept overnight at 4 °C in 4% glutaraldehyde in 0.1 M phosphate buffer. Eyes were trimmed and postfixed in 1% osmium for 1 hour. The epoxy embedded tissue was cut into 1 μm sections and stained with toluidine blue for light microscopy. All sections were cut along the vertical meridian of the eye passing through the optic nerve. Three serial sections of each eye were quantified for each experimental animal.

The degree of retinal degeneration was assessed by measuring the thickness of outer nuclear layer (ONL) and inner nuclear layer (INL). In each of the superior and inferior hemispheres, photographs of the retina were taken at nine defined points with a camera attached to a light microscope (E800, Nikon, Tokyo, Japan). The first photograph was made at approximately 500 μm from the center of optic nerve head, and subsequent photograph were taken every 400 μm more peripherally. The thickness of ONL and INL were measured on the photographs with the Scion Image analyzer (Scion Image; Scion Corp.). Three measurements were made at defined points separated from the adjacent photograph by 50 μm. The three measurements were averaged for the value plotted at each point. In this way, the 54 measurements in the two hemispheres were measured which represented the thickness over almost the entire retina. Each eye was coded so that the investigator was masked to treatment of the eye.

Electrophysiological Recordings from Superior Colliculus

Field responses evoked by TES were recorded from the surface of the superior colliculus (SC) of untreated 5-week-old RCS rats (N = 5). To do this, animals were anesthetized initially with urethane (1.75 g/kg, intraperitoneally),[21] and 0.5 g/kg was given intraperitoneally every 3 hours to maintain the anesthesia during the surgical preparations and electrophysiological experiments. A heating pad was used to maintain body temperature at approximately 37° C. The heart rate and electrocardiograms were continuously monitored throughout the experiments. The pupils were dilated with 0.1% atropine, 0.5% tropicamide, and 0.5% phenylephrine HCl, and the cornea was covered with a contact lens.

After tracheal cannulation, the head was fixed in a stereotaxic frame. Cerebrospinal fluid was drained through a small incision made in the dura on the obex. The temporal bone on the right side was removed, and the underlying occipital cortex was

removed by gentle aspiration to expose the dorsal surface of the superior colliculus.

A Ag:AgCl ball electrode (0.2-0.3 mm in diameter) was used to record the field potentials evoked by TES. The surface of the SC was covered with mineral oil to prevent drying and short-circuiting of the evoked potentials. The electrode was placed on the center of the exposed SC to record the field responses. Responses were amplified by 10 000 X and bandpass filtered from 0.08 Hz to 1000 Hz with a 60 Hz notch filter. The responses were digitized at a rate of 5 kHz, and 50 responses were averaged.

To investigate how TES stimulated retinal neurons, we recorded field responses from the surface of the superior colliculus (SC) of 5-week-old RCS rats which had not had TES or sham stimulation. Brief monophasic pulses (1 ms) were used because biphasic pulses of TES (1 ms/phase) evoked a large artifact which made it difficult to analyze the components of the evoked responses.

Statistical Analyses

Data were statistically analyzed with the SPSS 10.0J program (SPSS Inc, Chicago, IL). The data are expressed as the means ± standard deviations (SDs) or the standard error of the means (SEMs). Comparisons between two groups were made by Student's *t* test when data were normally distributed or by the Mann-Whitney Rank Sum test when the data were not normally distributed. Comparisons among many groups were made by one-way ANOVA followed by the Tukey test. The probability level is represented as the value "*P*"; statistical significance was set at $P < 0.05$.

Results

Transcorneal Electrical Stimulation and Survival of Photoreceptors *in vivo*

To evaluate the effect of TES on the survival of photoreceptors, we examined the thickness of the outer nuclear layers (ONLs) at 18 locations over the superior and inferior retina of 7-week-old RCS rats that had received 50 μA or 100 μA of TES, or were sham-stimulated, or were controls and not stimulated. Representative retinal sections from the superior retinas from 7-week-old RCS rats that had 100 μA of TES or had sham stimulation are shown in Figure 1. The number of rows of nuclei in the ONL layer was four or five in the retina receiving TES (Fig. 1A) and two or three in the retina with sham stimulation (Fig. 1B).

The extent of rescued photoreceptors was quantified by measuring the thickness of the ONL across the retina. Quantitative analyses showed that the ONL in the TES-treated eyes was significantly thicker than in the sham-stimulated and in control eyes (Figure 2A). The mean thickness of ONL in control retinas of 7-weeks-old RCS rats was 9.8 ± 2.46 μm (mean ± SD, n = 6) which was not significantly different from that in the sham-stimulated eyes at 10.9 ± 1.63 μm (n = 6). The mean ONL thickness in the retinas treated with TES at a current intensity of 50 μA was 13.7 ± 0.79 μm (n = 6), while that with a current intensity of 100 μA was 23.3 ± 3.69 μm. The ONL with 50 μA was not significantly thicker than that of sham stimulated eyes, but with 100 μA, the ONL was significantly thicker than that of the sham stimulated eyes ($P < 0.001$; n = 6; Fig. 2A).

To determine whether the differences in the thickness of the ONL was localized or widespread

across the retina, the mean thickness of the ONL was determined for 18 points along the superior-inferior plane of the eye in the three groups of RCS rats. The mean ONL thickness at every point in the superior and inferior hemispheres of the retinas treated with TES was significantly thicker than that treated with sham stimulation or in the controls (one-way ANOVA, $P < 0.001$; Fig. 2B). Thus, TES delayed the degeneration of the photoreceptors across the retina.

To determine whether the TES affected other layers of the retina, we measured the thickness of inner nuclear layer (INL). The mean thickness of the INL was: control = 29.0 ± 2.0 μm; sham = 28.1 ± 2.7 μm; 50 μA TES = 26.9 ± 0.8 μm; and 100 μA TES = 30.1 ± 1.9 μm, n = 6 each; Fig. 2C). None of these differences was significant.

Time Course of Survival of Photoreceptors Treated with TES

Because 100 μA of TES prolonged the survival of photoreceptors, we used this current intensity to follow the time course of the survival of photoreceptors. The ONL of the retina of RCS rats at 3-weeks-of-age is composed of 10 to 12 rows of nuclei (Fig. 3A). In the sham-stimulated rats, the ONL of 5-weeks-old RCS rat was made up of three rows, and at 9-weeks-old, only an occasional nucleus was seen in the ONL and the photoreceptors were scattered and disorganized (Fig. 3C, E). On the other hand, the ONL in 5-week-old RCS rats treated with TES had 5 to 6 rows, and at 9-weeks-old RCS rats, there were one-two rows and they were organized in a line (Fig. 3B, D).

Measurements of the ONL thickness showed that there was a significant difference in the mean ONL thickness between TES-treated and sham stimulated eyes in 5-week-old RCS rats (TES = 26.5 ± 2.9 μm; Sham = 17.1 ± 2.7 μm; mean ± SD, n = 6 each, *t* test; $P < 0.001$) , and at 9-weeks (TES = 8.34 ± 1.3 μm; Sham = 4.12 ± 1.2 μm; n = 6 each, *t* test; $P < 0.001$; Fig. 4A). The mean ONL thickness at every point in the superior and inferior hemispheres in the retinas treated with TES was thicker than that in the retinas treated with sham stimulation (Figure 4B).

There was no difference in the mean INL thickness between retinas treated with TES and sham stimulation at 5-weeks- and 9-weeks-of-age (data not shown).

The eye and fundus were examined at the end of the experiments, and neither retinal detachment nor vitreous hemorrhage was observed. In addition, cataracts or corneal opacities did not develop in all rats.

Retinal Function of RCS rats Treated with TES

Representative ERGs recorded from RCS rats at 3-, 5-, and 7-weeks-of age are shown in Figure 5. In the eye of the RCS rat at 3-weeks-of-age, the b-wave reached the criterion amplitude at an intensity of 1.9 log cd/m^2, and the a-wave was first detected at 1.1 log cd/m^2 (Fig. 5A). In 5-week-old RCS rats with sham stimulation, a negative response dominated the ERG over the whole intensity range, and a b-wave did not appear until nearly the maximum stimulus intensity (Fig. 5C). On the other hand, in the 5-week-old RCS rat treated with 100 μA TES, a b-wave appeared at –0.9 log cd/m^2 and the amplitude increased with increasing stimulus intensities. However, the amplitude of the a-wave was reduced (Fig. 5B).

Although 50 μA TES also preserved the retinal function, the effect of 100 μA was more consistent with less variation than that of TES at 50 μA (Data not shown). Therefore, we used TES at 100 μA in the following experiments.

The b-waves were used to assess retinal function of each RCS animal at 5-weeks-of-age because of the disappearance of a-wave in the sham stimulated animals. There was a 1.23-log-unit difference in the threshold of the b-wave between TES-treated eyes and sham stimulated eyes (TES: -0.07 ± 0.50 log cd/m^2; Sham: 1.16 ± 0.58 log cd/m^2; mean ± SEM, n = 6, *t* test; $P < 0.001$).

The intensity–response function curve for the b-wave in TES-treated eyes was shifted to the right by approximately 0.8 log units compared with the eyes at 3-weeks-of-age, whereas the curve from the eyes treated with sham stimulation was shifted to the right by 2.1 log units (Fig. 5A).

We also compared the mean amplitude of the b-waves elicited by the maximum intensity (2.1 log cd/m^2) from the TES-treated eyes with that from the sham-stimulated eyes. There was a significant difference between them (TES: 47.1 ± 9.90 μV; Sham: 19.2 ± 6.71 μV, mean ± SEM ; n = 6 each, Mann-Whitney Rank Sum test; $P = 0.038$).

At 7-weeks-of-age, the retinal function of RCS rat was more depressed, the b-wave was not present and a negative response called the "STR-like negative response" dominated the ERG over the whole intensity range. Therefore, the STR-like negative response was used to determine the neuroprotective effect of TES on the eye of 7-week-old RCS rats. In the eyes treated with sham stimulation, the STR-like negative response reached the criterion amplitude at 1.1 log cd/m^2 (Figure 5E). In the eye treated with TES, on the other hand, the STR-like negative response appeared at –0.4 log cd/m^2, and the amplitude increased with increasing stimulus intensities (Fig. 5D). There was a 0.88-log-unit difference in the threshold for the STR-like negative response between the TES-treated eyes and sham-stimulated eyes (TES: -0.45 ± 0.46 log cd/m^2; Sham: 0.43 ± 0.50 log cd/m^2; mean ± SEM; n = 6 each; Mann-Whitney Rank Sum test; $P = 0.027$).

In the eyes with sham stimulation, the intensity–response curve for the negative wave was shifted to the right by approximately 1.5 log units compared with the eyes with TES (Fig. 6B). The mean amplitude of the negative response at the maximum intensity in the TES-treated eyes was significantly larger than that in the sham-stimulated eyes (TES: 25.1 ± 2.79 μV; Sham: 11.9 ± 3.00 μV, mean ± SEM; t-test; $P < 0.001$).

We also recorded ERGs from 9-week-old RCS rats with TES or sham stimulation, but the responses from these animals were too weak to be assessed (Data not shown).

EER by TES from Surface of Superior Colliculus

Because the amplitudes of the responses elicited by TES depended on the recording site on the SC and the intensity of the current, we first identified the center of responsive area where the largest responses were recorded for light stimuli (Fig. 7A). Next the effect of stimulus current intensity of TES on the amplitudes of the potentials at this area of SC was determined. A typical series of responses recorded on the surface of SC for various intensities of TES in RCS rats is shown in Figure 7B. With suprathreshold levels of TES, the potentials were

composed of four potential changes: a sharp positive deflection (P1), a small negative wave (N1), a large positive wave (P2), and a small long-duration negative wave (N2). The peak latencies of the P1, N1, P2, and N2 components were; 3.1 ± 0.1 ms (mean ± SD; n = 19), 4.2 ± 0.7 ms (n = 13), 7.1 ± 0.7 ms (n = 13), and 14.0 ±1.4 ms (n = 10), respectively. When the electric current was increased from 50 μA to 300 μA, the P1 wave first appeared and the other waves appeared in sequence. The P1 and P2 waves became very clear with the increase of current intensity.

The amplitudes of P2, N1, and N2 were reduced by tetanic stimulation (approximately 50 Hz) leaving only P1 indicating that P1 was presynaptic and other waves were postsynaptic (data not shown). Therefore, we examined the P1 for the threshold of responses. With an increase of current intensity, a small but detectable P1 wave appeared at 75 μA (Fig. 7B). The mean amplitude of P1 for the electric current intensities is plotted in Figure 7C. The mean amplitude of P1 at 100 μA was greater than the criterion amplitude (7.7 ± 0.9 μV; mean ± SEM; n = 5). These results confirmed that TES at 100 μA stimulated retinal neurons to evoke responses on the surface of SC.

Discussion

Our morphologic and electrophysiological analyses showed that TES prolonged the survival of photoreceptors and retinal function against the inherited photoreceptor degeneration of RCS rats. As best we know, this is the first report to show that electrical stimulation alone has a neuroprotective effect on the photoreceptors.

Neuroprotective Effect of TES on Photoreceptors in Retinas with Inherited Retinal Degeneration

It has been reported that intravitreal injection of neurotrophic factors,[14,15,22-24] neuroprotective genes,[16,17,24,25] or transplantation of cells such as RPE cells,[18,26,27] exert strong neuroprotective effects on photoreceptors in animal models of RP. However, the survival of the photoreceptors was limited to the area of the injected site. Limited and localized protection of photoreceptors in the retina of RCS rat has also been demonstrated with mechanical injury alone[22] and by laser burns.[28]

For TES, the neuroprotective effect on the photoreceptors extended over the entire retina (Figs. 2, 4). This suggests that with our stimulating protocol, the electrical current may spread over the entire retina to exert neuroprotection on the entire retina, although we could not record EEPs at all sites of the SC. This neuroprotective effect was similar to the neuroprotective effect induced by light stress, which also has a neuroprotective effect on the photoreceptors over the whole retina.[19,29]

We also measured the thickness of the INL to determine whether TES also had a neuroprotective effect on the inner retinal cells. Unlike the significant effects on the photoreceptors, the inner retina appeared less affected by TES, although we have demonstrated that TES enhanced the survival of axotomized RGCs *in vivo*.[12]

Preservation of Retinal Function by TES

We used the b-wave and the STR-like negative response to evaluate retinal function, which are not direct indicators of the function of photoreceptors. In RCS rats, the a-wave is not always suitable for evaluating the efficacy of the treatment because of

the advanced photoreceptor degeneration.[24] Other researchers have demonstrated that the b-wave amplitude is related to the number of remaining photoreceptors.[32,33] The STR-like negative response was also used to evaluate retinal function in the RCS rat retina in which photoreceptor degeneration was advanced.[34]

The amplitudes of the b-waves (5-week-old) or STR-like negative responses (7-week-old) were significantly larger in TES-treated eyes than in sham-stimulated eyes of RCS rats (Fig. 5). These functional results were consistent with the histological results obtained by measuring the thickness of the ONL (Fig. 4).

However at the age of 9-weeks, there was no significant difference in the amplitudes of the b-waves of TES-treated and sham-stimulated animals although the mean thickness of ONL was still significantly thicker in the TES-treated eyes. These results show the limitation of TES for the treatment the retina with a rapid course of photoreceptor degeneration. In humans with RP, the degeneration is relatively slow, so other RP models such as RPE65 deficient mouse[35] may be more suited to investigate the effects of TES for slowly progressive photoreceptor degeneration.

SC Responses Evoked by TES

We measured the field response on the surface of SC evoked by TES and demonstrated that 100 μA TES could evoke response even in eyes with photoreceptor degeneration (5-weeks). These results indicate that TES probably stimulated the remaining inner retinal cells directly or indirectly, and if TES is to be used on RP patients, the strength of electric power of the TES must be strong enough to evoke light sensation called "phosphenes" to have a neuroprotective effect on the photoreceptors.

Possible Mechanism of TES-induced Neuroprotection of Photoreceptors

We have demonstrated earlier that TES induces a significant up-regulation of endogenous IGF-1 which is produced by Müller cells.[22] However, IGF-1 was reported to have low rescue effect on the survival of photoreceptors in light-damaged retinas.[15] IGF-1 may not be the key molecule that exerts the neuroprotective on photoreceptors.

The neuroprotective effect of TES on the photoreceptors was dependent on the intensity of the electric current (Fig. 2A). This suggests that the increase of electrical activity of the photoreceptors exerts neuroprotective effect on photoreceptors. However in this study, we could not determine whether TES activates photoreceptors or inner retinal neurons or Müller cells to induce the rescue effect.

A subretinal implant of an artificial retina has been shown to stimulate the retina and has a neuroprotective effect on the photoreceptors of the RCS rats[36] and the retinal function in patients.[37] Taken together, electrical activity may have a neuroprotective effect on photoreceptors. Additional experiments are needed to determine the mechanism of TES-induced neuroprotection for photoreceptors.

TES as New Clinical Technique

Our findings indicate that appropriate electrical stimulation is beneficial for the survival of photoreceptors in eyes with inherited photoreceptor degeneration. We applied TES once a week for 6

weeks, and the ocular side effects, such as cataracts, were not observed. These findings allow us to propose electrical stimulation as a new therapy for the diseases of photoreceptors such as in patients with RP for which there are only a few ways to prevent or halt the development of blindness. In RCS rats, a mutation of the gene coding for the receptor tyrosine kinase gene Mertk has been identified.[38] The same gene mutation has been identified in patients with autosomal recessive retinitis pigmentosa,[39] indicating that the RCS rat is a counterpart of one type of human RP. Therefore, the protective effect against photoreceptor degeneration in RCS rats suggests that TES could be considered as a treatment for patients with some forms of retinitis pigmentosa. Additional studies are needed to determine the optimal electrical stimulus parameters to obtain photoreceptor survival. We must also apply TES to other animals with different genetic mutations to evaluate the neuroprotective effects before consideration of TES for clinical use.

In summary, TES prolonged the survival of photoreceptors and preserved retinal function in RCS rats. These findings open the possibility that TES can be used to delay the photoreceptor degeneration in patients with inherited retinal degeneration such as RP.

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Figure legends

Figure 1. Photomicrographs of TES-treated and sham-stimulated retinas from 7-week-old RCS rats. A = 100 μA TES; B = sham stimulation. TES led to better structural preservation of the photoreceptors than sham-stimulated. Scale bar = 50 μm.

Figure 2. Effect of TES on the thickness of the ONL and INL of RCS rats. Data are the means ± SDs.

A. Mean thickness of the ONL in the retinas treated with TES (100 μA) is significantly thicker than that of control and sham-stimulated retinas (one-way ANOVA $P < 0.001$, followed by Tuky test, $^*P < 0.001$ vs Sham).

B. Mean thickness of the ONL along the vertical meridian of the retina of RCS rats. The data are the means ± SEMs. In all cases, the ONL in the eyes receiving 100 μA (□) TES was significantly thicker than that with sham stimulation (□) or controls (○) in both superior and inferior retina (one-way ANOVA, $P < 0.001$). ○; TES at the current intensity of 50 μA.

C. Mean thickness of the INL of the retinas of RCS rats at 7-weeks-of-age. There was no significant difference among them.

Figure 3. Photomicrographs of the retina from control, TES-treated and sham-stimulated RCS rats.

A. Retina from control RCS rats at 3-weeks-of-age.
B. Retina from TES eye at 5-weeks-of-age.
C. Retina from sham-stimulated eye at 5-weeks-of-age.
D. Retina from TES eye at 9-week-of-age.
E. Retina from sham-stimulated eye at 9-weeks-of-age.

Although the ONL thickness in the sham-stimulated retina decreases with age, the ONL was thicker in the retinas receiving TES.

Scale bar = 50 μm.

Figure 4. Time course of photoreceptor survival.

A. Mean thickness of ONL in the retina of 3-, 5- and 9-weeks-old RCS rats treated with TES or sham stimulation. The data are the means ± SDs. The differences in the mean ONL thickness between TES-treated retinas (black column) and sham stimulated retinas (grey column) at 5-week- and 9-weeks-of-age is not significant (*t* test $^*P < 0.001$).

B. Mean thickness of ONL along the vertical meridian of the eye in RCS rats. The data are the mean ONL thickness ±SEM. In all case treatment with TES at a current intensity of 100 μA led to ONL thickness that was significantly thicker than that with sham stimulation in both superior and inferior retina at each time point (one-way ANOVA, $P < 0.001$).

○; 3-week old RCS rat, □; 5-week old RCS rat treated with TES, □; 5-week old RCS rat treated with sham stimulation, △; 9-week old RCS rat treated with TES, △; 9-week old RCS rat treated with sham stimulation.

C. Measurement of mean INL thickness in the retinas at 5-week old and 9-week old RCS rats treated with TES (black column) or sham stimulation (grey column). There was no significant difference among them.

Figure 5. Effect of TES on the ERGs. Typical ERG responses elicited by different stimulus intensities from TES-treated eye and sham-stimulated eye of RCS rats at 5- and

7-weeks-of-age. Both eyes were recorded simultaneously.

A. ERGs from 3-weeks-old RCS control rat.

B. ERGs from 5-weeks-old RCS rat following sham stimulation.

C. ERG from 5-weeks-old RCS rat following TES

D. ERGs from 7-weeks-old RCS rat following sham stimulation.

E. ERGs from 7-weeks-old RCS rat following TES.

Figure 6. Intensity-response curves of the ERGs.

A. Average b-wave amplitudes versus stimulus intensity from TES-treated (○) and sham-stimulated retinas of 5-weeks-old (○) and 3-weeks-old (△) RCS rats on a log-log scale.

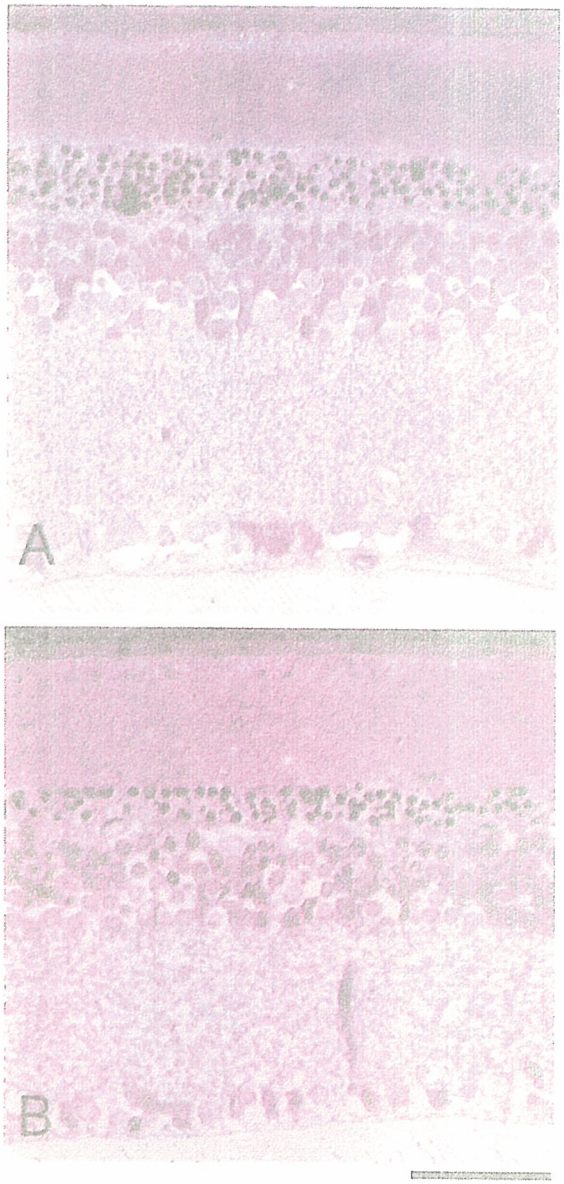
B. Average STR-like negative wave amplitudes versus stimulus intensity from TES-treated (○) and sham stimulated retinas (○) at 7-week old RCS rats on a log-log scale (B). Error bars, SEM.

Figure 7. Evoked potentials (EPs) recorded from the superior colliculus.

A. EPs elicited by light stimuli of 5-week-old RCS rats. Calibration, 50 μV, 50 ms.

B. EPs elicited by TES of 5-week-old RCS rats. Calibration, 50 μV, 10 ms.

C. Averaged amplitudes of SC responses versus current intensities of TES. Data are the means ± SDs.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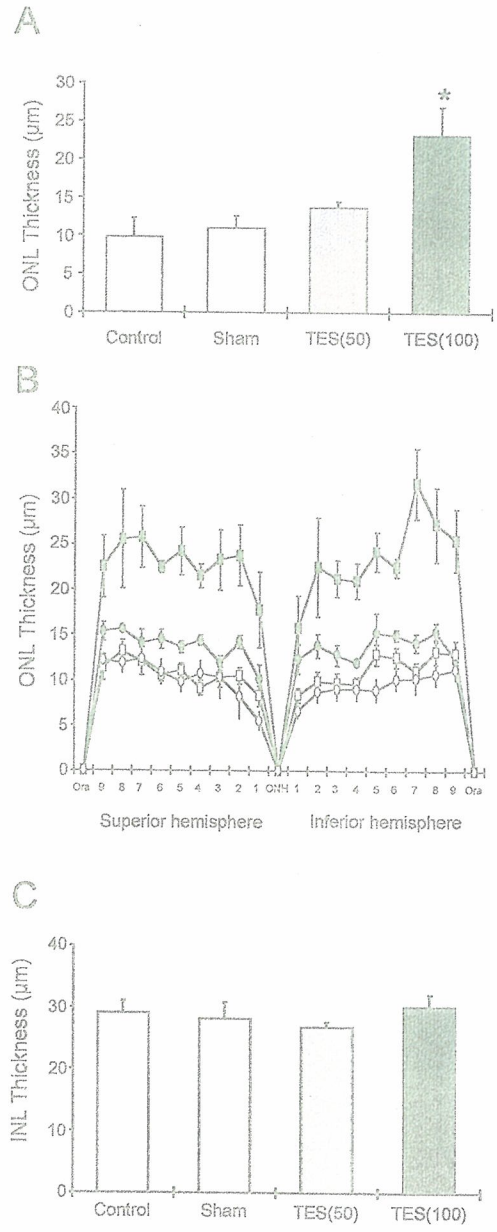
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