Topical Doxycycline Can Induce Expression of BDNF in Transduced Retinal Pigment Epithelial Cells Transplanted into the Subretinal Space

Toshiaki Abe,1 Ryosuke Wakusawa,2 Haruka Seto,1 Nobubaru Asai,1 Takae Saito,1,2 and Kohji Nishida2

PURPOSE. To determine whether topical doxycycline (DOX) induces the expression of brain-derived neurotrophic factor (BDNF) by BDNF-transduced retinal pigment epithelial (RPE) cells transplanted into the subretinal space of rats.

METHODS. A rat RPE cell line that can express BDNF by exposure to DOX was created (Tet-BDNF-RPE). The expression of BDNF was examined by ELISA, Western blot analysis, and real-time PCR. The expression of BDNF was controlled by exposure to DOX in vitro. Tet-BDNF-RPE cells were transplanted into the subretinal space of rats, and the rats were exposed to constant light 1 day or 1 month after the transplantation. The rats were followed with or without topical DOX and examined electrophysiologically and histologically.

RESULTS. The expression of BDNF was upregulated by exposure of Tet-BDNF-RPE cells to DOX in vitro. The optimal concentration for inducing BDNF expression was 0.5 to 1.0 μg/ml DOX. BDNF expression was also increased in vivo by topical DOX after subretinal transplantation of Tet-BDNF-RPE cells. Statistically significant protection of the electroretinogram amplitudes were found 3 days or 1 month after transplantation, and the outer nuclear layer was better preserved 7 days or 1 month after transplantation in the rats treated by 5 or 10 mg/ml/d topical DOX than rats treated by other conditions or sham-operation rats.

CONCLUSIONS. The expression of BDNF can be significantly increased by topical DOX after Tet-BDNF-RPE subretinal transplantation. Better photoreceptor protection against phototoxicity was achieved by DOX eye drops after the cell transplantation. (Invest Ophthalmol Vis Sci. 2008;49: 3631–3639) DOI:10.1167/iovs.07-0947

There are many retinal disorders, including retinitis pigmentosa, that have no effective treatments. Some of these diseases are inherited, and viral vectors have been used to transfer the normal gene to replace the mutated gene. Good results have been obtained in dogs1 and mice.2–4 However, a different viral vector must be constructed for each mutation, assuming that the mutation causing the defect is known.

Increasing evidence has shown that neurotrophic factors contribute not only to the survival but also to the protection of retinal neurons. These neurotrophic factors include basic fibroblast growth factor (bFGF),5 ciliary neurotrophic factor (CNTF),6–8 glial cell line–derived neurotrophic factor (GDNF),9 pigment epithelium-derived factor (PEDF),10,11 and brain-derived neurotrophic factor (BDNF).12 These factors have been administered by direct injection into the vitreous,13,14 viral vectors,6,9 and transgenic mice15; in explant retinal culture models16; and in transplantation of cells expressing a neurotrophic factor into the subretinal space.17

Some of the neurotrophic factors have been shown to block retinal degeneration, regardless of the cause. Thus, treatments with these neurotrophic factors may be an alternative strategy for treating retinal degeneration.

From the results of these experiments on animals, neurotrophic factors have been considered for clinical application, and clinical trials—for example, for CNTF (Clinical Trials of NT-501 for the Treatment of Retinitis Pigmentosa)—have begun.18 However, the pharmacodynamics of the factors in the eye, such as rapid elimination,19,20 would necessitate repeated injections of the factors. Such a delivery method would increase the possibility of retinal detachment, hemorrhage, endophthalmitis, cataract, and other complications.19

These difficulties may be overcome by injecting cells carrying vectors transduced with the gene of a neurotrophic factor. For example, we have constructed a vector that includes a tetracycline-responsive element (TRE) and the cDNA of BDNF. The TRE acts as a BDNF expression promoter (Tet-On expression vector), and the BDNF expression can be controlled by doxycycline (DOX), a tetracycline derivative.21–23 These vectors have been transduced into rat retinal pigment epithelial (RPE) cells (Tet-BDNF-RPE).

The purpose of this study was to determine whether topical DOX would induce the expression of BDNF from Tet-BDNF-RPE cells transplanted into the subretinal space of rats. To test the effectiveness of this strategy, we examined whether the expressed BDNF will protect photoreceptors of rats from phototoxicity.

MATERIALS AND METHODS

Animals, Biosafety, and Transplantation

All animals were used after receiving institutional approval and were handled in a humane manner. The procedures used complied strictly with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Before transplantation, the rats were anesthetized with ketamine hydrochloride (90 mg/kg body weight) and xylazine hydrochloride (10 mg/kg). The indicated cells or vehicle were injected into the superior...
subretinal space of the right eye of Sprague-Dawley (SD) male rats with a 30-gauge needle and syringe (Hamilton, Reno, NV). Two microliters containing $4 \times 10^3$ cells were injected. For control, the same surgical procedures were performed, but the cells were not injected.

A total of 178 rats were used; 120 were used for electroretinography and histologic examinations. For transplantation control, the same volume of Hank’s balanced salt solution (HBSS) was injected. All the rats were observed without immunosuppression.

After 24 hours of rearing in standard cyclic room light (light-dark, 14:10 hours), the rats were placed under continuous 2500-lux light for either 2 (group A) or 7 (group B) days. Some of the rats in these groups were also observed without exposure to light (group A’, B’, and B”, respectively). After exposure to light, the rats were killed by carbon dioxide, and the eyes were prepared for histologic analyses.

Some of the rats were also observed without light exposure (group D) for either 2 (group A) or 7 (group B) days. Some of the rats in these groups were also observed without exposure to light (group A”, B”, and B”, respectively). Other rats were kept in standard cyclic room light for 1 month and were placed under continuous 2500-lux light for either 2 (group C) or 7 (group D) days. Some of the rats were also observed without light exposure (group D”). The details of each group of rats is shown in Table 1. A total of 38 rats were in group A: 5 received Tet-BDNF1-RPE and were treated with 0 mg/mL DOX eye drops, 5 received 2 mg/mL DOX, 5 received 5 mg/mL DOX, 6 received 10 mg/mL DOX. 4 had only the RPE transplantation, 6 had only an HBSS injection, 4 were sham-operation control subjects, and 3 were untreated control. After exposure to light, the rats were killed by carbon dioxide, and the eyes were prepared for histologic analyses.

Eighty-eight rats were used for real-time PCR or ELISA: There were 15 rats in group A” for PCR 3 days after transplantation, 32 rats in group B” for PCR and B” for ELISA 8 days after transplantation, and 11 rats in group D” for PCR 38 days after transplantation.

### Preparation of Cultured Rat RPE Cells

RPE cells from Long-Evans (LE) rats were harvested and grown in DMEM/F-12 medium with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis MO). The medium was changed every 3 days. The cells were grown in Eagle’s MEM (EMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic solution at 37°C in a 5% CO₂/95% air humidified atmosphere.

Preparation of Cultured Rat RPE Cells

RPE cells from Long-Evans (LE) rats were harvested and grown in DMEM/F-12 medium with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis MO). The medium was changed every 3 days. The cells between passages 1 and 4 were first examined with anti-pancytokeratin (monoclonal antibody mixture; Sigma-Aldrich) to confirm that they were epithelial in origin.

Subtotal 58

Total 178

### Tet-On RPE Cell Line

The vector containing the reverse tetracycline transactivator (rtTA) gene under the control of the TetO promoter (BD Biosciences, San Diego, CA) was transduced into rat RPE cells by cationic liposomes according to the manufacturer’s instructions (Lipofectamine 2000; Invitrogen, Carlsbad, CA). Only the RPE cells that were resistant to geneticin, which is generated by G418 in the vector, were selected. One of the resistant cell lines (rtTA-RPE) was used for further examination.

Human BDNF in a vector (pBluescriptKs–)hBDNF; Stratagene, La Jolla, CA) was digested at the BamHI and Clal sites, and the hBDNF cDNA fragment was attached to the BamHI and Clal sites of the pTRE2hyg vector containing the rtTA response element (TRE), PmaxCMV, which promotes the expression of the downstream genes and the hygromycin resistance gene (pTRE2hygBDNF; BD Biosciences). Then, the pTRE2hygBDNF construct was further transduced into the rtTA-RPE cells by the same procedure.

The transduced cells were cultured in 600 μg/mL of G418 (Wako, Osaka, Japan) and 200 μg/mL of hygromycin B (Wako). Only cells that were resistant to G418 and hygromycin were selected for further experimentation (Tet-BDNF-RPE). More than 20 colonies were isolated and examined for BDNF expression by ELISA and Western blots in the presence of DOX (Sigma-Aldrich). One of the cell lines (Tet-BDNF-RPE) was used for further experiments. The transduced Tet-BDNF1-RPE cells were grown in Eagle’s MEM (EMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic solution at 37°C in a 5% CO₂/95% air humidified atmosphere.

### Cell Viability

Cell-viability assays were performed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), according to the manufacturer’s instructions (Promega Madison, WI). Tet-BDNF-RPE cells were cultured without DOX for 24 hours. Then, the medium was changed to the indicated medium for 48 hours. The absorbance at 490 nm was measured with a spectrophotometer (Spectra Max Gemini UVmax; Molecular Devices, Sunnyvale, CA).

### Preparation of Samples from Cultured Cells or Supernatant

The selected RPE cells were cultured with or without DOX in the indicated medium. Initially, the Tet-BDNF-RPE cells were cultured without DOX for 24 hours. The medium was changed to a medium with different concentrations (0.01–10 μg/mL) of DOX for 48 hours.

#### Table 1. Distribution of the Rats Used in the Study

<table>
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<tr>
<th>Group</th>
<th>Done</th>
<th>DOX</th>
<th>Sham Control</th>
<th>Untreated Control</th>
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<td>(0 mg/mL)</td>
<td>(2 mg/mL)</td>
<td>(5 mg/mL)</td>
<td>(10 mg/mL)</td>
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<td>ERG</td>
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n = 178. A total of 120 rats were used for either ERG or histology. Rats in group A were used for ERG studies 2 days after continuous light exposure (3 days after transplantation); group B for histology 7 days after continuous light exposure (8 days after transplantation); group C for ERG 2 days after continuous light exposure (3 days after transplantation); and group D for histology 7 days after continuous light exposure (38 days after transplantation). A total of 58 rats were used for PCR or ELISA. Rats in group A’ were used for PCR 3 days after transplantation; group B’ and B” for PCR and ELISA 8 days after transplantation, respectively; and group D’ for PCR 38 days after transplantation.
The cycle was repeated, to confirm that the expression of BDNF is DOX dependent.

**Enzyme-Linked Immunosorbent Assay (ELISA) for BDNF**

The level of BDNF peptides was quantified by ELISA, according to the manufacturer's instructions (Promega Co.). The cells were collected by trypsin digestion just after they had reached confluence. The concentration of each type of protein was expressed as the amount of protein in picograms/total protein in micrograms after homogenization of each type of cell. The color of the reaction products was measured with a microplate reader (Maxline; Molecular Devices).

The total protein concentration was determined by a protein assay kit (Pierce, Rockford, IL). The measurements were made in duplicate, and the mean was used.

**Western Blot Analysis for BDNF**

Tet-BDNF-RPE cells were collected by trypsinization and used for Western blot analysis. For this, the retina of each rat was isolated under a microscope, washed in ice-cold Dulbecco’s phosphate-buffered saline (DPBS) three times, and then immediately sonicated in lysis buffer (157 mM NaCl, 20 mM Tris-HCl, [pH 7.6], 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL aprotinin, and 1 μg/mL leupeptin). The supernatants were collected after centrifugation.

After the supernatants were blotted on the PVDF membranes (Immunoblot; BioRad Laboratories, Hercules, CA), the membranes were incubated overnight in goat anti-BDNF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C, and the bands were made visible with a peroxidase conjugated goat anti-goat IgG (Santa Cruz Biotechnology) and chemiluminescence detection system (ECL; Amersham Bioscience, Baie d’Urfe, Quebec, Canada). The Western blots were quantified with the ImageQuant software. The signals were expressed as the ratio between the bands and the GAPDH.

**Enzyme-Linked Immunosorbent Assay (ELISA) for BDNF**

DOX eye drops were also applied to the sham-operation rats in groups B and D. The eye drops were started on the selected day before the beginning of the constant light exposure for 3 days in each group. The rats were monitored closely and scored daily for 7 days until the rats were killed and the tissues prepared for histologic examination. The changes in the amplitude of electroretinograms and the thickness of ONL of the retina were analyzed. The statistical significance of any differences was determined by the Fisher protected least significant difference (PLSD); StatView, ver. 4.5, Abacus Concepts, Berkeley, CA). *P* < 0.05 was considered to be statistically significant.

**RESULTS**

**Western Blot and ELISA for BDNF with or without DOX**

Cultured rat RPE cells were confirmed to be epithelial in origin by anti-cytokeratin staining. Initially, we selected one of the clones that were resistant to G418. Then we selected more than 20 colonies that were resistant to both G418 for the rtTA vector and the hygromycin for the pTRE2hygBDNF vector. The Tet-BDNF-RPE cells selected by the antibiotics were sonicated in sample lysis buffer just after they had reached confluence. The PCR products were quantified (LightCycler FastStart DNA MasterPlus SYBR Green I; Roche, Basel, Switzerland), and the signals were detected with a specific thermal cycler (LightCycler3Tm; Roche).

The optimal conditions for BDNF and GAPDH were 95°C for 10 minutes, 1 cycle; of 95°C for 10 seconds, 57°C for 30 seconds, and 72°C for 10 seconds, 45 cycles (for BDNF); 95°C for 10 minutes, 1 cycle; and 95°C for 10 seconds, 62°C for 10 seconds, and 72°C for 10 seconds, 45 cycles (for GAPDH). The primers for the BDNF gene amplified 307 bp and GAPDH 307 bp.

The sequences were 5’-ACTCTGGAGAGGTGAAATG-3′ and 5’-TACTTGTCAACAGCCTAGC-3′ for the BDNF gene; and 5’-CATCAC-CACTTTCAGGAGC-3′ and 5’-CATGATCTTCCTCCAGATCC-3′ for the GAPDH.

**DOX Eye Drops**

The rats were treated with topical DOX once per day on the selected days by the same veterinarian (HS). The eye drops were started on the day before the beginning of the constant light exposure for 3 days in groups A and C. In groups B and D, the eye drops were continued for 7 days until the rats were killed and the tissues prepared for histologic examination. The concentration of the DOX eye drops was varied from 0 to 10 mg/mL. The eye drops were also applied to the sham-operation eyes. One eye drop (50 μL) was given to each eye.

**Electroretinography**

Electroretinograms (ERGs) were recorded simultaneously from both eyes 2 days after the continuous light exposure in groups A and C. A Ganzfeld stimulator was used to stimulate the eyes, and a data acquisition system (Universal Testing and Analysis System-Electrophysiology 3000; UTAS-E 3000: LKC Technologies, Inc., Gaithersburg, MD) was used to record the ERGs. The rats were anesthetized, and the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine. The rats were dark adapted for 40 minutes at 37°C, and the contact lens electrodes carrying light-emitting diodes (LEDs) were placed on the corneas. The reference electrode was inserted subcutaneously on the nose, and the animal was grounded by another needle electrode. The stimulus intensities were 0.01, 2.4, and 44.0 cd-s/m², and for comparison, ERGs elicited by 44.0 cd-s/m² were used.

The a-wave amplitude was measured from the baseline to the trough of the corneal negative wave, and b-wave amplitudes from the corneal negative trough to the major corneal positive peak.

**Histologic Procedures and Quantification of Photoreceptor Rescue**

Rats in groups B and D were used for histologic examination. The eyes were enucleated, fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 3 μm along the vertical plane through the optic nerve head and the transplant and nontransplant sites. The sections were stained with hematoxylin-eosin (H&E). The degree of photoreceptor protection was estimated by measuring the thickness of the outer nuclear layer (ONL). The thickness of the ONL was measured in three serial sections at 600, 700, 800, 1200, 1300, 1400, 2100, 2200, 2300, 2700, 2800, 2900, 3500, 3600, 3700, 4100, 4200, and 4300 μm from the optic nerve head to the ora serrata through the region of the transplantation and a nontransplantation site in the same sections (total of 36 sites). The same methods were used at 1 week and 1 month after transplantation.

**Statistical Analyses**

The changes in the amplitude of electroretinograms and the thickness of ONL of the retina were analyzed. The statistical significance of any differences was determined by the Fisher protected least significant difference (PLSD); StatView, ver. 4.5, Abacus Concepts, Berkeley, CA). *P* < 0.05 was considered to be statistically significant.
When we examined cell viability, the DOX concentrations used were not toxic to the selected cells, although 10 μg/mL of DOX resulted in lower cell viability than the other concentrations (Fig. 2). The results of Tet-RPE and the Tet-BDNF1-RPE at 10 μg/mL showed significantly lower activity than at other concentrations. Higher concentrations of DOX may be toxic to the cells. Some of the transduced cells, such as the Tet-BDNF10-RPE cells, showed low cell viability, whether the culture medium included DOX or not. On the other hand, Tet-BDNF1-RPE cells were not significantly different from nontransfected control RPE cells or rtTA vector-transduced RPE cells. However, 10 μg/mL DOX also showed lower cell activity than the other concentrations (Fig. 2).

From these results, one of the colonies (Tet-BDNF1-RPE) that was induced to express BDNF by DOX in the supernatant and had almost the same cell viability as those of nontransduced RPE cells or rtTA-RPE cells was selected for further experiments.

Next, we determined the optimal concentration of DOX in the supernatant for the induction of BDNF expression by the Tet-BDNF1-RPE cells. When we altered the DOX concentration between 0.05 and 10 μg/mL in the culture medium, the maximum BDNF expression was observed with 0.5 to 1 μg/mL DOX.
The amount of BDNF was estimated to be 35 to 90 pg/mg protein in the supernatant.

We continued the Tet-BDNF1-RPE culture with or without DOX at a concentration of 0.5 μg/mL. When the cells were cultured without DOX for 1 day after 48 hours of DOX, we found 1.5- to 3-fold more BDNF expression in the supernatant than in Tet-BDNF1-RPE cells without DOX by ELISA (baseline; Fig. 4). When we removed the DOX from the medium, the expression of BDNF by the Tet-BDNF1-RPE cells returned to the baseline level. When we reapplied the DOX, we found an induction of BDNF expression that then returned to the baseline level when we removed the DOX. The magnitude of BDNF expression after the second or third DOX addition was higher than that of the initial DOX addition.

**Transplantation of the Tet-BDNF-RPE Cells**

We transplanted the Tet-BDNF1-RPE cells into the rat subretinal region as we reported.13 One day after the transplantation, the rats were placed under continuous light for either 2 (group A) or 7 (group B) days. One month after the transplantation, we performed the same procedures as just described (groups C and D, respectively).

When we examined the BDNF expression using the total retina-RPE-choroid complex 3 days after transplantation (2 days after continuous light exposure), we found higher levels of BDNF gene expression in the rats receiving transplants of Tet-BDNF1-RPE cells, although the increase was not significant (Fig. 5A). Similar findings were made at 8 days (Fig. 5B). When the DOX eye drops were started 1 month after transplantation and continued for 3 days, we also found higher BDNF gene expression in the group receiving 10 mg/mL DOX eye drops than those receiving 0 mg/mL DOX eye drops and the sham-operation group (Fig. 5C).

When we examined the BDNF protein expression 8 days after transplantation, a statistically significant increase in the BDNF expression was observed in the rats treated with 10 mg/mL DOX eye drops every day compared with the untreated control or rats that received only RPE cell transplants (Fig. 5D).

We then compared the a-wave amplitudes of the ERG among the rats and statistically significant larger amplitudes...
were recorded in the eyes after Tet-BDNF1-RPE cell transplantation with 5 or 10 mg/mL DOX eye drops than in eyes with sham surgery, vehicle injection, RPE cell transplantation only, or Tet-BDNF1-RPE transplantation with 0 mg/mL DOX eye drops. Rats that received Tet-BDNF1-RPE cells with 10 mg/mL DOX eye drops showed significant preservation of the ERG b-waves, similar to that of a-waves (Fig. 6A). Rats receiving transplants of Tet-BDNF1-RPE cells with either 2- or 5-mg/mL DOX eye drops showed significant preservation of the b-wave amplitudes compared with that of sham-operation rats.

When the DOX eye drops were given only once (on the day of the transplantation) or three times (every day including the day of the transplantation), the ERG b-wave amplitudes were larger in the rats treated with eye drops three times than in those with only one eye drop treatment (Fig. 6B).

We started the DOX eye drops (0 or 10 mg/mL DOX) in other rats (group C) 1 month after transplantation for 3 days. When we compared the a-wave amplitudes of the ERG after 2 days of constant light exposure, statistically significant larger amplitudes were observed in the eyes after Tet-BDNF1-RPE cell transplantation with 10 mg/mL DOX eye drops than that of Tet-BDNF1-RPE transplantation with 0 mg/mL DOX eye drops. Higher b-wave amplitude of the ERG was also observed in the rats with Tet-BDNF1-RPE transplantation with 10 mg/mL DOX eye drops, although the results of statistical analysis are not significant (Fig. 6C).

Histologic examination of the rats treated with the indicated DOX eye drops 7 days after Tet-BDNF1-RPE transplantation (group B), showed that the degree of preservation of ONL thickness was variable (Fig. 7A). Although histologic examination showed photoreceptor loss in each experiment, statistically significant preservation of the thickness of the ONL was observed at many points in the rats treated with DOX eye drops when compared among the rats as the indicated treatment (Fig. 7B). Statistically significant photoreceptor protection was observed at four mean measurement points in the rat receiving Tet-BDNF1-RPE transplants with 2 mg/mL DOX eye drops (shown as squares), when compared with that of the sham-operation control. A significantly better preservation of ONL thickness was found at 19 and 22 mean measurement points in the rats receiving Tet-BDNF1-RPE transplants with 5 or 10 mg/mL DOX eye drops, respectively. When we compared the rescue points among the cell transplants, statistically significant rescue effects were observed in the rats treated by 5 or 10 mg/mL DOX eye drops when compared with those of 0 ($P < 0.0001$, both 5 and 10 mg/mL) or 2 ($P = 0.0002$, 5 mg/mL and $P < 0.0001$, 10 mg/mL) mg/mL DOX eye drops.

When we performed the same experiments 1 month after transplantation (group D), we found statistically significant preservation of the thickness of the ONL at some points in the rats treated with 0 or 10 mg/mL DOX eye drops compared with those with the treatments indicated in Figure 7C. A significantly better preservation of ONL thickness was found at 7 and 11 mean measurement points (shown as asterisks) in the rats receiving Tet-BDNF1-RPE transplants with 0 or 10 mg/mL DOX eye drops, respectively. Although a statistically significant difference between the mean measurement points was not observed in the rats treated by 0 and 10 mg/mL DOX eye drops ($P = 0.0801$), better preservation at the mean measurement points was observed in the rats treated by 10 mg/mL DOX eye drops. Although the number of statistically significant rescue points was fewer in group D than in group B, our results showed that ONL preservation was observed by DOX eye drops started even 1 month after transplantation.

**DISCUSSION**

Tetracycline derivatives can induce transgene expression in adenovirus and AAV vectors. These viral vectors were directly injected into the subretinal space or vitreous cavity, and the transgene expression was well controlled by DOX. The investigators in these studies also reported that the gene was delivered to the targeted cells using their system. However, the introduction of viral vectors into the eye can result in systemic dissemination, and for safety, systemic dissemination should be avoided. Thus, we transplanted RPE cells subretinally, and these cells were stably transduced by a tetracycline-responsive nonviral vector.
DOX has been administered in drinking water for Tet-on or Tet-off systems.\textsuperscript{21–27} This method produces highly variable results in individuals. First, it is known that attaining a sufficient amount of DOX in the specialized site, such as the brain or eye, may be difficult.\textsuperscript{22} Second, drinking water cannot provide equal amounts of DOX to each individual. In addition, systemic minocycline treatment, which has been used in patients with rheumatoid arthritis,\textsuperscript{29} has adverse side effects, such as gastrointestinal disorder, hyperpigmentation, and intracranial hypertension. Zhang et al.\textsuperscript{30} also reported a 15% weight loss in mice after 8 days (90 mg/kg) of minocycline treatment.

Eye drops are applied only to the eyes and can provide equal amounts in each individual with minimal systemic dissemination. Eye drops are also convenient, because they can be used only when needed. Furthermore, the control of the transgene expression may prevent transgene-mediated side effects. Thus, if the target gene could be induced from the subretinally transplanted cell by eye drops, there would be many advantages to the treatment.

As shown, we were able to induce BDNF expression in our Tet-BDNF1-RPE cells by DOX in vitro, as well as from subretinally transplanted Tet-BDNF1-RPE cells by DOX eye drops in vivo. Because we examined the BDNF expression in the whole retina-RPE-choroid complex, the BDNF expression was not extremely high. However, BDNF expression was always higher in the retina receiving Tet-BDNF1-RPE cell transplants with 10 mg/mL DOX eye drops than in sham-operation retinas, eyes with RPE cell transplant only, or eyes with Tet-BDNF1-RPE transplants with 0 mg/mL DOX eye drops.

We examined BDNF expression by ELISA or real-time PCR and its functional effects by ERG or histology after continuous light exposure. The continuous light exposure damaged the retina, and we could not repeat the same experiments in the same rats. Thus, we could not turn on or off the target gene expression. Conversely, we performed the same Tet-BDNF1-RPE transplantation and placed the rats under standard light cycle for 1 month without DOX. Then, we started the 0 or 10 mg/mL DOX eye drops for 5 or 7 days. We found better preservation of the ERG a-wave amplitude in the rats treated with 10 mg/mL DOX eye drops than with 0 mg/mL DOX eye drops. Better preservation of ONL was also observed in the rats treated with 10 mg/mL DOX eye drops than with 0 mg/mL DOX eye drops 1 month after transplantation. These results showed that although we could not perform on and off experiments in the same rats, we could turn on BDNF expression on the indicated days from the subretinally transplanted Tet-BDNF1-RPE by applying DOX eye drops, in our experimental conditions.

As we reported,\textsuperscript{10} the functioning of subretinally transplanted allogenic RPE cells (Tet-BDNF1-RPE) may gradually decrease. The lower preservation of the ERG amplitudes and ONL thickness at 1 month than at 3 or 7 days after transplantation may be influenced by the number of cells at the transplanted subretinal space.

Minocycline was reported to protect photoreceptors from phototoxicity by inhibiting microglial activation.\textsuperscript{30} Doxycycline has also been reported to suppress inflammation and show neuroprotection in some situations, such as brain injury or inflammation.\textsuperscript{31} Minocycline inhibits TNFα and IL-1β expression from microglia, whereas doxycycline suppresses IL-1β.\textsuperscript{51} From these reports, we also examined the effects of DOX eye drops without Tet-BDNF1-RPE cell transplantation in our experimental model. The animals included untreated control rats and sham-operation rats. However, we could not detect any neuroprotective effects by DOX eye drops in our experimental conditions.

Our experimental conditions were different from those of Zhang et al.\textsuperscript{50} who used mice in phototoxicity experiments with a different light-exposure system. They used mice after 2 days of dark adaptation before light exposure. In addition, they used high doses (45 mg/kg; twice daily) of intraperitoneal minocycline. Minor differences between minocycline and DOX may have also affected the results. Although DOX and minocycline are structurally related analogues, both have shown different protective role against light-induced stress.\textsuperscript{52}
Of note, the BDNF expression in our Tet-BDNF1-RPE cells tended to be enhanced even after a second or third time DOX administration in vitro, although it may be influenced by an increasing number of cells.

The ERG b-waves were also larger when we used DOX eye drops daily than with only one application. Folliot et al.\(^2\) reported that a GFP signal was undetectable 7 days after DOX withdrawal in rat eyes, although they used the Tet-off system, and induction was through the drinking water. Continuous administration of DOX in a Tet-on system or withdrawal of the DOX in a Tet-off system may be necessary for the maximum effect.

Increasing the concentration of DOX in the eye drops to 5 to 10 mg/mL also resulted in better protection of photoreceptors from phototoxicity than did 2 or 0 mg/mL DOX eye drops 3 or 7 days after transplantation.

Mild inflammation developed in the anterior segment of two eyes that received 10 mg/mL DOX eye drops. When we used eye drops of higher concentrations than 10 mg/mL, we found obvious irritation of the anterior segment and severe corneal epithelial erosion. We also found that DOX concentrations between 0.01 to 10 mg/mL showed almost same degree of gene expression, if we used Tet-EGFP-transduced cell transplantation in the subretinal space (data not shown). Although the optimal concentration of DOX eye drops has to be determined, these were the reasons that the maximum concentration of the eye drops was 10 mg/mL in our experiments. Because we applied eye drops once a day, repeated applications during the day may improve the results.

Although some investigators have reported a tight regulation of rTA-controlled transgene expression,\(^2\)\(^5\) rTA-controlled transactivators have been reported to show weak transgene expression in vivo without DOX administration.\(^2\)\(^3\)\(^5\)\(^6\) We also found a weak expression of BDNF in the supernatant of Tet-BDNF1-RPE cells without DOX, which was not observed in only RPE cells. The ERG amplitudes and the ONL thickness after Tet-BDNF1-RPE transplantation with 0 mg/mL DOX eye drops showed better results than that of only RPE transplantation. These results suggest a weak expression of BDNF in our experimental conditions. However, statistically significant preservation of the ERG expression was observed only at higher concentrations of DOX eye drops than that with no treatment or a lower DOX concentration. Although our Tet-on system may have slight leaky transgene expression, the expression level may not be high enough for photoreceptor protection in our experimental conditions.

rTA has also been reported to have some potential for inducing immune responses and may affect the transgene expression in monkeys. Although one of six monkeys showed persistent transgene expression, five monkeys showed decreased transgene expression probably due to immune reaction against rTA after an intramuscular injection of the transgene and intravenous DOX induction.\(^2\)\(^3\)\(^5\) Although TA metabolism in the cell is not clear, it is known that TA expression may be toxic to the expressing cells, and it may implicate improvement of the vector system as reported.\(^2\)\(^4\)

The results show that the vector system, the DOX concentration, and the eye drop frequency remain to be determined. However, together with the results of in vitro experiments, we have demonstrated that BDNF expression can be controlled by DOX eye drops from Tet-BDNF1-RPE cells transplanted into the subretinal space and protect photoreceptors from phototoxicity. Our system is safer not only because it is a nonviral system but also because the transgene expression is controlled as desired by eye drops.

References


