Effect of the Rho-Associated Kinase Inhibitor Eye Drop (Ripasudil) on Corneal Endothelial Wound Healing

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PURPOSE. Ripasudil (Glunatec), a selective rho-associated coiled coil-containing protein kinase (ROCK) inhibitor, was approved as a glaucoma and ocular hypertension treatment in Japan in 2014. The purpose of this study was to investigate the feasibility of using ripasudil eye drops to treat corneal endothelial injuries.

METHODS. Cultured human corneal endothelial cells (HCECs) were treated with ripasudil, and 5-bromo-2'-deoxyuridine (BrdU) incorporation was evaluated by ELISA. A rabbit corneal endothelial damage model was also created by mechanically scraping the corneal endothelium, followed by topical ripasudil eye drop application for 2 weeks. The anterior segment was evaluated by slit-lamp microscopy, and central corneal thickness was measured by ultrasound pachymetry. Corneal specimens were evaluated by phalloidin staining and immunohistochemical analysis using antibodies against Ki67, N-cadherin, and Na+/K+-ATPase.

RESULTS. Many more BrdU-positive cells were observed among the HCECs treated with ripasudil (0.3-30 μM) than among the control HCECs. Ripasudil-treated eyes in a rabbit model showed 91.5 ± 2.0% Ki67-positive cells after 48 hours, whereas control eyes showed 52.6 ± 1.3%. Five of six corneas became transparent in ripasudil-treated eyes, whereas zero of six corneas became transparent in the control eyes. Regenerated cell densities were higher in the eyes treated with ripasudil than in eyes treated with vehicle. Eyes treated with ripasudil expressed N-cadherin and Na+/K+-ATPase in almost all CECs, whereas this expression was decreased in control eyes.

CONCLUSIONS. Ripasudil promoted corneal endothelial wound healing, supporting its development as eye drops for treating acute corneal endothelial damage due to eye surgeries, especially cataract surgery.

Keywords: corneal endothelial cells, bullous keratopathy, Rho kinase inhibitor

The corneal endothelium regulates corneal transparency by balancing corneal hydration through pump and barrier functions. Hexagonal corneal endothelial cells (CECs) form a monolayer sheet at a cell density of approximately 2500 cells/mm² in late adulthood, but corneal endothelial damage—due to Fuchs' endothelial corneal dystrophy, cataract surgery, and corneal trauma—induces compensatory migration and spreading, which results in a cell density drop. When corneal endothelial cell density reaches a critical level, typically 500 cells/mm², the corneal endothelial function is not compensated, and the cornea then exhibits haziness, together with edema. The only therapy is corneal transplantation, as no pharmaceutical intervention has yet been introduced into clinical settings.

In 2009, we reported that the rho-associated kinase (ROCK) inhibitor, Y-27632, promoted in vitro cell proliferation of cultured CECs. Subsequently, we demonstrated that topical application of Y-27632 in the form of eye drops enhances in vivo corneal endothelial wound healing in rabbit and monkey models. Following the animal experiments, we performed clinical research on corneal endothelial dysfunction patients who were treated with Y-27632 eye drops following a 2-mm-diameter transcorneal freezing procedure. Rh-ROCK inhibitor eye drops showed effectiveness in reducing the central corneal thickness in patients with early stage Fuchs' endothelial corneal dystrophy (these patients still had residual healthy corneal endothelium at the peripheral cornea), whereas the ROCK inhibitor did not reduce central corneal thickness in patients with an advanced stage of corneal endothelial dysfunction with diffuse edema. These results from our clinical research suggest that ROCK inhibitor eye drops enhance corneal endothelial wound healing by promoting residual CEC proliferation, as long as some relatively healthy CECs remain. However, despite accumulating evidence, no commercially available ROCK inhibitor has been developed for the treatment of corneal endothelial injury.

In the field of ophthalmology, ROCK inhibitors have attracted much interest among investigators as potent drugs for glaucoma treatment, because ROCK inhibitors reduce the intraocular pressure by increasing the outflow facility of the aqueous humor. Ripasudil hydrochloride hydrate, a selective ROCK inhibitor, has been developed as a glaucoma drug and has been shown in phase 1 clinical trials to reduce IOP in healthy adult volunteers. Randomized clinical studies have demonstrated that 0.4% ripasudil eye drops reduced intraocular pressure.
pressure in patients with primary open-angle glaucoma and ocular hypertension. Subsequently, in 2014, a commercial preparation of ripasudil hydrochloride hydrate eye drops (Glantece ophthalmic solution 0.4%, Kowa Company Ltd., Nagoya, Japan) was approved in Japan for the treatment of glaucoma and ocular hypertension. The 50% inhibitory concentration (IC50) of ripasudil is 0.051 μM for ROCK 1 and 0.019 μM for ROCK 2, whereas the IC50 for PKAcz, PKC, and CaMKIIz are 2.1, 27, and 0.37 μM, respectively. This high selectivity for ROCK is considered to contribute to the safety of ripasudil, which shows minimal off-target effects through inhibition or activation of other signaling pathways.

In the present study, we tested the feasibility of repositioning ripasudil as a treatment for corneal endothelial injuries. Drug repositioning (i.e., the repurposing of an existing drug for new indications) is one possible strategy for drug development that could have a faster approval time and lower investment cost than traditional drug discovery. Here we showed that ripasudil treatment promotes in vitro CEC proliferation in cultured human CECs (HCECs) in a similar fashion to that shown by the conventional ROCK inhibitors Y-27632 and fasudil. We also demonstrated that ripasudil eye drops promoted wound healing and facilitated regeneration of the corneal endothelium in a rabbit wound model.

**Methods**

**Animal Experiment Approval**

In all experiments, animals were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbit experiments were performed at Doshisha University (Kyoto, Japan) according to the protocol approved by the University’s Animal Care and Use Committee (Approval A15012). Human donor corneas were obtained from SightLife (Seattle, WA, USA) for research purposes.

**Cell Culture**

A total of four human donor corneas (from persons >40 years of age) were used for cultivation of HCECs by the protocol described previously. Briefly, Descemet's membranes containing the HCECs were stripped from donor corneas, and the membranes were digested with 1 mg/mL collagenase A (Roche Applied Science, Penzberg, Germany) at 37°C for 12 hours. The HCECs were seeded in 1 well of a 48-well plate coated with laminin E8 fragments (iMatrix-511; Nippi, Inc., Tokyo, Japan) (0.5 μg/cm²). The culture medium was prepared according to published protocols.

First, bone marrow mesenchymal stem cells (BM-MSCs) were cultured according to previously reported protocols. Briefly, basal medium for BM-MSCs was prepared (OptiMEM-I [Life Technologies Corp., Carlsbad, CA, USA] containing 8% fetal bovine serum, 5 ng/mL epidermal growth factor [Sigma-Aldrich Corp., St. Louis, MO, USA], 20 μg/mL ascorbic acid [Sigma-Aldrich Corp.], 200 mg/L calcium chloride, 0.08% chondroitin sulfate [Wako Pure Chemical Industries, Ltd., Osaka, Japan], 50 μg/mL gentamycin, and 10 μM SB431542 [Merck Millipore, Billerica, MA, USA]) and conditioned by culturing BM-MSCs for 24 hours. The basal medium conditioned with BM-MSCs was then collected for use as the culture medium for HCECs. The HCECs were cultured with Y27632 (Wako Pure Chemical Industries, Ltd.), fasudil (Wako Pure Chemical Industries, Ltd.), or ripasudil (Kowa Company, Ltd., Nagoya, Japan) to evaluate the effect of each ROCK inhibitor on HCEC proliferation.

**Immunohistochemistry**

Cultured HCECs or rabbit corneal specimens were fixed in 4% formaldehyde and incubated in 1% BSA to block nonspecific binding. The samples were investigated by conducting immunohistochemical analyses of Ki67 (Sigma-Aldrich Corp.), N-cadherin (1:300; BD Biosciences, San Jose, CA, USA), and Na+/K+-ATPase (1:300; Upstate Biotechnology, Lake Placid, NY, USA) antibodies. Alexa Fluor 488–conjugated goat anti-mouse (Life Technologies Corp.) was used as a secondary antibody at 1:1000 dilution. Cell morphology was evaluated after actin staining with a 1:400 dilution of Alexa Fluor 594–conjugated phalloidin (Life Technologies Corp.). Proliferative cells were evaluated by 5-ethyl-12 Click-IT 5-ethyl-2′-deoxyuridine (EdU) imaging kit (Life Technologies Corp.) according to the manufacturer’s instructions. Briefly, the HCECs (1 × 10⁴ cells per well) were cultured in a 96-well plate and incubated with 10 μM EdU for 6 hours at 37°C. Following fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton X-100 (Nacalai Tesque, Kyoto, Japan), the HCECs were incubated with a reaction cocktail. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). The specimens were viewed with a fluorescence microscope (TCS SP2 AOB5; Leica Microsystems, Wetzlar, Germany).

**Cell Proliferation Assay**

Human CECs were cultured at a density of 5000 cells per well in a 96-well plate. Cell proliferation was determined by evaluating incorporation of 5-bromo-2′-deoxyuridine (BrDU) into DNA by use of the Cell Proliferation Biotrak ELISA system, version 2 (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) according to the manufacturer’s instructions. Briefly, HCECs were incubated with 10 μM BrdU for 24 hours. Cultured cells were incubated with fixation solution and incubated with 100 μL monoclonal antibody against BrdU for 30 minutes. The BrdU absorbance was measured by a spectrophotometric microplate reader.

**Rabbit Corneal Endothelial Damage Model by Corneal Freezing**

As an in vivo wound model, the corneal endothelium of 48 eyes of 24 Japanese white rabbits was damaged in a modified protocol, as described previously. Briefly, a stainless steel 7-mm-diameter probe was immersed in liquid nitrogen for 3 minutes, and the center of the rabbit cornea was cryofrozen with the probe for 15 seconds under general anesthesia. This procedure was carefully confirmed not to induce complete blindness or any severe general adverse effect. One 0.4% ripasudil eye drop (four times daily) or 0.8% ripasudil eye drop (two times daily) was topically instilled (50 μL) in one eye of each of six rabbits, whereas vehicle was instilled in the fellow eye of each rabbit as a control (n = 6). Ripasudil eye drops and vehicles were provided by the Kowa Company. After 48 hours of treatment, the rabbits were euthanized, and the corneal endothelium wound area was evaluated by Alizarin red staining and examined with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The Ki67-positive cells located at the leading edge (3.5 mm distant from the center of the cornea), middle area (4.5 mm distant from the center of the cornea), and periphery (5.5 mm distant from the center of the cornea) were also evaluated in the same specimens. These experiments were performed in duplicate.
A rabbit corneal endothelial damage model was created to mimic surgical trauma. Half the area of the corneal endothelium of 24 eyes of 12 Japanese white rabbits was mechanically scraped from the Descemet's membrane with a 20-gauge silicone needle (Soft Tapered Needle; Inami & Co., Ltd., Tokyo, Japan) while the animals were under general anesthesia. The scraped area was confirmed as damaged by 0.04% trypan blue staining. The experimenter who created the endothelial damage model was blinded to subsequent treatment with ripasudil or vehicle. Before this procedure, the lenses were removed with an Alcon Series 20000 Legacy Surgical System (Alcon, Inc., Fort Worth, TX, USA) to deepen the anterior chamber. One eye of each rabbit was used for 0.4% ripasudil treatment and the contralateral eye served as the control. The corneal endothelium of both eyes was used in the experiment to reduce the number of rabbits required for the study and to provide a correct evaluation of the effect, because wound healing varied with the individual rabbits. This procedure was confirmed not to induce complete blindness or any severe general adverse effects.

**Rabbit Corneal Endothelial Damage Model by Mechanical Scraping**

A rabbit corneal endothelial damage model was created to mimic surgical trauma. Half the area of the corneal endothelium of 24 eyes of 12 Japanese white rabbits was mechanically scraped from the Descemet's membrane with a 20-gauge silicone needle (Soft Tapered Needle; Inami & Co., Ltd., Tokyo, Japan) while the animals were under general anesthesia. The scraped area was confirmed as damaged by 0.04% trypan blue staining. The experimenter who created the endothelial damage model was blinded to subsequent treatment with ripasudil or vehicle. Before this procedure, the lenses were removed with an Alcon Series 20000 Legacy Surgical System (Alcon, Inc., Fort Worth, TX, USA) to deepen the anterior chamber. One eye of each rabbit was used for 0.4% ripasudil treatment and the contralateral eye served as the control. The corneal endothelium of both eyes was used in the experiment to reduce the number of rabbits required for the study and to provide a correct evaluation of the effect, because wound healing varied with the individual rabbits. This procedure was confirmed not to induce complete blindness or any severe general adverse effects.

**Rabbit Corneal Assessment**

Anterior segments were evaluated by slit-lamp microscopy for 2 weeks. Scheimplug images and a corneal thickness map were obtained with a Pentacam instrument (OCULUS; Optikgeräte GmbH, Wetzlar, Germany). Corneal endothelium was evaluated by contact specular microscopy (Konan scanning slit specular microscope; Konan Medical, Nishinomiya, Japan). Corneal thickness was determined with an ultrasound pachymeter (SP-2000; Tomey, Nagoya, Japan), and the mean of 10 measured values was then calculated (up to a maximum thickness of 1200 μm, the instrument’s maximum reading).

**Statistical Analysis**

The statistical significance (P value) of differences in the mean values of the two-sample comparison was determined with the Student's t-test. Values shown on the graphs represent the mean ± SEM.

**RESULTS**

**Effect of Ripasudil on HCECs Proliferation In Vitro**

Cultured HCECs were seeded on a culture plate and cultured for 24 hours, and then HCECs were treated with the following ROCK inhibitors for 48 hours: Y-27632 (10 μM), fasudil (10 μM), and ripasudil (0.3–100 μM). Representative phase contrast images showed that higher numbers of cells were observed when cells were treated with ROCK inhibitors (Fig. 1A). The proliferative potential was assessed by BrdU incorporation into the newly synthesized DNA. An ELISA showed that concentrations of ripasudil from 0.3 to 30 μM significantly enhanced BrdU incorporation (Fig. 1B). The numbers of EdU- and Ki67-positive cells were significantly higher following treatment of HCECs with ripasudil. **P < 0.01.

**FIGURE 1.** Effect of ripasudil on in vitro corneal endothelial cell proliferation. (A) Cultured HCECs were seeded onto the culture plate and incubated for 24 hours, followed by treatment with the ROCK inhibitors Y-27632 (10 μM), fasudil (10 μM), and ripasudil (0.3–100 μM) for 48 hours. Representative phase contrast images are shown. Scale bar: 50 μm. (B) The HCECs were cultured at the density of 5000 cells per well in a 96-well plate for 24 hours, and DNA synthesis was detected by ELISA as incorporation of BrdU into DNA. BrdU incorporation was significantly enhanced at the ripasudil concentrations from 0.3 to 30 μM. **P < 0.01. (C, D) The effect of ripasudil on proliferation of corneal endothelium was evaluated by EdU and Ki67 staining after 48 hours of treatment. The numbers of EdU- and Ki67-positive cells were significantly higher following treatment of HCECs with ripasudil. **P < 0.01.
Effect of Ripasudil Eye Drops on Corneal Endothelial Proliferation in a Transcorneal Freezing Rabbit Model

We tested the effect of 0.4% ripasudil eye drop (four times daily) and 0.8% ripasudil (two times daily) in the rabbit partial corneal endothelial wound model, because the topical and systemic safety of doses lower than these was already confirmed in commercially available ripasudil eye drops (Glanatec ophthalmic solution 0.4%; Kowa Company, Ltd.) in clinical trials. Slit-lamp microscopy showed that control rabbits with 7-mm-diameter corneal endothelial wounds created by transcorneal freezing exhibited hazy corneas after 48 hours. On the other hand, rabbits treated with 0.4% or 0.8% ripasudil eye drops exhibited less haze in their corneas, and no other adverse effects, such as delay of corneal epithelial wound healing, severe conjunctival injection, and corneal opacity, were observed (Fig. 2A). Coincidently, representative Alizarin red staining images showed that the wound area tended to be smaller in eyes treated with 0.4% or 0.8% ripasudil eye drops than in control eyes (Fig. 2B). The Ki67 staining demonstrated that 0.4% or 0.8% ripasudil eye drops promoted cell proliferation throughout the wound edge to the peripheral area of the residual corneal endothelium (Fig. 2C). The percentage of Ki67-positive cells in the leading edge was 72.8 ± 4.3% in the control, but it was 84.4 ± 2.5% and 90.5 ± 1.7% in the eyes treated with 0.4% and 0.8% ripasudil, respectively. In the peripheral area, Ki67-positive cells accounted for 33.5 ± 3.7% of the cells in the control and 40.4 ± 2.7% and 47.1 ± 3.1% of the cells in the eyes treated with 0.4% and 0.8% ripasudil, respectively (Fig. 2D).

Effect of Ripasudil Eye Drops on Corneal Endothelial Wounds in a Mechanical Scraping Rabbit Model

Next, we evaluated whether ripasudil eye drops are an effective treatment for severe corneal endothelial damage; for example, the damage occurring during ocular surgeries such as cataract surgery, which is one of the leading causes of bullous keratopathy. We mechanically scraped half the area of the rabbit corneal endothelium and applied 0.4% ripasudil eye drops (three times daily). Slit-lamp microscopic images showed that eyes treated with vehicle as a control exhibited corneal edema, whereas eyes treated with ripasudil exhibited almost clear corneas (Fig. 3A). Five of six eyes treated with ripasudil recovered corneal transparency in the pupil center, whereas six of six control eyes did not. Scheimpflug images and corneal

0.8% ripasudil eye drops exhibit less hazy corneas. No other adverse effects, such as the delay of corneal epithelial wound healing, severe conjunctival injection, and corneal opacity were observed.

The wound area of the corneal endothelium was evaluated by Alizarin red staining after 48 hours of treatment. Alizarin red staining images showed that eyes treated with vehicle as a control exhibited corneal edema, whereas eyes treated with ripasudil exhibited almost clear corneas (Fig. 3A). Five of six eyes treated with ripasudil recovered corneal transparency in the pupil center, whereas six of six control eyes did not. Scale bar: 50 μm. These experiments were performed in duplicate.
thickness maps obtained with a Pentacam HR instrument also demonstrated that control eyes exhibited corneal edema throughout the whole area. By contrast, ripasudil-treated eyes showed less edema in the cornea, including the pupillary area, although edema was still evident in the peripheral damaged area (Fig. 3B). The central corneal thickness of eyes treated with ripasudil was 392.8 ± 12.6 μm, but that of eyes treated with vehicle was 1200 μm. **P < 0.01. (D, E) After 48 hours of ripasudil eye drops, Ki67-positive cells in the wounded area at the center of cornea were evaluated. The control eye showed 52.6 ± 1.3% of the cell population as Ki67 positive, whereas the ripasudil-treated eyes showed 91.5 ± 1.97% Ki67-positive cells. Scale bar: 50 μm. **P < 0.01.

**Figure 3.** Effect of ripasudil eye drops on wound healing in a mechanical damage corneal endothelial rabbit model. (A) Half the area of the corneal endothelium was mechanically scraped in six rabbits, and 0.4% ripasudil eye drops were applied (three times daily) for 2 weeks. Vehicle was applied to the fellow eyes as a control. The damage to the scraped area was confirmed by 0.04% trypan blue staining, and the experimenter who created the endothelial damage model was blinded to the subsequent treatment with ripasudil or vehicle. Representative slit-lamp microscope images are shown. (B) Representative Scheimpflug images and corneal thickness maps obtained by Pentacam HR are shown. Values for the corneal thickness map are indicated in micrometers. (C) The central corneal thickness was evaluated by ultrasound pachymetry. The central corneal thickness of eyes treated with ripasudil was 392.8 ± 12.6 μm, but that of eyes treated with vehicle was 1200 μm. **P < 0.01. (D, E) After 48 hours of ripasudil eye drops, Ki67-positive cells in the wounded area at the center of cornea were evaluated. The control eye showed 52.6 ± 1.3% of the cell population as Ki67 positive, whereas the ripasudil-treated eyes showed 91.5 ± 1.97% Ki67-positive cells. Scale bar: 50 μm. **P < 0.01.

Effect of Ripasudil Eye Drops on Regenerated Corneal Endothelium

Contact specular microscopy showed that control eyes exhibited blurred corneal endothelial images from the border (center of the cornea) to the damaged area. On the other hand, regenerated corneal endothelium was observed throughout whole area of the cornea with hexagonal and monolayer morphology (Fig. 4A). Cell density was significantly higher in the eyes treated with ripasudil than in eyes treated with vehicle, although the corneal endothelial cell density tended to be lower in the damaged area than in nondamaged areas in both treated eyes and control eyes (Fig. 4B).

Regenerated corneal endothelium in the damaged area of the control eye exhibited a fibroblastic phenotype with the formation of stress fibers, but that of the treated eye exhibited a hexagonal monolayer phenotype and actin fibers were distributed at the cell cortex (Fig. 4C). Eyes treated with ripasudil expressed N-cadherin (a marker of adherence junctions) and Na+/K+-ATPase (a marker of pump function) in almost all the CECs, but this expression was lower in the control eyes (Figs. 4D, 4E). The Ki67 expression was not observed in either the control or the ripasudil-treated eyes (Fig. 4F). This indicates that corneal endothelial cell
FIGURE 4. Effect of ripasudil eye drops on regenerated corneal endothelium in a mechanical damage corneal endothelial rabbit model. (A) Regenerated corneal endothelium was evaluated by contact specular microscopy after 14 days. Nondamaged areas, borders (pupil center), and damaged areas were evaluated. (B) The corneal endothelial cell density of the eyes treated with ripasudil was significantly higher in all areas (nondamaged areas, border, and damaged areas) than in the eyes treated with vehicle. (C) Regenerated corneal endothelium was evaluated histologically after 14 days. Morphology was evaluated using actin staining performed with Alexa Fluor 594-conjugated phalloidin. (D, E) Functional recovery was analyzed by immunostaining for N-cadherin (D) and Na+/K+-ATPase (E). (F) Cell proliferative status was analyzed by Ki67 immunostaining. No Ki67-positive cells were observed in either the ripasudil treated eyes or the control eyes after 14 days. Nuclei were stained with DAPI. Representative images of pupil centers are shown. Scale bar: 50 µm.
Effect of Ripasudil on Corneal Endothelium

Discussion

In many countries, bullous keratopathy caused by cataract surgery is one of the leading causes of corneal transplantation due to corneal endothelial dysfunction. In the United States, 12.2% of the corneas are used for postcataract surgery corneal edema. As in western countries, 20–40% of corneal transplantation in Asian countries is performed to treat bullous keratopathy caused by cataract surgery. The World Health Organization reported that 51% of blindness is caused by cataracts on a global basis; therefore, it is reasonable to estimate that numerous patients will undergo cataract surgery in the near future, and a certain percentage of those patients will unfortunately experience severe corneal endothelial damage during their surgeries. The development of pharmaceutical treatments is urgently needed.

Wound healing is a combined effect of cell migration, spreading, and proliferation in various tissues. However, wound healing of the corneal endothelium is mainly caused by migration and spreading due to the severely limited proliferation ability of corneal cells. Consequently, critical damage induces impairment of wound healing and loss of function. For that reason, researchers, including our group, have devoted much effort in the search for pharmaceutical agents (e.g., epidermal growth factor, platelet-derived growth factor (PDGF), PGE2, and ROCK inhibitors) that can promote proliferation of CECs for the treatment of corneal endothelial disease.

Here, we showed that ripasudil eye drops are potent enhancers of corneal endothelial wound healing in a rabbit model that mimics severe corneal endothelial injury. Thus, we propose that drug repositioning of ripasudil for treating corneal endothelial diseases would be a judicious strategy for delivering ROCK inhibitor eye drops in the clinical setting.

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SK holds patent no. 5657252, regarding the application of ROCK inhibitors for treating corneal endothelial injury. NO and NK are listed as inventors on the patent.

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References


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