Enhancement of corneal endothelium wound healing by Rho-associated kinase (ROCK) inhibitor eye drops

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ABSTRACT

Aim To demonstrate the efficacy of Rho-associated kinase (ROCK) inhibitor Y-27632 for corneal endothelial wound healing both in in vitro and in vivo models.

Methods As an in vitro model, cultivated cynomolgus monkey corneal endothelial cells were scraped to create a linear defect. The wound distance was then determined during a 24-h culture in the presence or absence of 10 μM of Y-27632. As an in vivo model, central corneal endothelium of Japanese white rabbits was damaged by transcorneal freezing, then 10 mM of Y-27632 was applied topically six times daily for 48 h. The wound area of the corneal endothelium was evaluated after 48 h.

Results The mean wound distance in the cultured corneal endothelial cells was significantly shorter in the Y-27632 group than in the control group. In the rabbit model, the mean wound area of the Y-27632 group was significantly smaller than that of the control group.

Conclusion This study demonstrated that ROCK inhibitor Y-27632 promotes corneal endothelial wound healing both in in vitro and in vivo.

INTRODUCTION

Corneal endothelial cells (CECs) are essential for the maintenance of corneal transparency. Since human corneal endothelial cells (HCECs) do not proliferate sufficiently in vivo, CEC loss causes a compensatory migration and enlargement of the remaining endothelial cells. When CEC density decreases to a critical level as a result of corneal endothelial disorders such as Fuchs' endothelial dystrophy, pseudophakic bullous keratopathy or trauma, the lack of sufficient pump function in the remaining endothelial cells results in irreversible corneal haziness. Although penetrating keratoplasty has been widely performed for corneal endothelial dysfunction, alternative methods for replacing corneal endothelium have been developed such as posterior lamellar keratoplasty, deep lamellar endothelial keratoplasty and Descemet's stripping endothelial keratoplasty. In the USA, approximately 12 000 patients receive keratoplasty for endothelial dysfunction each year.[1] Although these methods provide considerable clinical benefits, allograft rejection and primary graft failure are problems associated with these procedures that have yet to be overcome. Moreover, the shortage of donor corneas is a critical issue in many countries, especially in Japan. Since the establishment of new therapies is the key to solving these problems, several researchers, including us, have reported transplantations of cultivated corneal endothelium in an animal model in an attempt to establish a new clinical intervention for corneal endothelial dysfunction.[2–5]

To the best of our knowledge, no pharmacological therapy is clinically used for the treatment of corneal endothelial dysfunction. In this study, in order to establish a new pharmacological intervention as a form of eye drops, we focused our investigation on regulating the activity of Rho-associated kinase (ROCK). The Rho/ROCK pathway is involved in regulating the cytoskeleton, cell migration, cell apoptosis and cell proliferation.[6–9] In terms of cell proliferation, Rho GTPases play a critical role in cell-cycle progression. However, and in contrast to the previous reports,[6 9] we recently showed that a specific ROCK inhibitor, Y-27632, increased the cell proliferation of cultivated primate corneal endothelium in vitro.[10] HCECs in vivo are known to possess limited proliferative ability throughout their lifespan.[11 12] However, there have been several reports of successful CEC cultivation.[13 14] The fact that HCECs show proliferation activity in in vitro cultivation suggests that corneal endothelium has the possibility to proliferate under an appropriate condition, even in vivo. Thus, we hypothesised that the use of a ROCK inhibitor is applicable for the treatment of corneal endothelial dysfunction in the clinical setting.

In this current study, we investigated whether the use of ROCK inhibitor Y-27632 promotes corneal endothelial wound healing both in vitro and in vivo, and assessed the usefulness of Y-27632 for the treatment of corneal endothelial dysfunction.

MATERIALS AND METHODS

Animal experiment approval

In all experiments, animals were housed and treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The animal experiments were performed at Doshisha University according to the protocol approved by the Animal Care and Use Committee of Doshisha University (Approval No. 0831).

Primary cultures

We used four corneas from two cynomolgus monkeys (3–5 years old; estimated equivalent human age 5–20 years). The monkeys were euthanized for other research purposes, after which the corneas were recovered. We cultivated the cynomolgus monkey CECs (MCECs) as described previously.[4 5] Descemet's membrane with MCECs was stripped and incubated in 0.6 U/ml of Dispase

II (Roche Applied Science, Penzberg, Germany) to release the MCECs. The MCECs were then re-suspended in culture medium. All primary cell cultures and serial passages of MCECs were performed in growth medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 ng/ml basic fibroblast growth factor (bFGF; Invitrogen Corp., Carlsbad, California, USA).

In vitro wound healing assay

The MCECs were cultured at confluence for 14 days in six-well culture dishes, and the cultivated MCECs were then scraped with a 1000-μl plastic pipette tip to create wounds. Six wounds were created in each culture dish, both for the Y-27632 group and for the control. The culture medium was then replaced with fresh medium containing 10 μM of Y-27632.[10] Culture medium without Y-27632 was used for the control. The wound distance, that is, the distance between the cells existing at one edge of the linear defect and those existing at the opposite edge of the defect, was then determined by use of Image J (National Institutes of Health (NIH); http://rsbweb.nih.gov/ij/) software after 12, 18 and 24 h of incubation. Experiments were performed in triplicate.

In vivo wound model

As an in vivo wound model, the corneal endothelium of four Japanese white rabbits was damaged, under general anaesthesia, by transcorneal freezing with a probe (7-mm diameter) for 15 s. A stainless steel probe was immersed in liquid nitrogen for 3 min to stabilise its temperature at approximately −196°C. The rabbits were euthanised following to transcorneal freezing and the wound area of the corneal endothelium was evaluated by Alizarin red staining after enucleation. Reproducibly of the diameter, area, and shape of the wound was then evaluated and calculated by use of Image J (NIH) software.

In vivo wound healing after Y-27632 treatment

The corneal endothelium of six Japanese white rabbits was damaged by transcorneal freezing as above. Then, 10 mM of Y-27632 diluted in phosphate-buffered saline (PBS) (50 μl) was applied topically in one eye of each animal six times daily, while PBS was applied in the other eye as a control. Corneal transparency was assessed by use of a slit-lamp microscope. Corneal thickness was determined with an ultrasound pachymeter (model SP-2000; Tomey, Nagoya, Japan), and the mean of 10 measured values calculated (to a maximum of 1200 μm, which is the maximum reading for the ultrasound pachymeter). The rabbits were euthanised after 48 h of treatment and the wound area of the corneal endothelium was evaluated by Alizarin red staining after enucleation.

Statistical analysis

Statistical analysis was performed use of the Excel software program (Microsoft Co., Redmond, Washington). The statistical

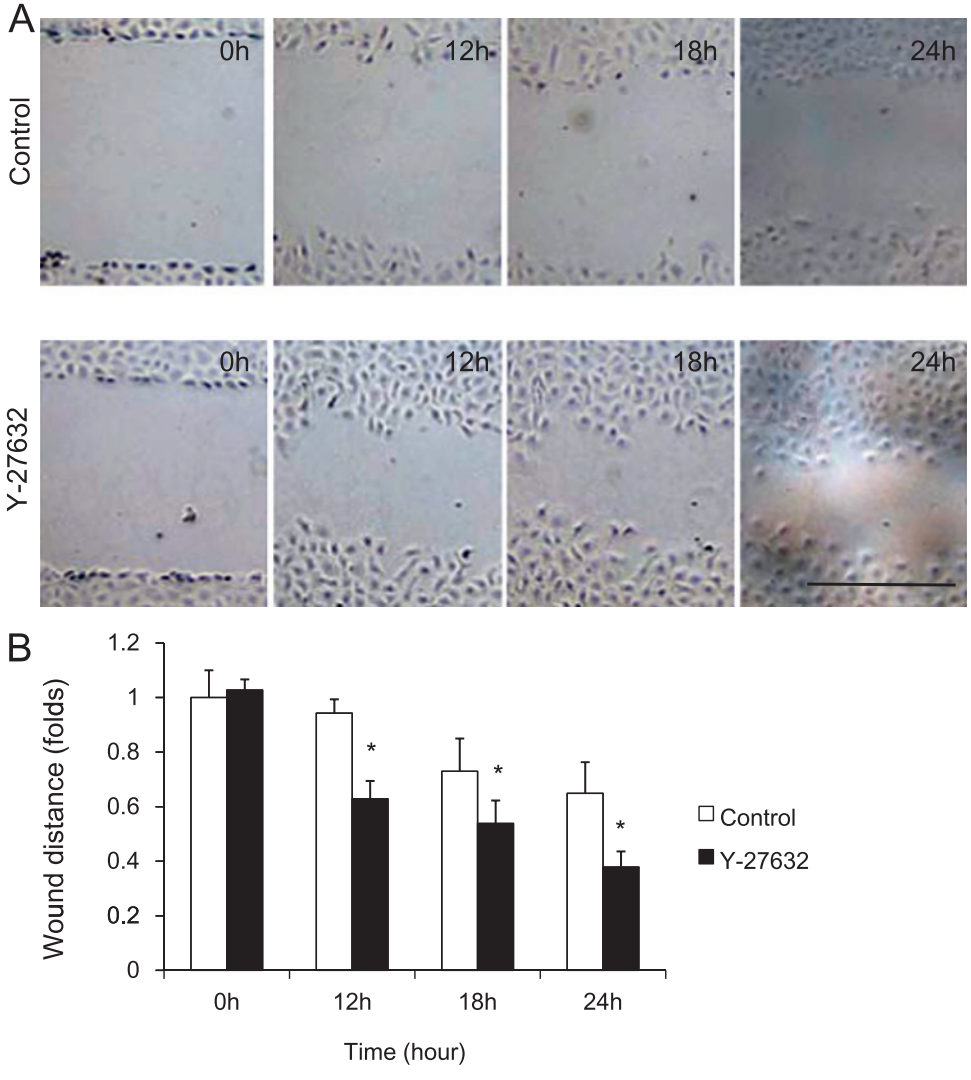

Figure 1 Promotion of wound healing by Rho-associated kinase (ROCK) inhibitor Y-27632 in an in vitro model of cultured monkey corneal endothelial cells (MCECs). (A, B) The mean wound distance was significantly shorter in the Y-27632 group than in the control group after 24 h (37.9±5.7% and 64.9±11.4% as a ratio of the initial wound distance, respectively; *$p<0.01$). Scale bar, 500 μm.

significance (p value) in mean values of the two-sample comparison was determined with the Student t test. $p<0.05$ was considered statistically significant. Values shown represent the mean±SD.

RESULTS

Promotion of wound healing by Y-27632 in an in vitro model of cultured MCECs

MCECs cultured to confluence for 14 days were scraped with a 1000-μl plastic pipette tip to create six linear defect sites and the culture was then continued for 24 h with fresh medium containing 10 μM of Y-27632. The mean wound distance was found to be significantly shorter in the Y-27632 group than in the control group (37.9±5.7% and 64.9±11.4% as a ratio of the initial wound distance, respectively; *$p<0.01$) (figure 1A,B), thus showing that Y-27632 promoted wound healing in the in vitro model of cultured MCECs. Representative data from triplicate experiments are shown.

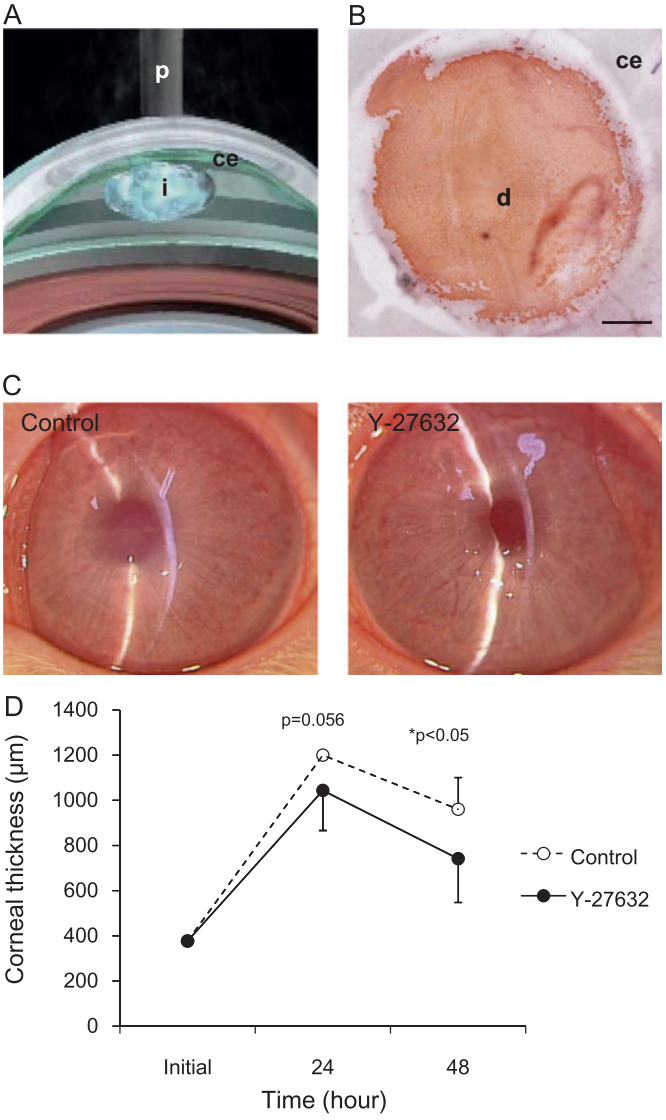

Figure 2 Effects of Rho-associated kinase (ROCK) inhibitor Y-27632 eye drops in a rabbit model. (A) Schematic image of the corneal endothelial wound created by transcorneal cryogenic injury (p, stainless steel probe; i, ice ball; ce, corneal endothelium). (B) Centre of the corneal endothelium is damaged and has a circular profile (d, area of wounded corneal endothelium; ce, area of intact endothelium). (C) Slit-lamp microscopy revealed that the corneal transparency was higher in the Y-27632 group compared with the control group. (D) Ultrasound pachymetry revealed that the corneal thickness was significantly thinner in the Y-27632 group compared with the control group after 48 h. Scale bar, 1 mm.

In vivo wound healing after Y-27632 treatment

To test whether Y-27632 would actually promote corneal endothelial wound healing in an in vivo model, we used rabbits in which we made the corneal endothelial wound by transcorneal cryogenic injury. First, we confirmed that the transcorneal cryogenic injury damaged the centre of corneal endothelium in a round shape reproducibly (diameter 6.4±0.2 mm; area 23.8±2.4 mm^2; n=4) (figure 2A,B). Next, the wounded rabbits were treated with the topical instillation of 10 mM of Y-27632 6 times daily in the form of eye drops. Slit-lamp microscopy examination revealed that the corneal transparency was higher and that the corneal thickness was thinner in the Y-27632 eye-drop treated group compared with the control group (figure 2C). Ultrasound pachymetry revealed that the corneal thickness was significantly thinner in the Y-27632 group compared with the control group after 48 h of treatment (figure 2D). The mean wound area of the Y-27632 group was significantly smaller than that of the control group, expressed as 23.1±22.9% as a ratio of control (*$p<0.05$) (figure 3A,B). These results demonstrated that the topical administration of ROCK inhibitor, Y-27632, as eye drops could enhance endothelial wound healing. In addition, no severe side effects were observed during the experiments.

DISCUSSION

We recently reported that the inhibition of Rho/ROCK signalling by the ROCK inhibitor Y-27632 inhibited dissociation-induced apoptosis and promoted the adhesion and proliferation of MCECs.[10] In this present study, our findings were extended to

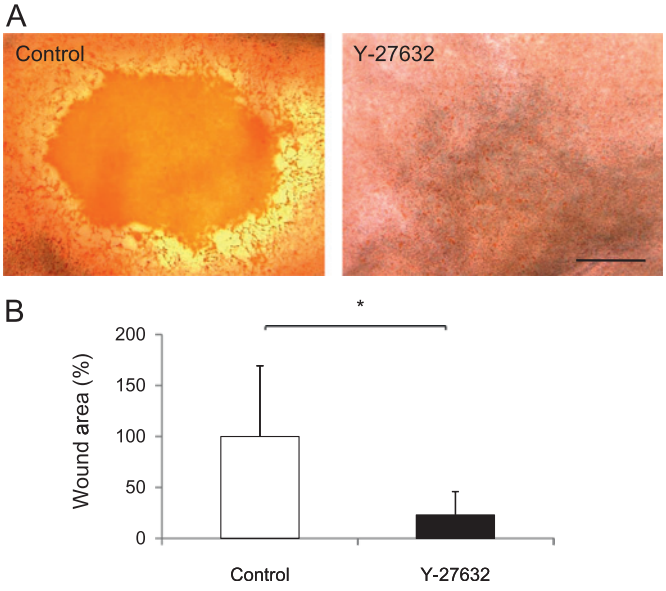

Figure 3 Rho-associated kinase (ROCK) inhibitor Y-27632 eye drop enhanced wound healing in a rabbit model. (A) Alizarin red staining shows the corneal endothelial wound. (B) The mean wound area of the Y-27632 group was significantly smaller than that of the control group after 48 h (23.1±22.9% as a ratio of control; *$p<0.05$). Scale bar, 500 μm.

demonstrate that the topical application of Y-27632 in the form of eye drops promoted corneal endothelial wound healing in both an in vitro and an in vivo model.

The results of our experiments showed that Y-27632 promoted in vitro wound healing of cultivated MCECs. Then, to determine whether a ROCK inhibitor is applicable for the treatment of corneal endothelium dysfunction, Y-27632 was topically instilled in an animal model. Our findings revealed that the topical administration of Y-27632 enhanced corneal endothelial wound healing in an in vivo rabbit model. However, wound healing may be the integrated outcome of the improved cell migration and the cell proliferation. In terms of cell motility, ROCK is known to play a role through the regulation of actomyosin contraction.[7 8 15] ROCK activity is increased in tumour metastasis and overexpression of constitutively activated ROCK promotes tumour invasion.[16] Conversely, ROCK inhibitor or overexpression of dominant negative mutant ROCK inhibits invasion of rat hepatoma cells and metastatic breast cancer cells.[17] Contrary to the findings of those reports, ROCK inhibitor Y-27632 reportedly enhanced the cell motility of trabecular meshwork cells[18] and corneal epithelial cells[19] during wound healing, that suggesting the effect of Rho/ROCK signalling is fully cell-type-dependent.[9 20] Although we reported that ROCK inhibition promotes the proliferation of MCECs in vitro, the mechanisms of cell migration and cell proliferation involved in the enhancement of wound healing by ROCK inhibitor are in need of further study.

To establish the application of a ROCK inhibitor in clinical settings, the effect on corneal endothelial cell density will need to be verified. Since rabbit corneal endothelial cells retain high proliferative ability in vivo, rabbit corneal endothelium might proliferate rapidly after injury[21] and return to a state of high cell density. However, the proliferation of the monkey corneal endothelium is severely limited, as is the case in humans,[22] rendering this a clinically applicable model for corneal endothelial cell disorders.[4 5] In fact, our preliminary findings demonstrated that the topical administration of Y-27632 following a cryo injury enabled the cornel endothelium to retain a high cell density in a cynomolgus monkey during a 1-month observation period (data not shown). Since in vivo wound healing animal models retain an undamaged peripheral corneal endothelium, aligned to the fact that the experimental animals were likely to be significantly younger than the equivalent age of typical human patients presenting with endothelial dysfunction, the viability of the human corneal endothelium and patient's age must be considered before clinical application. Further, modulation of contact inhibition, which is one of the most important factors that prevents primate corneal endothelial cells from proliferation, might be necessary. Nevertheless, our observations in a primate model as well as findings in rabbit model suggest that the topical administration of Y-27632 eye drops might represent a possible treatment regimen for human corneal endothelial disorders.

As to future clinical applications, ROCK inhibitors are expected to be useful for a wide range of diseases such as cardiovascular disease, pulmonary disease and cancer.[15 20] The clinical use of fasudil, one of the ROCK inhibitors, has already been approved in Japan and China for the prevention and treatment of cerebral vasospasm, and to date, has been used in over 124 000 cases in Japan.[15] Furthermore, in the ophthalmology field, ROCK-inhibitor eye drops have been developed for the treatment of glaucoma[18 23] and are currently undergoing clinical trial.[15] Although ROCK inhibitor reportedly inhibits cell proliferation of corneal epithelial cells,[24] the severe ocular toxicological effect associated with the topical administration of Y-39983, which is also one of the ROCK inhibitors derived from Y-27632, was reportedly not observed in rabbit and cynomolgus monkey models.[23] This suggests that the ROCK inhibitor eye drops are a clinically applicable and safe tool for the treatment of corneal endothelium dysfunction.

In summary, our results demonstrate that the ROCK inhibitor Y-27632 promotes corneal endothelium wound healing in an animal model. The topical instillation of a ROCK inhibitor is a less invasive and novel therapy that is promising for the treatment of corneal endothelium dysfunction.

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