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The stimulatory effect of ROCK inhibitor on bovine corneal endothelial cells

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ABSTRACT

Reagents which can promote the proliferation, adhesion and migration of cultured corneal endothelial cells (CECs) will be helpful for the treatment of reduced visual acuity due to CECs deficiency. The objectives of this study were to investigate the potential use of an inhibitor of Rho-associated protein kinase (ROCK), Y-27632, to cultured bovine corneal endothelial cells (B-CECs) and evaluated its effects on the proliferation, adhesion and migration of B-CECs. The proliferation of cultured B-CECs was moderately enhanced by 10 μM Y-27632. Y-27632 induced fibroblast-like morphological changes in the cultured B-CECs and normal cell morphology could recover after Y-27632 removal. In addition, Y-27632 was found to significantly enhance the adhesion and migration of B-CECs. Furthermore, the hanging drop aggregation assay showed that Y-27632 promoted B-CECs to form cellular networks and sheets, which proliferated along the liquid–air interface and migrated to the surface of the lid of dish. Our study demonstrated that Y-27632 is a potentially powerful reagent which can enhance the proliferation of cultured B-CECs. Y-27632 will be useful in CEC injection therapy and topical application for CEC deficiency.

1. Introduction

The corneal endothelial cell-layer is a single layer of cells forming a boundary between the corneal stroma and the anterior chamber. Corneal endothelial cells (CECs) are responsible for maintaining corneal transparency by regulating stromal hydration via their barrier and pump functions. However, the density of human CECs often decreases with the increase in age or various diseases, such as bullous keratopathy, Fuchs' syndrome and endothelium decompensation (Bourne, 2003). If a large number of CECs are lost, irreversible corneal endothelial dysfunction often occurs. The current solution to restore vision is to replace the dysfunctional endothelium with cornea of healthy donor through penetrating keratoplasty or endothelial keratoplasty (Engelmann et al., 2004; Tan et al., 2012). But the shortage of good quality donors for keratoplasty is the major limiting factor in many nations. Eye banks cannot match the demands worldwide, resulting in long waiting lists for corneal transplantation in most developing countries (Tan et al., 2012). Endothelial keratoplasty may provide significantly better visual outcomes. Yet, some complications, such as graft dislocation, primary graft failure and so on, can occur. The future of endothelial keratoplasty may well entail the use of cultured endothelial cells (Anshu et al., 2012).

Y-27632 is a protein kinase inhibitor selective for Rho-associated kinase (ROCK) isoforms ROCK-I and ROCK-II, which regulate the phosphorylation of the regulatory myosin light chain to promote actomyosin-driven cell contractility (Uehata et al., 1997; Ishizaki et al., 2000). Y-27632 was implicated in various cellular functions including actin cytoskeleton organization, cell adhesion, cell motility, vascular and smooth muscle contraction and cytokinesis (Uehata et al., 1997; Takahara et al., 2003). Previous experiments demonstrated that ROCK inhibitor enhanced the in vitro survival of mouse embryonic stem (ES) cell derived neural precursors (Koyanagi et al., 2008), mouse intestinal stem cells (Sato et al., 2009), and murine prostate stem/progenitor cells (Zhang et al., 2011). There are several reports on the effects of Y-27632 in three corneal cell types. Okumura and co-workers (Okumura et al., 2011b, 2013) displayed that Y-27632 eye drops were able to promote corneal endothelial wound healing on corneal endothelial dysfunction models in rabbit and monkey. Y-27632 promoted adhesion, inhibited apoptosis, and increased the number of proliferating CECs. It enhanced corneal endothelial wound healing both in vitro and in vivo in these animal models (Okumura et al., 2009, 2012). However, Pipparelli and co-workers (Pipparelli et al., 2013) reported that Y-27632 had no effect on human CECs proliferative

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capacities, just induced changes in cell shape, increased cell adhesion and enhanced wound healing ex vivo and in vitro. ROCK negatively regulate human corneal epithelial wound healing via modulating cell behaviors including cell migration, proliferation, cell–matrix adhesion, and cell–cell junctions (Yin and Yu, 2008). Rabbit corneal epithelial cell cycle progression was influenced by Rho/ROCK signaling and Y-27632 delayed the progression of G1 to S phase and led to a decrease in the number of rabbit CECs entering the S phase (Chen et al., 2008). A study showed that Y-27632 inhibited keratocyte-to-myofibroblast transition. Y-27632 topical application after a superficial keratectomy suppressed the expression of α-SMA at the center of the wound (Yamamoto et al., 2012). In view of these diversified reports, we, thus, examined the effect of ROCK inhibitor Y-27632 on another corneal endothelial cell model, namely, cultured bovine corneal endothelial cells (B-CECs). We investigated the effect of Y-27632 on the proliferation, adhesion, and migration of B-CECs maintained in vitro as monolayer cultures or in hanging drop culture, which was specific for CECs. The goal was to understand if Y-27632 could be a specific and appropriate reagent for cultivated B-CECs and its implication for CEC treatment and engraftment in regenerative medicine.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's media, fetal bovine serum (FBS), penicillin-streptomycin and trypsin (0.25%)-EDTA (0.02%) were purchased from Invitrogen-Gibco (Grand Island, NY, USA). Cell Counting Kit-8 (CCK-8) was from Dojindo (Kyushu, Japan), Cell Cycle and Apoptosis Analysis Kit, Acridine Orange (AO) and AnnexinV-FITC/PI apoptosis detection kit were from KeyGEN (Nanjing, China). Alexa Fluor 488-labeled Goat Anti-Rabbit IgG(H + L) secondary antibody were from Beyotime (Jiangsu China). Rabbit polyclonal anti-AQP1 antibody, Rabbit polyclonal anti-Na^+/K^+-ATPase antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Y-27632, a selective Rho-associated kinase inhibitor was obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Isolation and culture of B-CECs from bovine corneal tissue

Bovine eyes were obtained from a local abattoir (Shipai, Guangzhou, China). Eyes were processed within 6 h of death. The tissues were obtained in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Experimental Committee at Jinan University, Guangzhou, China. We cultivated the B-CECs as described previously (Chifflet et al., 2003; Satpathy et al., 2004) with some modifications. In detail, the corneal explants were washed three times with ice-cold phosphate-buffered saline (PBS) contained 2% penicillin-streptomycin and 50 μg/ml gentamicin. Then Descemet's membrane was stripped from the posterior surface of the corneal tissue with sterile surgical forceps under a dissecting microscope. The strips were incubated in trypsin (0.25%)-EDTA (0.02%) at 37 °C for 8–10 min. The cells were then centrifuged ($300 \times g$, 5 min) and placed on a gelatin-coated 6-well dish containing low-glucose DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 2.5 μg/ml fungizone in a humidified incubator at 37 °C containing 5% CO_2. Fresh media were replenished every two days. When B-CECs were grown to form a confluent monolayer (5–7 days after plating), subsequent subcultures were propagated in the same media. First and third passage cells were used as indicated for all experiments.

2.3. Scanning electron microscopy

Briefly, samples were fixed in 2.5% glutaraldehyde for 2 h, washed 3 times for 15 min in PBS. After dehydration through a graded ethanol series (70, 90, 100, 100, and 100%), specimens were transferred to isoamyl acetate for 30 min, critical point dried, coated with gold, and mounted for viewing in the JSM-T300 scanning electron microscopy (SEM) (JEOL Technics Co. Ltd., Tokyo, Japan).

2.4. Immunofluorescence assay

Immunofluorescence was used to identify the B-CECs and examine the protein expression of AQP1 and Na^+/K^+-ATPase treated with Y-27632. Cells were treated with 10 μM Y-27632 for 24 h before the immunofluorescence assay was performed. Control cells were not treated with Y-27632. Briefly, after fixation in 4% paraformaldehyde for 30 min at room temperature, cells were washed three times with PBS and incubated with PBS containing 5% fetal bovine serum for 30 min at room temperature. The cells were incubated with the primary antibodies rabbit polyclonal anti-AQP1 antibody (1:200), rabbit polyclonal anti-Na^+/K^+-ATPase antibody (1:200) overnight at 4 °C, and then with the secondary antibodies for 60 min before staining with DAPI. The cells were examined by fluorescence microscope.

2.5. Cell proliferation assay

We first determined the optimal concentration of Y-27632 for the growth of B-CECs. A CCK-8 was employed in this experiment to quantitatively evaluate B-CECs proliferation according to the manufacturer's instructions. Briefly, cells were seeded in 96-well flat-bottomed plates at 1×10^4 cells/well and incubated at 37 °C for 24 h. Subsequently, the cells were then treated with increasing concentrations of Y-27632 (at 0 μM, 1 μM, 5 μM, 10 μM, 20 μM and 40 μM) for 72 h. After 10 μl dyes were add to each well, cells were incubated at 37 °C for 2 h. The absorbance of each well at 450 nm was measured using Multimode Reader. Six different wells per plate were used to assess the cell proliferation.

2.6. Flow cytometry

B-CECs were incubated in culture media with or without 10 μM Y-27632 for 3 days. Cells (1×10^6) from both groups were collected by centrifugation at $500 \times g$ for 5 min, washed twice with ice-cold PBS, and then fixed with 70% ice-cold ethanol and stored at 4 °C for overnight. Cells were centrifuged again, washed with ice-cold PBS twice, incubated with PI (0.1 mg/ml) solution containing RNase A (0.1 mg/ml) for 30 min at 37 °C in the dark. The DNA content was measured by flow cytometry (FACSCalibur, BD, USA), the excitation wavelength was 488 nm, and the percentage of cells in each phase of the cell cycle was evaluated using the ModFit software. Three parallel experiments in each group were used to assess the cell cycle.

Annexin V and PI were analyzed by flow cytometry to detect apoptosis in B-CECs challenged with H_2O_2, which was performed using previously described methods (Zhao et al., 2012). An Annexin V-FITC/PI Apoptosis Detection Kit (Key-GEN) was used according to the manufacturer's instructions. B-CECs were cultured in media with or without 10 μM Y-27632 till cells were about 60–70% confluent (2–3 days after plating). Then media were replaced by fresh media and challenged with 125 μM H_2O_2 for 12 h. The cells were then harvested and washed twice in ice-cold PBS. B-CECs were re-suspended in 200 μl of Annexin V binding buffer at a concentration of 1×10^6 cells/ml. Then the samples were stained with 2 μl FITC-labeled Annexin V and 2 μl PI, the samples were immediately analyzed by flow cytometry (FACSCalibur, BD, USA). Data analysis