LABORATORY SCIENCE

Prophylactic exposure of human corneal endothelial cells to Rho-associated kinase inhibitor reduced apoptosis rate after phacoemulsification: Ex vivo study

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Purpose: To evaluate whether prophylactic exposure of corneal endothelial cells (CECs) to a selective Rho-associated kinase (ROCK) inhibitor will inhibit CEC apoptosis after phacoemulsification.

Setting: Laboratory evaluations at the Edith Wolfson Medical Center, Holon, Israel and the Chaim Sheba Medical Center, Tel-Hashomer, Ramat-Gan, Israel and the Chaim Sheba Medical Center, Tel-Hashomer, Ramat-Gan, Israel.

Design: Experimental study.

Method: Human donor corneolimbal rings were divided into fragments that were stored in commercial storage media with or without the addition of 10 mM ROCK inhibitor for 1 week and were then exposed to phacoemulsification energy. Samples were dissociated into single cells by trypsin digestion and CECs were targeted using the antihuman CD166 antibody, a new biomarker. The CEC survival was evaluated for early and late apoptosis rate with flow cytometric analysis of annexin-V and propidium iodide (PI) double staining.

Results: Six corneoscleral rings from 4 donors were studied. After phacoemulsification, CEC exposed to ROCK inhibitor demonstrated a 37.06% reduction in early apoptosis rate (29.36% ± 4.33% [SD] versus 46.65% ± 1.51%, $P = .006$) and 45.27% reduction in late apoptosis rate (17.6% ± 16.81% versus 32.16% ± 26.30%, $P = .007$), compared with controls. Subsequently, ROCK levels in apoptotic CECs were significantly lower in cells incubated with ROCK inhibitor than the control medium.

Conclusions: In this ex vivo study, ROCK inhibitor reduced endothelial loss and thus, could be used to limit or slow down CEC loss. Rho-associated kinase inhibitor might be used before cataract surgery, especially in high risk patients. This might be a promising new method for preventing pseudophakic bullous keratopathy.

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Cataract surgery is a widely performed procedure with a high success rate.[1] Phacoemulsification during surgery offers a fast visual recovery and a predictable postoperative result. Corneal endothelial cell loss after phacoemulsification is quite low (1.7% to 2.0% at 3 months) in straightforward cases with the use of advanced energy delivery systems, modified surgical techniques to reduce energy, and the use of ophthalmic viscosurgical devices (OVDs).[2] However, endothelial cell loss is significant in high-risk patients with shallow anterior chamber depth (12.9%), advanced cataract (28.0%), small pupil size (16.2%), or intraoperative floppy-iris syndrome risk (12.0%) (Supplemental Table 1, available at: http://jcrsjournal.org).

The endothelium, which dynamically regulates corneal hydration, is crucial for corneal transparency and refractive

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function. In humans, damaged corneal endothelial cells (CECs) cannot regenerate because they are locked in the Gap 1 (G1) phase of the cell cycle. Tight cell-to-cell contact and antigrowth factors in the aqueous prevent cell proliferation.[3] However, under some circumstances, human CECs can regenerate in vivo via cell divisions.[4–6] In addition, the presence of corneal endothelial stem cells has been proposed to induce this corneal endothelial repair process by cell proliferation.[7,8]

Corneal endothelial cell recovery can occur, to some degree, by cellular enlargement and migration of surviving adjacent endothelial cells. These repair mechanisms, however, are limited and severe injury to the endothelium will cause corneal edema and subsequently, reduced visual acuity and pain.[9]

During the past 2 decades, a changing trend from penetrating keratoplasty (PKP) to endothelial lamellar techniques has occurred, and the latter are becoming the standard treatment for endothelial failure.[10–12] However, the complexity and high cost of the procedure and the shortage of donor corneal tissue in some areas of the world have created the incentive to find alternative solutions to this global public health problem.[13] Selective Rho-associated kinase (ROCK) inhibitors in combination either with cryoablation of central diseased endothelial cells or with central descemetorhexis have been studied as an alternative to corneal transplant surgery.[14,15] The cytoplasmic Rho family of guanosine triphosphate phosphohydrolases (GTPases) and its downstream effectors, the Rho-associated protein kinases, are associated with diverse cellular signaling pathways. These proteins play a critical role in regulating actin cytoskeleton organization, cell adhesion, and apoptosis.[16] In addition, Rho GTPases suppress cell-cycle progression from the G1 phase into the synthesis (S) phase.[17] In vitro, ROCK inhibitor enhanced proliferation of primate and human cultured CECs.[3] In in vivo models of rabbits and cynomolgus monkeys, the central corneal endothelium was damaged by transcorneal freezing. When ROCK inhibitor was applied topically 6 times per day for 48 hours, the mean of the corneal endothelium defect was significantly smaller than the control groups.[17,18] This ROCK inhibitor effect on slowing CEC loss might also be useful in preventing cell loss after cataract surgery. Therefore, the aim of this study was to evaluate whether prophylactic exposure of CECs to ROCK inhibitor will inhibit CEC apoptosis after phacoemulsification.

MATERIALS AND METHODS

The study followed the guidelines for experimental investigations required by the Institutional Review Board or Ethics Committee of the Wolfson Medical Center and the Sheba Medical Center.

Tissue Preparation

Six donor corneoscleral rings obtained from corneal grafts prepared for corneal transplantation, PKP, or Descemet-stripping automated endothelial keratoplasty were used.

The corneoscleral rings were placed in the same storage medium (Optisol-GS, Chiron Vision) that was used before transplantation. The corneoscleral rings were divided and samples were stored at 4°C with or without 10 mM of ROCK inhibitor (Y-27632 dihydrochloride, Tocris Biochemicals) for 7 days. This concentration of Y-27632 ROCK inhibitor resulted in the highest cell-survival enhancement in cultivated monkey CECs in a previously published study.[19] On the eighth day, samples were exposed to phacoemulsification energy. A Stellaris PC machine (Bausch & Lomb, Inc.) with the probe held approximately 2.0 mm from the corneal samples was used with no irrigation or aspiration. To simulate real-life energy delivery during surgery for advance nuclear cataract, an effective phacoemulsification time of 30 seconds was planned (100% continues energy for 30 seconds).[20–22]

Corneal Endothelial Cell Evaluation

On the ninth day, corneoscleral segments were dissociated into single cells by 2 mg/mL collagenase digestion at 37°C for 4 hours.[23] After digestion, all corneal cells (epithelial, stromal, and endothelial cells) were collected by centrifugation at 2000 rpm for 3 minutes and stained by antihuman CD166 antibody (BD Bioscience). Antihuman CD166 antibodies were used because they identify CECs.[24]

Three levels of quantitative analysis were prepared: (1) estimation of the fraction of CD166-positive cells, (2) evaluation of the annexin-V and propidium iodide (PI) content in CD166-positive cells population, and (3) measurement of the ROCK distribution within CD166-positive cells population. The cells were subjected to an annexin V and PI assay (Annexin V-FITC Apoptosis Detection Kit Plus, MBL International Corp.), which has been used for assessment of early phases of apoptosis in a broad range of cell types, including human CECs.[25,26] The apoptotic phases could be time framed according the staining pattern: (1) Early apoptotic cells can be detected using fluorescein-labeled Annexin V, which is a Ca2+ dependent phospholipid-binding protein with high affinity for the plasma membrane phosphatidylserine, as alterations in phosphatidylserine occur during the initial stages of apoptosis. (2) Late apoptotic and necrotic cells are stained with both PI and Annexin V because these cells demonstrate impaired plasma and nuclear membranes, which allow the PI to pass and to intercalate into nucleic acids and to display red fluorescence.[26] A histopathological examination of the corneoscleral segments was also conducted. The tissue was fixated in formalin and embedded in paraffin. Sections were then prepared from the paraffin block using a microtome, mounted on glass slides, and stained by hematoxylin–eosin for microscopic evaluation.

Assessment of Rho-Associated Kinase Level in the CD166+ Cells by Fluorescence Activated Cell Sorting

For evaluation of ROCK levels in CECs that had apoptosis during their incubation with ROCK inhibitor, the cultures were stained for fixable viability dye 660 (FVD660, Affymetrix, Inc.) and ROCK antibody–fluorescein isothiocyanate (FITC) conjugated (Bioss Antibodies). Unlike PI staining, cells labeled with fixable viability dye 660 can be fixed, permeabilized, and stained for intracellular antigens without any loss of staining intensity of the dead cells.[A]

The CECs were washed twice in phosphate-buffered saline, resuspended and 1 μL/1 mL of fixable viability dye 660 was added. The CECs were incubated for 30 minutes at 2°C to 8°C, washed, and permeabilized for 15 minutes at 37°C with 0.2% Tween 20 detergent in permeabilization buffer. At the next step, cell culture was stained for 1 hour with 0.05 μg/μL of human anti-ROCK antibody FITC-conjugated, washed by permeabilization buffer, and collected by centrifugation at 2000 rpm for 5 minutes. Pellet was resuspended in 250 μL of permeabilization buffer and analyzed.[27]

Data were analyzed using SPSS for Windows software (version 22.0, IBM Corp.) Continuous data are presented as means ± SD. The paired Student *t* test was performed to compare CEC ROCK inhibitor levels and early and late apoptosis rates. A *P* value less than 0.05 was considered statistically significant.