The Effect of a p38 Mitogen-Activated Protein Kinase Inhibitor on Cellular Senescence of Cultivated Human Corneal Endothelial Cells

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PURPOSE. We have begun a clinical trial of a cell-based therapy for corneal endothelial dysfunction in Japan. The purpose of this study was to investigate the usefulness of a p38 MAPK inhibitor for prevention cellular senescence in cultivated human corneal endothelial cells (HCECs).

METHODS. HCECs of 10 donor corneas were divided and cultured with or without SB203580 (a p38 MAPK inhibitor). Cell density and morphology were evaluated by phase-contrast microscopy. Expression of function-related proteins was examined by immunofluorescent microscopy, membrane blotting array, and ELISA.

RESULTS. Phase-contrast microscopy showed a significantly higher cell density for HCECs cultured with SB203580 than without SB203580 (2623 ± 657 cells/mm² and 1752 ± 628 cells/mm², respectively). The HCECs cultured with SB203580 maintained a hexagonal morphology and expressed ZO-1, N-cadherin, and Na⁺/K⁺-ATPase in the plasma membrane, whereas the control HCECs showed an altered staining pattern for these marker proteins. HCECs cultured without SB203580 showed high positive SA-β-gal staining, a low nuclear/cytoplasm ratio, and expression of p16 and p21. Senescence-associated factors were evaluated by membrane blotting array, quantitative PCR, and ELISA.

CONCLUSIONS. Activation of p38 MAPK signaling due to culture stress might be a causative factor that induces cellular senescence; therefore, the use of p38 MAPK inhibitor to counteract senescence may achieve sufficient numbers of HCECs for tissue engineering therapy for corneal endothelial dysfunction.

Keywords: corneal endothelial cells, p38 MAPK, tissue engineering therapy
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Pathways, which are among the most widespread mechanisms of eukaryotic cell regulation. MAPK has four main signaling modules: extracellular-signal-regulated kinase (ERK), ERK5, Jun-NH2-terminal kinases (JNK), and p38 MAPK. The JNK, ERK5, and p38 MAPK signaling pathways are activated in response to physical stress signals, whereas the ERK pathway is activated by mitotic stimuli. Signaling by p38 MAPK is associated with various cellular activities, such as cell proliferation, differentiation, migration, and apoptosis. In addition, p38 MAPK plays an important role in cellular senescence.

In this study, we determined whether cell density drop of HCECs under culture condition is a phenotypic feature associated with cellular senescence. In addition, we evaluated whether inhibition of p38 MAPK could suppress cellular senescence.

Materials and Methods

Ethics Statement

The human donor corneas used in this study were handled in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from the next of kin of all deceased donors with regard to eye donation for research. Donor corneas were obtained from SightLife (Seattle, WA, USA). All tissue specimens were recovered under the tenets of the Uniform Anatomical Gift Act of the particular state in accordance with the tenets of the Declaration of Helsinki.

Cell Culture

The 10 human donor corneas were used for culturing HCECs. The mean donor age was 52.9 ± 11.7 years. All corneas were stored at 4°C in storage medium (Optisol-GS; Chiron Vision, Irvine, CA, USA) for less than 14 days before use. The HCECs were cultured according to published protocols, with some modifications. Briefly, Descemet’s membranes containing the HCECs were stripped from the 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 resulting HCECs were suspended in Opti-MEM I (Life Technologies, Carlsbad, CA, USA) and divided equally into two tubes. The HCECs in one tube were then seeded in culture medium with p38 MAPK inhibitor (10 μM SB203580; Cayman Chemical, Ann Arbor, MI, USA) in a well of a 48-well plate, and cells in another tube were seeded with culture medium without p38 MAPK inhibitor, as a control. The plates were washed with PBS, either Alexa Fluor 488-conjugated goat anti-mouse (Life Technologies) or Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Life Technologies) was used as the secondary antibody at a 1:1000 dilution. F-actin was stained with a 1:400 dilution of Alexa Fluor 546 conjugated phalloidin (Life Technologies). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kumamoto, Japan). The samples were examined by fluorescence microscopy (BX-900; Keyence, Osaka, Japan). More information about the antibodies is presented in Supplementary Table S1.

The nuclear/cytoplasmic area ratio was determined using the ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) software and images of F-actin and DAPI staining. The cell borders determined by F-actin staining were manually traced using ImageJ, and the cytoplasmic area was measured. Likewise, the nuclear area determined by DAPI staining was manually traced using ImageJ and measured. The average of the nuclear area/cytoplasmic area for 10 cells was used as the nuclear/cytoplasmic area ratio.

β-Galactosidase Senescence Staining

HCECs were seeded at a density of 1 × 10^4 cells per well in 48-well culture plates coated with laminin E8 fragments. The cells were cultured for 24 hours, using the Senescence Detection Kit (Merck Millipore), according to the manufacturer’s instructions. Briefly, cultured HCECs were washed with PBS once and fixed with the fixative solution for 10 minutes. The samples were then washed twice with PBS and stained for SA-β-gal with a staining solution mix at 37°C. Cells (both SA-β-gal–positive cells and all cells) that showed the whole area of the nucleus in the images were counted manually in triplicate images for each passage (passages 3 to 5) using ImageJ software. The average from three images was used to calculate the percentage of SA-β-gal–positive cells.

Immunoblotting

Cultured HCECs were washed with ice-cold PBS and lysed with ice-cold RIPA buffer containing phosphatase inhibitor cocktail 2 (Sigma-Aldrich Corp.) and protease inhibitor cocktail (Roche Applied Science). Samples were centrifuged and the supernatant containing the proteins was fractionated by SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% nonfat dry milk. The samples were incubated overnight at 4°C with the primary antibodies: p16 (1:1000; BD Biosciences), p21 (C-19) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p38 MAPK (Thr180/Tyr182) (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), p38 MAPK (1:1000; Cell Signaling Technology), phospho-ATF-2 (Thr71) (1:1000; Cell Signaling Technology), ATF-2 (1:1000; Merck Millipore), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Medical & Biological Laboratories Co., Ltd., Aichi, Japan). The blots were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000;
GE Healthcare, Piscataway, NJ, USA), developed with luminal for enhanced chemiluminescence using the highly sensitive luminal-based chemiluminescence assay kits for Western blotting (Chemilumi One Ultra; Nacalai tesque, Kyoto, Japan), and documented with an Amersham Imager 600 (GE Healthcare) for high-resolution digital imaging of protein in membranes. Molecular weight markers (Bio-Rad, Hercules, CA, USA) were used alongside all samples. More information about the antibodies is presented in Supplementary Table S1. The relative density of the immunoblot bands was determined using Image J software. Relative fold differences were compared with the control values.

Membrane Blotting Array

The release of cytokines and chemokines was measured using the Proteome Profiler Array (Human cytokine arrays Panel A; R&D Systems, Inc., Minneapolis, MN, USA), according to manufacturer’s protocol. Briefly, after culturing HCECs with or without SB203580 for 6 weeks, the culture medium was replaced with a fresh medium and further cultured for 72 hours. The culture medium was then recovered and centrifuged. Human cytokine array detection antibody cocktail was added to the recovered culture medium and incubated at room temperature for 1 hour. Membranes were blocked with blocking reagents, and incubated with sample overnight at 4°C. The membranes were washed and then streptavidin-HRP and chemiluminescent detection reagents were added. The data were analyzed by measuring the pixel density in each spot of the array using the LAS4000S (GE Healthcare) cooled charge-coupled-device camera gel documentation system.

Quantitative Real-Time PCR

Gene expression levels were analyzed using TaqMan real-time PCR. Total RNA was extracted from the corneal endothelium from cultured HCECs using the RNeasy Mini Kit (250) (QIAGEN, Hilden, Germany), and cDNA was synthesized with ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). TaqMan probes for IL-6, Hs00985639_m1; IL-8, Hs00174105_m1; CCL2, Hs00234140_m1; CXCL1, Hs00236937_m1; CXCL10, Hs01124252_g1; MIF, Hs00236988_g1; and GAPDH, Hs00266705_g1 were used. The PCR was performed using the StepOne (Thermo Fisher Scientific, Inc.) real-time PCR system. GAPDH was used as an internal standard.

Enzyme-Linked Immunosorbent Assay

The amount of IL-6 and IL-8 in the supernatants of HCECs was determined by Human DuoSet ELISA kit (R&D Systems), according to the instructions of the manufacturer. Briefly, samples were collected using the same protocol as previously described for the membrane blotting array. A 96-well plate, precoated with a monoclonal antibody specific for human IL-6 or IL-8, was covered with the diluted capture antibody overnight at room temperature. The plate was washed with wash buffer three times and blocked with 1% BSA for 1 hour. After BSA removal, samples and standards were pipetted into the wells and incubated for 2 hours at room temperature. After washing the well, an enzyme-linked polyclonal antibody specific for human IL-6 or IL-8 was added to the wells and incubated for 2 hours at room temperature. The reagent was removed from the well and a substrate solution added. The absorbance was measured at 450 nm and the concentrations were determined by interpolation from a standard calibration curve.

Statistical Analysis

The statistical significance (P value) of the mean values for two-sample comparisons was determined with the Student’s t-test. The statistical significance for the comparison of multiple sample sets was determined with the Dunnett’s multiple-comparisons test. All data represent the mean ± SE.

RESULTS

Effect of a p38 MAPK Inhibitor on Cell Density and Functional Phenotype of HCECs

We investigated the effect of p38 MAPK inhibitor on endothelial phenotypes using 10 human donor corneas to establish 10 respective HCECs cultures (Table). Phase-contrast microscopy showed a contact-inhibited monolayer of hexagonal endothelial cells both in control and SB203580-treated cells (Fig. 1A; Supplementary Fig. S1). Of interest, HCECs were smaller in size when cultured with SB203580 than without SB203580. The mean cell density was significantly higher for HCECs treated with SB203580 than without SB203580 (2623 ± 657 cells/mm² and 1752 ± 628 cells/mm², respectively) (Fig. 1B). The HCECs cultured with SB203580 expressed ZO-1, N-cadherin, and Na⁺/K⁺-ATPase in the plasma membrane, along with F-actin in the cytoskeleton of the polygonal cells, whereas the control HCECs cultured without SB203580 showed a greatly altered staining pattern for these marker proteins. F-actin was observed in the cortex of the cells cultured with SB203580, whereas cortical F-actin distribution was irregular in the control HCECs (Fig. 1C). In addition, long-term cultivation of HCECs showed that cells maintained a higher cell density when cultured with SB203580 than without SB203580 throughout the 18 months (Fig. 2).

Effect of a p38 MAPK Inhibitor on Senescence of HCECs

A decrease in cell density typically accompanies senescence in clinical settings. Therefore, we examined if culture stress might induce cellular senescence in HCECs and we further tested if the p38 MAPK signaling pathway is involved in this cellular senescence. The percentage of SA-β-gal-positive cells was significantly lower in HCECs treated with SB203580 than in control cells (25.2% and 64.9% [passage 3], 25.5% and 71.3% [passage 4], and 35.2% and 63.1% [passage 5], respectively) (Figs. 3A, 3B). The ratio of nuclear/cytoplasmic areas was significantly higher in HCECs cultured with SB203580
than in control cells (0.22 and 0.10 [passage 3], respectively) (Fig. 3C). We evaluated the expression level of p16 and p21, as these inhibitory proteins are associated with senescence. Western blotting analysis demonstrated that the expression of p16 and p21 were reduced in the cells cultured with SB203580, whereas HCECs expressed high levels of p16 and p21 in the absence of SB203580 (Figs. 3D, 3E). Taken together, HCECs exhibit a senescent phenotype under culture conditions and inhibition of p38 MAPK signaling counteracts cellular senescence.

**Suppression of the Senescence and Senescence-Associated Secretory Phenotype (SASP) by a p38 MAPK Inhibitor**

Acquisition of an SASP, which includes secretion of inflammatory, growth-regulating, and tissue-remodeling factors, is one of the typical phenotypes of various senescent cell types. We evaluated the expression of 36 cytokines and chemokines related to SASP in culture medium cultured with or without SB203580 for 72 hours. The expression of six cytokines and chemokines (CXCL10, CCL5, CXCL1, IL-8, CCL2, and IL-6) was lower in HCECs treated with SB203580 than in control cells, whereas the expression of MIF was higher than in the control cells (Fig. 4A; Supplementary Fig. S2).

We further examined the expression levels of these cytokines by quantitative PCR (Figs. 4B–G). The expression levels of IL-6, IL-8, CCL2, and CXCL1 were significantly downregulated in HCECs treated with SB203580 when compared with the control cells. The expression of CXCL10 at the protein level was suppressed, as shown in Figure 4A; however, the expression level of the CXCL10 transcript was not statistically reliable (Fig. 4F). In addition, ELISA showed that the expression of IL-6 and IL-8 in culture medium was significantly downregulated by SB203580 (Figs. 4H, 4I). Thus, inhibition of p38 MAPK signaling pathway in HCECs counteracts the acquisition of SASP due to the culture conditions.
The Role of the p38 MAPK Signaling Pathway in HCECs

The p38 MAPK inhibitor blocked cellular senescence; therefore, we tested whether activation of the p38 MAPK signaling pathway was associated with cellular senescence in HCECs. Western blotting showed that anisomycin upregulated the phosphorylation of p38 MAPK and ATF-2, suggesting that anisomycin activated the p38 MAPK signaling pathway (Figs. 5A, B). HCECs treated with anisomycin exhibited a hexagonal and monolayer morphology, but the cell size was larger than the control cell size (data not shown). The cell density was significantly lower for the HCECs treated with anisomycin than for the control cells (Fig. 5C). Expression of p16 and p21 was greatly upregulated by anisomycin, whereas SB203580 was able to block this effect of anisomycin (Figs. 5D–F). Taken together, the p38 MAPK inhibitor, as shown in the schematic presentation in Figure 5G, is able to block the culture stress and the induction of p38 MAPK signaling-mediated senescent phenotypes.

Discussion

Since 2013, we have been performing a clinical trial of injection of a combination of cultured HCECs and a ROCK inhibitor as a treatment for corneal endothelial dysfunction. However, the in vitro expansion of HCECs proved surprisingly difficult and we often had an insufficient number of cells with adequate cell quality for clinical use. HCECs are difficult to maintain under culture conditions, and even if they survive, they show limited proliferative ability and undergo massive fibroblastic transformation with loss of functional phenotypes. Consequently, our research group and others have continuously strived to develop a successful culture method. For instance, we reported the effect of a ROCK inhibitor and conditioned medium obtained from good manufacturing practice (GMP)-grade human bone marrow-derived mesenchymal stem cells that enhanced HCEC proliferation. We also showed that activation of TGF-β signaling causes the fibroblastic transformation in HCECs, and that a TGF-β signaling inhibitor enables HCECs to avoid fibroblastic changes and maintain the HCEC phenotype.

One remaining challenge is that HCECs tend to decrease their cell density, especially after several cell passages, leading to failure to obtain sufficient quantities of cells. The CEC density is the most important indicator for corneal endothelial health in clinical settings, so low cell density has become a concern with respect to transplanting cultured HCECs in humans. Recently, we have demonstrated that transplanted CECs of low cell density could regenerate corneal endothelium, but the cell density of the regenerated corneal endothelium was lower than that of the eye transplanted with high cell density CECs in a rabbit corneal endothelial dysfunction model. These results suggested that obtaining high cell density CECs is essential for good prognosis after tissue engineering therapy.

Senescence, as defined by Hayflick, is a loss of replicative ability due to the shortening of telomeres. A later study showed that senescence is induced by exhaustion of replicative capacity as well as by stress and oncogenes. Cellular senescence is heterogeneous and cell-type dependent, no single biomarker can identify cellular senescence. We used several biomarkers in the present study to show that the cell density drop in HCECs is a phenotypic feature of cellular senescence. We demonstrated that low cell density HCECs, which were cultured without a p38 MAPK inhibitor, showed high positive SA-β-gal staining, a low nuclear-cytoplasm ratio, and high expression of p16 and p21. SA-β-gal is the biomarker most frequently used to show upregulation of the lysosome mass induced by the loss of capabilities to respond to cellular damage and stress. However, the sole use of SA-β-gal as marker often yields false-positive results, necessitating the use of a combination of several markers. We evaluated cell size, because membrane lipid composition and membrane biophysical properties are altered by cellular senescence, resulting in an alteration of cell size. In addition, cyclin dependent kinase inhibitors (CKIs), such as p16 and p21, have been used as biomarkers, because cell cycle arrest is recognized as a hallmark of cellular senescence. Indeed, overexpression of p21 promotes cellular senescence, and downregulation of...
p21 in senescent cells restores replicative capacity. The expression levels of p16 and p21 in the corneal endothelium were higher in older donors than in young donors, suggesting that these CKIs might be suitable biomarkers. Collectively, the results showing that low cell density HCECs exhibited multiple biomarkers of senescence indicated that the cell density drop during cell culture is induced by cellular senescence.

Consistently, we also showed that the low cell density HCECs exhibited SASP. The SASP phenomenon has been observed in various cell types, such as aged human fibroblasts and epithelial cells, and it is recognized as a robust marker of senescence. The secreted factors include inflammatory mediators, growth factors, and detached cell surface molecules, but SASP is cell-type and senescence-stage dependent. Here, we demonstrated that IL-6, IL-8, CCL2, and CXCL1 were expressed at high levels in low cell density HCECs. This finding suggests that these molecules are markers of SASP in HCECs and that they could be useful quality control markers in regenerative medicine, as they provide a contact-free and nondestructive evaluation.

In the current study, we showed that inhibition of the p38 MAPK signaling pathway counteracted the cell density drop observed during cell culture by suppressing senescence. In addition, activation of p38 MAPK signaling mediated by anisomycin induced the senescence associated with the cell density drop. Iwasa and his colleagues induced four different types of senescence: Ras-induced, replicative, oxidative stress–induced, and culture shock–induced senescence, and they demonstrated that p38 MAPK is a senescence-executing molecule. In addition, inhibition of p38 MAPK signaling counteracted the senescence program. Recently, other researchers have shown that inhibition of p38 MAPK decreased p16 levels and restored replicative capacity in aged HCECs. This finding suggests that these molecules are markers of SASP in HCECs and that they could be useful quality control markers in regenerative medicine, as they provide a contact-free and nondestructive evaluation.
muscle stem cells. We showed that these antisenescent effects of inhibition of p38 MAPK signaling are consistently shown in HCECs, and could be applicable in tissue engineering therapy.

In conclusion, activation of p38 MAPK signaling due to culture stress may be a causative factor that induces cellular senescence, whereas the use of a p38 MAPK inhibitor can counteract this senescence and allow the generation of

![Figure 4](image)

**Figure 4.** Suppression of the senescence and the SASP by a p38 MAPK inhibitor. (A) Expression of 36 cytokines and chemokines in HCECs (passage 0) cultured with or without SB203580. Representative data from experiments using three HCEC cultures derived from three independent donor corneas are shown. (B–G) Expression of the cytokines and chemokines that differ in HCECs (passage 1) cultured with and without SB203580, evaluated by quantitative PCR. The experiments were performed in duplicate using six HCEC cultures established from six independent donor corneas. *P < 0.05, **P < 0.01. (H, I) ELISA evaluation of IL-6 and IL-8 in the culture medium derived from HCEC cultures established from four independent donor corneas (passage 0) cultured with or without SB203580. *P < 0.05, **P < 0.01.
FIGURE 5. The role of p38 MAPK signaling pathway on HCECs. (A) Western blot analysis of the expression of phosphorylated p38 MAPK, p38 MAPK, phosphorylated ATF-2 and ATF-2 in HCECs (passage 15) treated with or without 10 μM anisomycin. Representative data from triplicate experiments using three HCEC cultures established from three independent donor corneas are shown. (B) Expression levels of phosphorylated p38 MAPK, p38 MAPK, phosphorylated ATF-2, and ATF-2 were evaluated by Western blotting and assessed by densitometry. *P < 0.05. (C) Cell density of HCECs (passage 15) treated with or without anisomycin (0.001 μM, 0.01 μM, 0.1 μM) with or without SB203580 (10 μM). Representative data from triplicate experiments using three HCEC cultures established from three independent donor corneas are shown. *P < 0.05, **P < 0.01. (D) Western blot analysis of the expression of p16 and p21 in HCECs (passage 11) treated with or without anisomycin (0.01 μM, 0.1 μM) with or without SB203580 (10 μM). Representative data from triplicate experiments using four HCEC cultures established from four independent donor corneas are shown. **P < 0.01. (E, F) Expression levels of p16 and p21 were evaluated by Western blotting and assessed by densitometry. **P < 0.01. (G) A model of the involvement of p38 MAPK signaling pathway in cellular senescence of HCECs under culture conditions, showing how SB203580 maintains a nonsenescent phenotype.
sufficient numbers of HCECs for tissue engineering therapy for treatment of corneal endothelial dysfunction. Furthermore, the antisenescent effects of p38 MAPK inhibitors may be worth evaluating in different cell types in other settings of regenerative medicine.

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