Original Paper

LY2109761, Transforming Growth Factor β Receptor Type I and Type II Dual Inhibitor, is a Novel Approach to Suppress Endothelial Mesenchymal Transformation in Human Corneal Endothelial Cells

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Background/Aims: Preventing undesirable endothelial-mesenchymal transformation (EnMT) with repetitious *in vitro* expansion of human corneal endothelial cells (CECs) is a pivotal issue in cornea regeneration. Previous studies have shown that inhibition of the TGF- β pathway reduces epithelial-mesenchymal transformation. However, its potential role in EnMT remains poorly understood. As such, the effect of LY2109761, a novel TGF- β receptor type I and type II dual inhibitor, was investigated on EnMT. **Methods:** CECs cultured with various concentrations of LY2109761 were evaluated for their growth rate and phenotype. Additionally, the expression of functional markers (sodium-potassium pump Na⁺/K⁺-ATPase and the tight junction protein ZO-1) and mesenchymal markers (CD73, fibronectin, and vimentin) was detected using immunostaining and western blot. The mRNA expressions were also assayed by real-time polymerase chain reaction analysis. **Results:** At a 1 μ M concentration, LY2109761 did not influence the proliferation of CECs and subsequent experiments were therefore performed using this concentration. Furthermore, CECs cultured in the presence of 1 μ M LY2109761 maintained their ability to grow as a monolayer of hexagonal-shaped cells. The expression of functional markers increased in LY2109761-treated CECs, while the expression of mesenchymal markers decreased (both in protein and mRNA levels). **Conclusion:** Inhibition of TGF- β receptor type I and type II by LY2109761 maintained the phenotype of CECs and inhibited the EnMT process. These results indicate the possible continuous *in vitro* expansion of CECs with normal function.

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Introduction

The corneal endothelium layer maintains corneal transparency by balancing its hydration through barrier and pump functions [1, 2]. Damage of the hexagonal-shaped corneal endothelial cells (CECs) can cause severe visual impairment and can even lead to blindness [3]. As no pharmaceutical intervention has yet been introduced into clinical settings, the only treatment available is corneal transplantation, which includes penetrating keratoplasty and endothelial keratoplasty. However, both transplantations require grafts containing functional CECs of high density, which is highly restricted by the shortage of donor corneas. Hence, successful engineering of surgical grafts with functional CECs will have a significant impact on the treatment of corneal blindness.

In 2015, we demonstrated that CECs isolated from discarded human corneal tissues could form a monolayer on a scaffold and manifest normal physiological function [4]. We also found that CECs were vulnerable to morphological fibroblastic change *in vitro* and eventually underwent massive endothelial-mesenchymal transformation (EnMT) and lost function-related marker expression after long-term culture or multiple subculture during expansion. Thus, the crucial goal is to prevent these undesirable cellular changes, so that the continuous *in vitro* expansion of CECs from discarded tissues can be achieved.

Recently, studies have suggested that the TGF- β signaling pathway might be an important pathway involved with the fibroblastic change of CECs [5, 6]. Furthermore, additional studies have also shown that CECs were prone to acquire fibroblastic phenotypes in response to TGF- β stimulation, and that blocking the TGF- β signaling pathway maintained the cell morphology [6-9]. LY2109761 is. Previous studies have showed that LY2109761, a novel TGF- β receptor type I (T β RI) and type II (T β RII) dual inhibitor, could effectively reverse the epithelial-to-mesenchymal transition (EMT) *in vitro* as well as *in vivo* [10, 11]. In the present study, we investigated the potential role of LY2109761 on EnMT *in vitro* by evaluating LY2109761-treated CECs for their proliferation, morphology, and function-associated marker expression.

Materials and Methods

Ethical statement

Discarded cornea tissues were obtained from the Department of Ophthalmology, Shanghai General Hospital. This study was performed in accordance with the tenets of the Declaration of Helsinki and approved by the ethics committee of Shanghai General Hospital. For all donors, written consent was obtained from the next of kin regarding eye donation for research.

Cell culture

A total of six human donor corneoscleral rims were used for the cultivation of CECs based on the protocol described previously [4]. Briefly, the limbal Descemet's membrane was scraped and immersed in 0.2% collagenase II (278 U mg⁻¹, Worthington Biochemical Co., Lakewood, NJ, USA), which was dissolved in endothelial growth medium-2 (EGM-2; Lonza, Walkersville, MD, USA; Catalog No CC-4176), for 1 h at 37°C. The cells were then released by gently pipetting the dissolved membrane with a Pasteur pipette, followed by resuspending the cell pellets in TryPLE Express (Invitrogen, Camarillo, CA, USA) for 5 min at 37°C. After that, the cells were resuspended and seeded in 6-well tissue-culture plates pre-coated with FNC Coating Mix (Athena Environmental Sciences, Baltimore, MD, USA). The culture media was supplemented with 0.1% epidermal growth factor (EGF), 0.1% vascular endothelial growth factor (VEGF), 0.4% fibroblast growth factor (FGF), 0.1% insulin-like growth factor (IGF), 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, 5 μ g/ml gentamicin, 0.25 μ g/ml amphotericin-B and 2% FBS. The culture medium was changed every 3 days. Cells were sub-cultured by treating with TryPLE Express upon reaching confluency. Passages 2 to 5 were used for all experiments.

Treatment of TGF- β receptor inhibitor in CECs

LY2109761 (Selleck, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted to 0.1, 1 and 10 μ M with EGM-2 [DMSO final concentration, 0.1% (v/v)]. The CECs were passaged at a ratio of 1:2 with culture medium supplemented with or without LY2109761 (0.1, 1, and 10 μ M). LY2109761 was only added to the medium when the cells were passaged. After 3 to 5 days, cell proliferation and gene and protein expression were evaluated as described below.

Cell proliferation

CECs were seeded at a density of 130 cells/mm² and cultured for up to 4 days with or without LY2109761 (0.1, 1, and 10 μ M). Cell proliferation was determined at day 1, 2 and 4 post-seeding with the use of CyQUANT[®] NF Cell Proliferation Assay Kit (Invitrogen, Camarillo, CA, USA). Cell proliferation was measured by the intensity of fluorescence detected from each sample with a fluorescence microplate reader at an excitation of 485 nm and an emission detection of 530 nm. All the samples were tested in triplicate, and the results expressed as a proliferation curve.

Characterization and immunostaining of CECs

Cell morphology was evaluated by using an inverted light microscope (Olympus IX-71, Tokoyo, Japan). The CECs were further characterized for function-related markers, including Na⁺/K⁺-ATPase (a pump function associated protein [12]), and zona occludens-1 (ZO-1, a tight junction associated protein [13]). Changes of the EnMT were also evaluated by investigating CD73 (a marker to determine the fibroblastic phenotype of CEC [14]), and fibronectin [15, 16] and vimentin [17, 18] (two mesenchymal markers). Briefly, the cultured cells were first fixed with cold methanol for 20 min. After blocking with Protein Block Serum-Free solution (DAKO, Carpinteria, CA, USA), the cultured cells were incubated overnight with the following primary antibodies: anti-Na⁺/K⁺-ATPase (1:300 dilution, Millipore), anti-ZO-1 (1:150 dilution, Invitrogen), anti-CD73 (1:300 dilution; BD Pharmingen Stain Buffer), anti-fibronectin (1:200 dilution, Sigma-Aldrich), and anti-vimentin (1:100 dilution; Santa Cruz). Fluorescein isothiocyanate (FITC) conjugated antibodies (1:200 dilution, Vector Laboratories, Burlingame, CA) were used as the secondary antibodies.

Western blot analysis

Cells treated with and without LY2109761 (0.1 and 1 μ M) were washed with phosphate buffered saline (PBS) and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Thermo Fisher, Rockford, IL, USA) containing 1% phenylmethyl-sulfonyl fluoride (PMSF). The protein concentrations of the samples were then assessed with the BCATM Protein Assay Kit (Takara Bio Inc., Otsu, Japan). Cell lysates (containing each an amount of 30 μ g protein) were separated by 10 % SDS-PAGE gels electrophoresis and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was then blocked in Tris-buffered saline/Tween20 (TBST) buffer containing 5% non-fat milk powder for 2 h at room temperature. The membranes were then probed overnight at 4 °C with the following primary antibodies: anti-Na⁺/K⁺-ATPase, anti-ZO-1, anti-CD73, anti-fibronectin, and anti-vimentin. Furthermore, the primary antibodies anti-phosphorylated Smad2 (Cell Signaling Technology), anti-phosphorylated Erk1/2 (BD), and anti-phosphorylated p38 (BD) were used to determine the inhibition effect of LY2109761 on the TGF- β signaling pathway (0.1 and 1 μ M LY2109761-treated samples). For all samples, anti-GAPDH was used as a housekeeping gene. After incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature, protein signal was detected with the ECL chemiluminescent detection system (Bio-Rad). All sample protein values were normalized with the GAPDH expression.

Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from CECs using Trizol (Invitrogen). The cDNA was synthesized with the PrimeScript[®] RT reagent Kit (Perfect Real Time) (Takara Bio Inc., Toyobo, Osaka, Japan). Gene transcription levels were quantified using the SYBR[®] Premix Ex Taq[™] (Perfect Real Time) (Takara Bio Inc., Toyobo, Osaka, Japan) according to the manufacturer's recommended cycling conditions. The specific primers used were listed as follows:

GAPDH, forward: 5'- GGAGCGAGATCCCTCCAAAT-3'

reverse: 5'- GGCTGTTGTCATACTTCTCATGG-3'

Na⁺/K⁺-ATPase, forward: 5'-CTTCCTCCGATTTATGCTCATTTTCTCACCC-3'

reverse: 5'-GGATGATCATAAACTTAGCCTTGATGAACTC-3'

ZO-1, forward: 5'-GGACGAGGCATCATCCCTAA-3'

reverse: 5'-CCAGCTTCTCGAAGAACCAC-3'

CD73, forward: 5'- CTCCTCTCAATCATGCCGCT-3'

reverse: 5'- CATCAATGGGCGACCGGATA-3'

Fibronectin, forward: 5'- GAGGCACCACGAGAAGTGAC -3'

reverse: 5'- GCACTCCAGTGTGAGGGTTT-3'

Vimentin, forward: 5'-GGACCAGCTAACCAACGACA-3'

reverse: 5'-AAGGTCAAGACGTGCCAGAG-3'

GAPDH was used as an endogenous control. All the samples were detected in triplicate for each experiment. All results shown are representatives of three independent experiments. Expression levels of mRNA were calculated with the delta-delta Ct method ($2^{-\Delta\Delta C_t}$).

Statistical analysis

Data analyses were performed by the SPSS software (Version 13.0, Chicago, IL, USA). Statistical significance between two groups was determined by using the Student's t-test. The statistical significance in the comparison of multiple groups was carried out using the one-way analysis of variance. Additionally, comparisons between each two groups were made by least significant difference (LSD) analysis. All values were expressed as the mean $\pm$ standard deviation (SD). P values ≤ 0.05 were considered statistically significant.

Results

Cell growth of CEC cultures and inhibition of the TGF- β signaling pathway with and without LY2109761

The growth rates of CECs cultured with LY2109761 (0.1, 1, and 10 μ M) and without (control group) were evaluated and compared to each other (Fig. 1). Two days after seeding, there were significantly more CECs for the 1- μ M LY2109761 group compared to the control group ($P = 0.044$) but no difference thereafter. For the 10- μ M LY2109761 group, significantly less CECs were found 4 days after seeding compared to the control group ($P = 0.019$). Next, western blot analysis showed that phosphorylation of Smad2, Erk1/2, and p38 decreased in cells cultured with LY2109761 in a concentration-dependent manner compared to cells cultured without (Fig. 2). Overall, LY2109761 did not influence CECs the proliferation of CECs at a 1- μ M concentration but did inhibit the TGF- β signaling pathway. Therefore, for additional experiments, CECs were cultured with 1 μ M of LY2109761 to evaluate its effect on the EnMT process.

Fig. 1. The effect of TGF- β signaling inhibition on CECs proliferation. The CECs were cultured with the TGF- β inhibitor LY2109761 in concentration groups of 0.1, 1, and 10 μ M or without as a control (DMSO only). The cell number of each treatment was measured by a fluorescence-based cell proliferation assay on days 1, 2, and 4. Each concentration group was tested in three independent experiments. The mean value of the fluorescence signal of each group is shown with SD as error bar (*, $P < 0.05$ compared with control group).

LY2109761 at a 1 μ M concentration inhibits EnMT in human CECs

Firstly, the phenotypes of CEC cultures treated with and without LY2109761 (1 μ M) in the culture medium were examined. Representative microscopic images of CECs cultured with and without LY2109761 at passages 3 and 5 are shown in Fig. 3. During the whole culture period, CECs cultured in the presence of LY2109761 maintained their ability to grow as a monolayer of hexagonal-shaped cells, while the control CECs gradually exhibited the fibroblastic morphology after subsequent subcultures. Secondly, to confirm EnMT inhibition effect of LY2109761 in human CECs, the expression of functional proteins and fibroblastic proteins were evaluated by immunostaining. The results showed an increased expression for both the sodium-potassium pump Na⁺/K⁺-ATPase and the tight junction protein ZO-1 in the LY2109761-treated (1 μ M) CECs (Fig. 4). In

contrast, the expression of mesenchymal proteins CD73, fibronectin and vimentin decreased in the LY2109761-treated CECs (Fig. 5). Together, these data indicated that inhibition of T β RI and T β RII by LY2109761 maintained the polygonal monolayer properties of CECs and decreased the EnMT process.

Moreover, western blot analysis of CEC cultures treated with and without 1 μ M of LY2109761 was performed (Fig. 6). Consistent with the immunostaining data, the results showed a 2.03-fold upregulation of the Na⁺/K⁺-ATPase protein level and a 1.78-fold upregulation of the ZO-1 protein level in the LY2109761-treated CECs compared to the control. These results confirmed that LY2109761-treated CECs were associated with a significantly higher expression of function-related proteins. Also, 1 μ M LY2109761 significantly downregulated the expression of fibronectin, CD73, and vimentin by 1.70-fold, 2.69-fold, and 2.71-fold, respectively.

Fig. 2. The inhibition effect of LY2109761 on the TGF- β signaling pathway. The CECs were cultured with LY2109761 (0.1 and 1 μ M) and without (control). Phosphorylation of Smad2, p38, and Erk1/2 was decreased by LY2109761 in a concentration-dependent manner. GAPDH was used as an endogenous control.

Fig. 3. In vitro culture of CECs at different passages. Representative light microscopy images were taken of CECs cultured with 1 μ M LY2109761 and without (control) at culture passages 3 and 5 (scale bar = 100 μ m).

Fig. 4. Characterization of function-related markers by immunostaining. CECs cultured with 1 μ M LY2109761 and without (control) were immunostained with antibodies against Na⁺/K⁺-ATPase and ZO-1 (both in green).

Cells at passage 4 were used for all the stainings. Immunostaining was performed 2 days after the cells had reached confluency (scale bar = 25 μ m).

Fig. 5. Investigating of changes of the EnMT by immunostaining. CECs cultured with 1 μ M LY2109761 and without (control) were immunostained with antibodies against CD73 (in green), fibronectin (in red), and vimentin (in red). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). Cells at passage 5 were used for the fibronectin staining, and the result showed that cells cultured without LY2109761 had fibroblastic cell shape. Cells at passage 3 were used for all the other stainings. Immunostaining was performed 3 days after seeding (scale bar = 25 μ m).

Fig. 6. Western blot analysis of CECs treated with 1 μ M of LY2109726 and without (control). The expression of functional-markers Na⁺/K⁺-ATPase and ZO-1, as well as mesenchymal markers CD73, fibronectin and vimentin, was assessed at protein level. The relative expression of proteins was calculated by using GAPDH as an endogenous control for normalization, followed by normalizing the protein levels in the control group to 1.0. Each group was tested in three independent experiments. The mean value of relative protein expression of each group is shown with SD as error bar (*, P<0.05; **, P<0.01). For all experiments, CECs at passage 4 were used 2 days after having reached confluency.

Fig. 7. Real time PCR analysis of CECs. CECs were cultured with 1 μ M LY2109726 or without (control). The expression of functional-markers Na⁺/K⁺-ATPase and ZO-1, as well as mesenchymal markers CD73, fibronectin and vimentin, was assessed at RNA level for both treatment groups. The relative expression of proteins was calculated by using GAPDH as an endogenous control for normalization, followed by normalizing the protein levels in the control group to 1.0. Each group was tested in three independent experiments. The mean value of relative RNA expression of each group is shown with SD as error bar (*, P<0.05; **, P<0.01). For all experiments, CECs at passage 3 were used 2 days after having reached confluency.

To further confirm the expression change of the indicated markers, real-time PCR analysis was employed to evaluate the mRNA levels. For functional proteins, a 1.55-fold increase for Na⁺/K⁺-ATPase and a 2.34-fold increase for ZO-1 were found. For mesenchymal proteins, a 1.78-fold decrease for fibronectin, a 1.31-fold decrease for CD73, and a 1.39-fold decrease for vimentin were found (Fig. 7). Taken together, these data supported that LY2109761 at 1 μ M could effectively block the EnMT.

Discussion

In the past decade, corneal transplantation has improved greatly. Advances in lamellar keratoplasty have made it possible to transplant only posterior corneas for corneal endothelial diseases, collectively termed “endothelial keratoplasty” [19, 20]. Along with technique improvements, more conditions have become treatable, and the demand for corneal tissue has therefore increased [21]. However, the number of donated corneas for transplantation has not increased along with demands [22, 23]. For this reason, there has become a need for tissue-engineered substitutes that can overcome this scarcity. For this reason, the establishment of an efficient cultivation technique for functional CECs is an essential and inevitable part for the tissue engineering of endothelium [24].

The role of TGF- β as a key molecule in the development and progression of EMT has been well characterized and studied [25, 26]. Recently, several studies have suggested that TGF- β signaling might also exert a similarly important role regarding the fibroblastic change of CECs [5-9]. TGF- β initiates mesenchymal transformation by binding to T β RI and T β RII, which allows for the constitutively active T β RII kinase to transphosphorylate and activate the T β RI kinase. This activation leads to the propagation of the signal by activating downstream Smad-dependent and Smad-independent pathways [27-30]. Okumura et al. [6] reported that the specific inhibitor to the T β RI was able to maintain CECs in a polygonal phenotype with a higher expression of function-related markers. However, T β RII possesses a tyrosine kinase activity and enables the phosphorylation of the Smad-independent signaling pathway [31]. So, if only T β RI was inhibited, a higher expression ratio of T β RII compared to T β RI would still activate the EnMT via the non-canonical Smad signaling pathway [32]. Therefore, in this study, we employed LY2109761, a dual inhibitor of T β RI and T β RII, to prevent CECs from acquiring fibroblastic phenotypes.

TGF- β regulates a wide range of cellular behavior. Studies have shown that blocking TGF- β resulted in various effects on cell proliferation [33-36]. To test LY2109761's effect on CECs proliferation, we evaluated the growth rates of CECs cultured with and without LY2109761. For CECs cultured with 1 μ M of LY2109761, there were significantly more CECs found compared to the control group on day 2 of post-seeding. Additional days after seeding, however, did not show a significant difference. For CECs cultured with 10 μ M of LY2109761, there were significantly less HCECs found compared to the control group on day 4 of post-seeding. These results indicate that LY2109761, at a concentration of 10 μ M, might influence the normal growth of CECs.

Next, we also found that the phosphorylation of Smad2, p38, and Erk1/2 was decreased by LY2109761 in a concentration-dependent manner. After evaluating the results, we continued with 1 μ M of LY2109761 as the focus point of this study; however, future studies should further determine the most optimal concentration to inhibit EnMT effectively without interfering with the cell growth.

To the best of our knowledge, this currently presented study is the first to focus on the potential effect of the dual inhibitor LY2109761 on EnMT *in vitro*. Our results demonstrated that LY2109761 completely abolished the undesirable cellular changes. For instance, when CECs were treated with 1 μ M of LY2109761, they maintained their authentic polygonal cell shape during continuous subcultures. Furthermore, the characteristic subcellular locations of ZO-1 and Na⁺/K⁺-ATPase were perfectly resumed at the plasma membrane in the 1- μ M LY2109761-treated group. What's more, the expression of these two functional proteins was significantly increased at both the mRNA and protein levels. These results suggest that the barrier and pump function in LY2109761-treated CECs were better preserved than in the control group. Finally, the expression of mesenchymal markers, including fibronectin and vimentin, was significantly reduced in response to the 1- μ M LY2109761 treatment. These results indicate that the EnMT process was effectively inhibited during the cultivation of CECs treated with LY2109761.

Conclusion

In conclusion, LY2109761, a novel T β RI and T β RII kinase selective inhibitor, can inhibit EnMT *in vitro* and enable the continuous expansion of CECs with a normal phenotype and function, which would be an ideal property for the engineering of functional corneal endothelium.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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