Tropical Journal of Pharmaceutical Research March 2023; 22 (3): 493-498
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v22i3.5

Original Research Article

Eriocalyxin B mediates the migration and inflammation of TGF-β2-induced human lens epithelial cells by inhibiting JAK/STAT3 pathway

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Sent for review: 18 December 2022 *Revised accepted: 28 February 2023*

Abstract

Purpose: *To study the role of Eriocalyxin B (EriB) in the migration and inflammation of TGF-β2-induced human lens epithelial cells (hLECs), and to elucidate the molecular mechanisms involved.*
Methods: *The hLECs cultured in vitro were divided into 5 groups, viz, control, TGFβ2, TGFβ2+2 μM EriB, TGFβ2+4 μM EriB, and TGFβ2+8 μM EriB groups. CCK-8, clone formation and Edu labeling assays were performed to assess the effect of EriB on the proliferation of hLECs cells. To determine the role of EriB in cell migration, Transwell and wound healing assays were used. The levels of vimentin, α-SMA, snail, TNF-α, IL-1β, IL-6, P65, p-P65, p-JAK2, JAK2, p-STAT3, STAT3, and β-catenin in hLECs cells were evaluated by enzyme-linked immunosorbent assay (ELISA) and western blot analysis in order ascertain the signaling pathways involved.*
Results: *The rate of cell proliferation significantly decreased in TGFβ2+2μM EriB, TGFβ2+4μM and TGFβ2+8μM groups compared with TGFβ2 group ($p < 0.001$). In addition, the migration of hLECs cells and epithelial mesenchymal transition were inhibited by EriB in a dose-dependent way ($p < 0.001$). ELISA results showed that compared to TGFβ2 group, TNF-α, IL-1β and IL-6 levels in EriB group significantly decreased ($p < 0.001$). The levels of TNF-α, IL-1β, IL-6, p-P65/P65, p-JAK2/JAK2, p-STAT3/STAT3 and metastasis-associated proteins (α-SMA and snail) in hLECs cells were downregulated by EriB ($p < 0.001$). Furthermore, vimentin level was increased by EriB ($p < 0.001$).*
Conclusion: *The results show that EriB inhibits the growth, metastasis and inflammation of hLECs cells by inhibiting JAK/STAT3 pathway, thus indicating that this pathway is a potential therapeutic target for treating cataract*

Keywords: *Eriocalyxin B, Posterior capsular opacification, Janus kinase, Signal transducer, Activator of transcription 3, Transforming growth factor beta 2*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cataract caused by lens opacity leads to serious visual impairment [1], and surgical intervention is the current management strategy for it. However, surgery can still result in cataract recurrence. Following cataract surgery, posterior capsular opacification (PCO) is the most common

postoperative complication [2]. The incidence of PCO in adults is greater than 25 %. PCO is thought to be mainly caused by the activity and epithelial mesenchymal transformation (EMT) of lens epithelial cells (LECs). However, the pathogenesis underlying PCO is still unknown. As a multifunctional growth factor, TGF-β2 plays a central role in cell vitality, proliferation, differentiation, migration, invasion, inflammation and wound healing [3]. As a result, TGF-β2-induced hLEC is a common cell model for studying the pathogenesis of PCO. Chinese medicine has assumed an increasingly important role in the field of ophthalmology. For example, Ganoderic acid A protects lens epithelial cells from UVB radiation and delays lens opacity [4]. Eriocalyxin B (EriB) is a bioactive component extracted from *Isodon eriocalyx var,* which has long been used as an anti-inflammatory treatment in traditional Chinese medicine. Research shows that EriB possesses anti-cancer effects, and can inhibit the proliferation, migration, invasion and other malignant phenotypes of pancreatic cancer, colon cancer and other malignant tumor cells. In terms of anti-inflammation, EriB therapy plays an anti-inflammatory role by inhibiting the differentiation of T helper cells (Th) 1 and Th17 cells, thereby improving experimental autoimmune encephalomyelitis [5]. However, EriB is rarely used in ophthalmology, and its mechanism is unclear. Studies have shown that EriB can effectively slow down the development of PCO and ECM synthesis in the lens capsule of rats by inhibiting the JAK/STAT3 signal transduction [6,7], and promote TGF-β2 activation and ECM synthesis, so as to promote the development of PCO by promoting JAK/STAT3 signal dependence mechanism. This study sought to examine EriB and its ability to mediate the migration and inflammation of human lens epithelial cells.

EXPERIMENTAL

Cell culture

Human lens epithelial SRA01/04 cell line from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences was generously grown in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10 % FBS (Invitrogen, Carlsbad, CA). 10 ng/mL TGFβ2-induced SRA01/04 cells were maintained in an incubator containing 5 % CO_2 at 37 °C.

Cell counting kit 8 (CCK8) assay

CCK8 assay was performed using CCK8 Kit (Dojindo, Shanghai, China). hLECs cells were inoculated separately by adding 0 μM, 2 μM, 4 μM and 8 μM EriB in 96-well plates. After incubation in CCK8 solution (Dojindo Molecular Technologies, Inc) at 37 °C for 2 h according to the manufacturer's protocol, absorbance was measured at a wavelength of 490 nm.

Clone formation assay

The hLECs cells were cultured in each dish and cultured at 37 °C in 5 % CO_2 for 72 h. Then, the medium was replaced with PBS. The cells were fixed for 15 min and then stained with 0.1 % crystal violet. The number of clones were quantified by taking a photograph.

5-Ethynyl-2’-deoxyuridine (EdU) labeling assay

3×10^3 cells taken in logarithmic growth period were seeded into 96 well plates and cultured separately by adding 0, 2, 4 and 8 μM EriB to a density of 60 ~ 70 %. After 48 h of treatment, 100 μL, 50 μM Edu culture medium was added per hole, and after incubation for 2 h, and the remaining steps were followed according to the instructions in the Edu cell proliferation kit (RiboBio, Guangzhou, China), and finally, photos were taken under a fluorescence microscope.

Wound healing assay

The cells in good growth condition were inoculated in 6-well plate for 24 h. 1000 μL lance head was used to draw a straight line in the culture plate to form a cell-free area, and then the cells were cultured separately by adding 0, 2, 4 and 8 μM EriB, and images were collected after incubation at 0 h and 24 h, respectively. The width of the "bare" area of the cell at the top, middle and bottom of the scratch in the image were measured, and its mean value was calculated [8].

Transwell assay

The cells were incubated for 24 h in serum-free medium, digested using trypsin and counted. Transwell chamber (8.0 μM polycarbonate microporous membrane) was added into a 24 well culture plate. Appropriate amounts of cells were added into the upper chamber, and cultured with a medium containing 0.1 % serum. The lower chamber medium contained 10 % serum and 2.5 mg/L fibronectin. After 24 h of treatment by EriB (0, 2, 4, 8 μM), the microporous membrane was taken out, fixed in 90 % ethanol for 10 min, and then the upper layer cells of the membrane were wiped off with a degreased cotton ball. The number of cells attached to the

lower layer of the membrane was counted under an inverted microscope after trypan blue staining. Randomly, 6 visual fields were counted and mean value calculated.

Western blot analysis

Total protein was collected with RIP lysis buffer (Genesent, Shanghai, China) and quantified using the BCA method. The remaining samples were added with loading buffer and boiled for denaturation. The proteins were separated by 10 % SDS-PAGE and then blotted onto a PVDF membrane. After 5 % skimmed milk powder was sealed for 1 h, the primary antibody was incubated overnight, and then the secondary antibody was incubated for 1 h. ECL solution was added for chemiluminescence to detect protein bands, and β-actin was used as the internal reference [9,10].

Enzyme-linked Immunosorbent Assay (ELISA)

TNF- α, IL-1 β, IL-6 kits were provided by Shenzhen Chuanghoyuan Industrial Co. Ltd. (Genzyme, USA). Enzyme-linked immunosorbent assay (ELISA) was performed according to the kit manufacturer's instructions. The cells were inoculated into six-well cell culture plates and cultured for 3 days. The supernatant was collected to determine the concentration of TNF-α, IL-1 β and IL-6 in the supernatant[11], while the cells were digested with Trypsin EDTA and counted under the microscope with cell counting plate. The above tests were repeated 3 times.

Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) and reported as mean ± SD (n = 3) [12]. Significance was set at $p < 0.05$ [13].

RESULTS

EriB treatment reduces the proliferation of hLECs cells

Research assessed the effect of the levels of EriB on hLECs cells proliferation by CCK-8, clone formation and Edu labeling assays. The data in Figure 1 A showed that the absorbance of TGFβ2 group was significantly increased compared to those of NC group ($p < 0.05$), and that EriB inhibits the proliferation of hLECs cells. In addition, clone formation and Edu labeling data also showed similar results that compared with the TGFβ2 group. Also, the rate of cell proliferation significantly decreased in TGFβ2 + 2 μM EriB, TGFβ2 + 4 μM and TGFβ2 + 8 μM groups (Figure 1 B, B1, C and C1, respectively). Thus, EriB significantly inhibited the proliferation of hLECs.

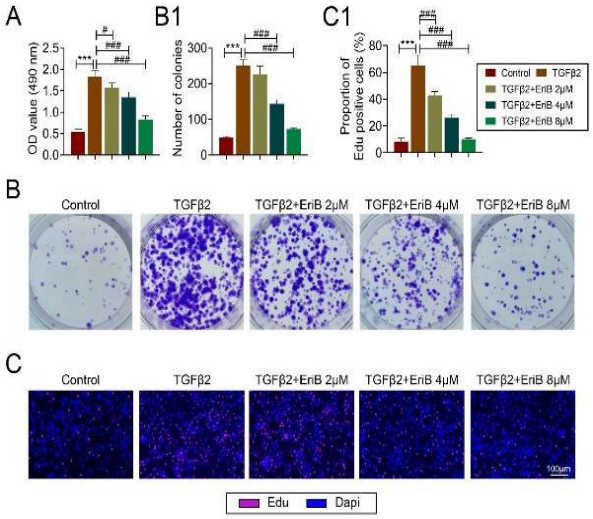

Figure 1: EriB treatment reduces the proliferation of hLECs cells. A: CCK8 migration assay was performed with control, TGFβ2, TGFβ2 + EriB 2 μM, TGFβ2 + EriB 4 μM and TGFβ2 + EriB 8 μM treatments; B: Clone formation assay was used to explore the cell proliferation of the control, TGFβ2, TGFβ2 + EriB 2 μM , TGFβ2 + EriB 4 μM and TGFβ2 + EriB 8 μM. B1, A quantitative analysis of the number of colonies in the hLECs cells; C: EdU-stained cells were measured by EdU labeling assay; C1: A quantitative analysis of the Edu positive cells in the hLECs cells. $^{***}P < 0.001$ vs. control group, $^{\#}p < 0.05$ vs. TGFβ2 group, $^{\#\#\#}p < 0.001$ vs. TGFβ2 group

EriB treatment inhibits TGF β2-induced hLECs cell migration and epithelial mesenchymal transition

To evaluate the effect of EriB on cell migration, transwell, wound healing and western blot assays were conducted. This study found that the migrated surface in TGFβ2 group was significantly higher compared to those in NC group ($p < 0.05$), and EriB significantly increased the migrated surface of the hLECs cells compared to the TGFβ2 group (Figure 2 A and B1). Transwell assay results in Figure 2 B and C1 showed that EriB significantly decreased the number of invaded cells compared to that of TGFβ2 group cells. Figure 2 C shows that the expression of E-cadherin was notably increased in the EriB group, while the expression of Vimentin, α-SMA and snail was significantly decreased ($p < 0.05$). These findings imply that EriB inhibits the activity of hLECs cell and epithelial mesenchymal transition.

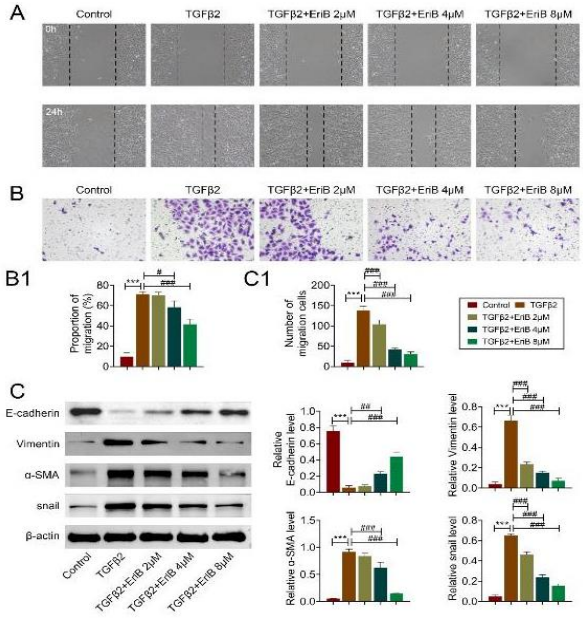

Figure 2: EriB treatment inhibits TGF β2-induced hLECs cell migration and epithelial mesenchymal transition. A: Wound healing assay was performed with control, TGFβ2, TGFβ2 + EriB 2 μM, TGFβ2 + EriB 4 μM and TGFβ2 + EriB 8 μM treatments in 0 h and 24 h; B1: Quantitative analysis of the proportion of migration were shown in each lower panel; B: Cell migration was analyzed by Transwell; C1: A quantitative analysis of migration cells in the hLECs cells; C: Western blot was used to test the levels of E-cadherin, Vimentin, α-SMA and snail. $^{***}P < 0.001$ vs. control group, $^{\#}p < 0.05$ vs. TGFβ2 group, $^{\#\#}p < 0.01$ vs. TGFβ2 group, $^{\#\#\#}p < 0.001$ vs. TGFβ2 group

EriB treatment reduces TGFβ2-induced hLECs cell inflammation

To further determine the effect of EriB on inflammation and related pathways, the TNF-α, IL-1β, IL-6, P65 and p-P65 levels were measured. As shown in Figure 3 A, compared to the TGFβ2 group, TNF-α, IL-1β and IL-6 levels in the EriB group decreased (p<0.05), and treatment with EriB decreased the phosphorylation of P65 (Figure 3 B). Thus, EriB reduced the TGFβ2-induced hLECs cell inflammation.

EriB inhibits JAK/STAT3 pathway

This study also examined the effect of ErtiB treatment on the JAK/STAT3 pathway, to illustrate the regulating mechanism. A significant increase in p-JAK2/JAK2 and p-STAT3 /STAT3 was observed in TGFβ2 + EriB treatment, compared with TGFβ2 alone (Figure 4). Overall, these results demonstrate that JAK/STAT3 pathway contributes to the changes induced by EriB on hLECs cells.

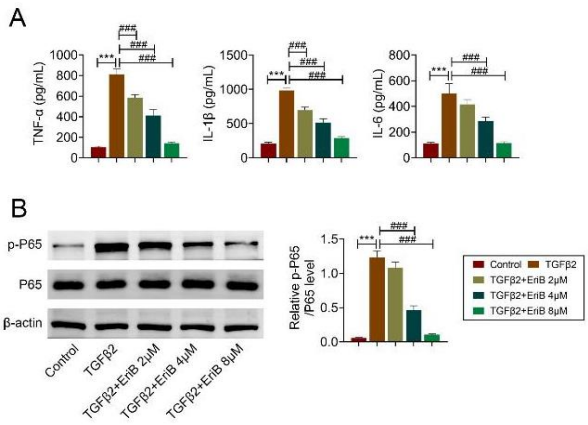

Figure 3: EriB treatment reduces TGFβ2-induced hLECs cell inflammation. A: ELISA was performed with control, TGFβ2, TGFβ2 + EriB 2 μM, TGFβ2 + EriB 4 μM and TGFβ2 + EriB 8 μM treatments; B: Western blot was used to explore the phosphorylation of P65 in control. TGFβ2, TGFβ2 + EriB 2 μM, TGFβ2 + EriB 4 μM and TGFβ2 + EriB 8 μM group. $^{***}P < 0.001$ vs. control group, $^{\#\#\#}p < 0.001$ vs. TGFβ2 group

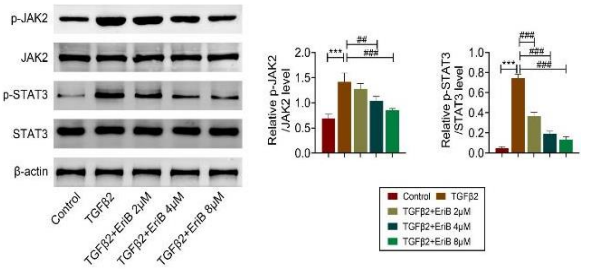

Figure 4: EriB inhibits the JAK/STAT3 pathway. Western blot was used to explore the phosphorylation of JAK and STAT3 in control, TGFβ2, TGFβ2 + EriB 2μM, TGFβ2 + EriB 4 μM and TGFβ2 + EriB 8 μM group. $^{***}P < 0.001$ vs. control group, $^{\#\#\#}p < 0.001$ vs. TGFβ2 group

DISCUSSION

Posterior cataract is the most common long-term complication after phacoemulsification, and is also an important factor affecting the visual quality of patients after surgery. PCO is mainly caused by the proliferation, migration, fibrosis and epithelial mesenchymal trans-differentiation of some residual lens epithelial cells, stimulated by some growth factors and cytokines [14,15]. More and more studies have confirmed that the occurrence and development of PCO is closely related to lens epithelial cells which are induced by various growth factors, among which TGF-β2 is one of the most important growth factors. This study revealed that TGF-β2 induced EMT in RPE cells and participated in the process of proliferative vitreoretinopathy [16]. The application of TGF-β2 induced the upregulation of matrix metalloproteinase expression in rat LEC and the EMT of LEC, and led to the occurrence

of cataract. Many growth factors and inflammatory mediators also participate in the formation of PCO after cataract surgery. It has been reported that residual equatorial LEC secretes TGF-β2 into the aqueous humor in the bag after surgery, while a previous research shows that TGF-β2 plays the predominant role in inducing EMT and activity of LEC [17]. In the present work, TGF-β2 was used to induce SRA01/04 cultured *in vitro*, in order to establish the PCO model. The results indicate an increase in cell proliferation, migration and inflammation.

Snail family proteins are a group of highly conserved zinc finger structure transcription inhibitors. At present, three Snail family proteins have been found: Snail (Snail), Snai2 (Slug) Snai3 (Smuc). In fibrotic diseases, Snail participates in the EMT process of renal tubular epithelial cells, resulting to interstitial renal fibrosis. In ocular fibrosis, Snail is involved in the formation of corneal scar, anterior subcapsular cataract, and proliferative vitreoretinopathy [18]. Snail and Slug binds to E-box sequence near E-cadherin promoter, inhibits E-cadherin transcription, downregulates E-cadherin expression, and reduces cell adhesion attribute. This experiment used Western Blot to observe Snail, α-SMA and E-cadherin expression. This study also confirmed that the expression of α-SMA decreased with increase in EriB concentration. In contrast, E-cadherin expression was up-regulated. The expression of Snail protein also decreased with increase in EriB concentration.

Proliferation, migration and EMT of LECs play an important role in the pathogenesis of many fibrotic lesions, including PCO. TGF- β2 is an important growth factor involved in this process [19]. This study mainly explored the role and mode of the action of EriB in the process of TGF-β2-induced migration and inflammation of human lens epithelial cells, and the relationship between EriB and JAK/STAT3 signaling pathway in the process of inhibiting the development of PCO. It was found that EriB blocked TGF-β2-induced activity and inflammation of human lens epithelial cells. JAK/STAT3 signaling pathway promotes TGF-β2-induced occurrence of EMT in lung cancer. A previous study reported that JAK/STAT3 signal-dependent mechanism is promoted to initiate TGF-β2 activation and ECM synthesis. On the other hand, the inhibition of JAK/STAT3 signal transduction effectively slows down PCO development and ECM synthesis in lens capsule of rats [20].

EriB has long been used in traditional Chinese medicine as an anti-inflammatory treatment. In the present study, a role for EriB has been established in TGF-β2-induced lens epithelial cell proliferation, migration and inflammation, and that EriB plays an inhibitory role in JAK/STAT3 signaling pathway. Therefore, EriB shows some promise as a new specific drug for the prevention and treatment of PCO. But whether it can also achieve ideal results in clinical application, the kind of toxic reactions it may produce, and the right approach in management still need to be further studied.

CONCLUSION

The findings of this study confirm that TGFβ2 induces hLECs cell migration and inflammation by activating JAK/STAT3 signal pathway, and that EriB inhibits the growth, migration and inflammation of hLECs cells by blocking JAK/STAT3 pathway. These findings provided a novel insight into the treatment of patients diagnosed with cataract.

DECLARATIONS

Acknowledgements

This work was supported by General Project of Yunnan Provincial Department of Science and Technology for Joint Chinese Medicine (no. 11372110213) and Health Research Project of Kunming Health Commission (no. 2021-08-01-001).

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaomei Feng designed the study and carried them out;

Xiaomei Feng, Wenjian Hu, Guangjin Wang and Wei Wang supervised the data collection, analyzed the data, interpreted the data; Xiaomei Feng and Wei Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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