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β-cellulin promotes the proliferation of corneal epithelial stem cells through the phosphorylation of erk1/2

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ABSTRACT

The proliferation of corneal epithelial stem cells (CESCs) is a very important process in the recovery of corneal wounds. Recent studies have shown that β-cellulin (BC) is effective in the repair of other tissues. However, its mechanism of action in corneal wound healing is not yet clear. The purpose of this study was to investigate how BC accelerates wound healing of the cornea. Here, we confirmed that the proliferation of CESCs was induced at a specific concentration (0.2, 2 and 20 ng/mL) by treatment with BC. Markers associated with proliferation activity (ΔNp63, bmi-1, abcg2) were also upregulated. *In vivo* experiments showed that the corneal wound healing rate was increased in mice. We found that BC stimulates the phosphorylation of the erk1/2 signaling pathway, which is triggered during the recovery of mouse corneal wounds. However, the inhibition of erk1/2 phosphorylation delayed the recovery of mouse corneal wounds in an organ culture assay. According to these results, BC may be a potential treatment factor for corneal wound healing.

1. Introduction

The cornea is composed of corneal epithelial cells, which are maintained by CESCs. The cornea protects against external infections [1–3]. Corneal epithelial cells are rapidly replaced by CESCs and maintained for visual function [4]. Deficiency or dysfunction of CESCs can be caused by disease or chemical and physical injuries. Once the CESCs are severely damaged, they cannot support the replacement and maintenance of the cornea [5]. The migration and proliferation of CESCs play an important role in maintaining the visual function of the cornea [6]. The proliferation of CESCs is relatively slow but is activated when the cornea is wounded [4,7]. Therefore, CESC proliferation is a critical process in replacement and maintenance of the cornea.

Recently, many studies have been carried out on the correlation between the eye and brain. Previous studies have suggested that neuronal growth factors promote wound healing in the cornea. Pigment epithelium-derived factor (PEDF) and ciliary neurotrophic factor (CNTF) promote self-renewal and migration of CESCs [8–10]. BC is an EGF family member that was first found in the cultured medium of murine beta cell carcinoma [11]. BC affects the growth in various cell types [12,13]. According to a previous research, BC binds to ErbB1, ErbB2, ErbB3, and ErbB4 receptors to form homodimers or heterodimers [14]. BC is similar to EGF but works a little differently. While EGF induces only erk1/2 signaling pathways, BC induces erk and akt pathways as the main signal. However, the signaling pathways involved vary depending on the cell type [13,15]. In particular, BC activates both erk and akt signals in neural stem cells [15]. However, it is not known which signals are promoted in CESCs through BC. Thus, the effects of BC on CESC proliferation and the mechanism involved were investigated in this study. We have demonstrated that BC accelerates the proliferation of CESCs and promotes corneal wound healing in mice. This process acts only on the erk1/2 pathway in CESCs through BC.

2. Materials and methods

2.1. Cell culture

Mouse CESCs (TKE2) were purchased from Public Health England. The cell line was incubated in Keratinocyte Serum-Free

Abbreviations: CESCs, Corneal epithelial stem cells; PEDF, Pigment epithelium-derived factor; CNTF, Ciliary neurotrophic factor; BC, β-cellulin; KSFM, Keratinocyte Serum-Free Medium; CFE, Colony Forming Efficiency.

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Medium (KSFM) (Invitrogen, Carlsbad, CA) with bovine pituitary extract and epidermal growth factor at 37 °C under 5% CO_2. The cells were cultured overnight in a basal medium before treatment with BC (Sigma, St. Louis, MO) and p-p38 (SB203580) and p-erk1/2(PD98059) inhibitors (Cell Signaling Technology, Danvers, MA) for the measurement of p-erk1/2 protein expression.

2.2. Proliferation assay

Cell proliferation assay was carried out using a Cell Counting Kit-8 (CCK-8) Kit (Dojindo Molecular Technologies, Kumamoto, Japan). The TKE2 cell line was incubated in a 96-well plate for 24 h and then the cells were starved overnight with basal medium. Cells were treated with varying concentrations of BC and then cultured for 24 h. The cells were incubated with WST-8 for 1 h and absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

2.3. Colony formation assay

1×10^3 cells were seeded into 12 well plates. The cells were cultured with basal KSFM containing 0.2, 2, and 20 ng/mL BC. The medium was replaced every 3 days. After 14 days of incubation, the colonized cells were fixed with 4% paraformaldehyde and stained using crystal violet. The Colony Forming Efficiency (CFE) was calculated as the percentage of the number of colonies per number of seeded cells.

2.4. Animal experiments

BALB/c mice (6–8 weeks old) were anesthetized by injection with ketamine (70 mg/kg) and xylazine (7 mg/kg). 20% ethanol soaked filter paper (2 mm diameter) was applied to the right cornea of the mice for 1 min, and then surgical punches and blades were used to create 2 mm diameter wounds under a microscope (Olympus, Waltham, MA). To administer eye drops, BC was mixed with basal medium and then applied to the damaged mouse eyes. The corneal wounds were stained using 0.25% fluorescein and captured using a microscope with a camera (Olympus, Tokyo, Japan). The corneal defective area was analyzed at each time point with ImageJ software (National Institutes of Health, Bethesda, MD).

2.5. Immunofluorescence

Cultured cells were fixed with 4% paraformaldehyde and then blocked with 5% BSA in TBS-T for 1 h. The cells were incubated with primary antibodies overnight at 4 °C (ΔNp63, abcg2, and bmi-1). After three washes with TBS-T, the cells were treated and incubated with Alexa488 donkey IgG and 568 goat IgG-conjugated secondary antibody for 1 h. The cells were visualized with a Zeiss LSM 700 confocal microscope (Carl-Zeiss, Oberkochen, Germany).

2.6. Western blot assay

Proteins were extracted with RIPA buffer (Sigma, St. Louis, MO). Proteins were loaded onto a 12% SDS-PAGE gel and electrophoresed for 1 h 30 min at 100 V; then the proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 10 min. The proteins on the membranes were treated with 5% BSA in TBS-T for 1 h 30 min for blocking. After blocking, the membranes were exposed to primary antibodies (Abcam, Cambridge, MA; Santa Cruz Biotechnology, Santa Cruz, CA) and incubated overnight at 4 °C. The membranes were incubated with horseradish-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h 30 min after three washes with TBS-T, and then the membranes were developed with ECL western blotting reagent (Bio-Rad, Hercules, CA) and captured with an ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare Life Sciences, Pittsburgh, PA). Bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

2.7. Organ culture of injured mouse eyes

To examine the effect of neutralizing p-erk1/2 MAPK, inhibitors SB202190 and PD98059 were used to treat the extracted eyes. Two inhibitors were used to avoid cross-talk between erk1/2 and p38. BALB/c mice were aestheticized to make corneal wound (2 mm in diameter) and then sacrificed. The mouse eyes were individually harvested and then cultured. The corneas were treated with SB203580 (20 μM) and/or PD98059 (50 μM) and then cultured with BC (2 ng/mL) for 24 h. Other corneas without any treatment during their culture were used as a control. The wound closure was determined by Richardson's staining after 24 h, and then images were captured using a microscope with a camera (Olympus, Tokyo, Japan). The defected corneal area was measured with ImageJ software.

2.8. Statistical analysis

All experiments were repeated at least in triplicate, and values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS Statistics 23 software (IBM, Armonk, NY). One-way analysis of variance (ANOVA) was used. Statistical significance was considered as $p < .05$.

3. Results

3.1. BC accelerates the proliferation of CESCs

To investigate the effects of BC on CESC proliferation, we conducted the CCK-8 assay. CESCs were incubated with BC for 24 h. After 24 h, proliferation increased at 0.2, 2, and 20 ng/mL BC (2.08, 2.25, and 1.89 fold, respectively) (Fig. 1A). A colony formation assay was also performed. The cells were cultured for 14 days in KSFM with 0.2, 2, and 20 ng/mL of BC. BC induced the CFE of the CESCs compared with the control (2.99, 2.95, and 4.04 fold for the 0.2, 2, and 20 ng/mL concentrations, respectively; Fig. 1B).

3.2. BC upregulates CESCs proliferation makers

To determine the effects of BC on CESCs proliferation, the cells were fluorescently stained for the markers abcg2 and bmi-1. The expression levels of Abcg2 and bmi-1 increased after BC treatment (11.41 and 8.57 fold, respectively; Fig. 2A). The increased expression levels of abcg2 and bmi-1 were quantified by western blot analysis (Fig. 2B). The cells were also treated with ΔNp63 antibodies, and expression was measured by western blotting. The ΔNp63 marker was strongly expressed (Fig. 2C), and western blot analysis showed a significant difference (6.95 fold) in ΔNp63 expression (Fig. 2D). These results indicate that BC stimulates the proliferation makers of CESCs.

3.3. BC enhances wound healing in the mouse corneal epithelium

To determine the efficacy of BC in mouse corneal epithelial wound healing, mice with physically or chemically damaged corneas were treated with BC via eye drop delivery every hour. The size of the wound was measured by fluorescein staining (Fig. 3A). After

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