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ORIGINAL ARTICLE

Red Seaweed (*Eucheuma cottonii*) Extract Promotes Human Keratinocyte Migration via p38 Mitogen-Activated Protein Kinase Phosphorylation

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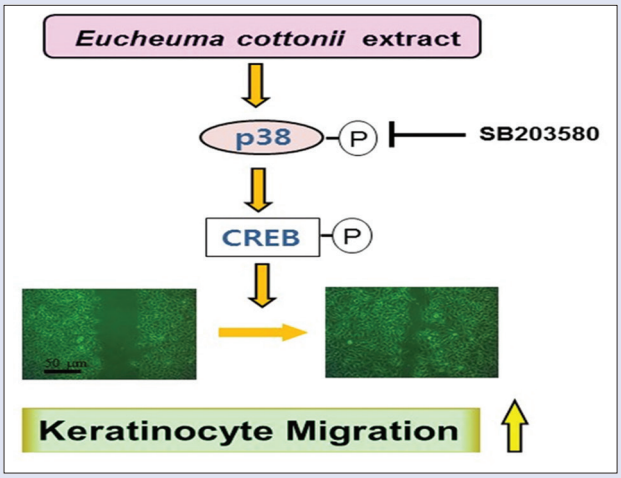
ABSTRACT

Background: *Eucheuma cottonii* (EC) is a rapidly growing red seaweed in Southeast Asia. **Objectives:** This study was performed to investigate the effects of EC extract on human keratinocyte migration. **Materials and Methods:** Cell migration was assessed using the scratch wound assay. Western blot analysis was performed to investigate the EC extract-induced signaling pathways. **Results:** EC extract promoted HaCaT keratinocyte migration in a concentration-dependent manner. To investigate the mechanism of EC extract-induced migration, we examined the migration-related signaling pathways. Western blot analysis showed that the EC extract showed no changes in extracellular signal-activated kinase (ERK) but showed slight Akt activation. In contrast, the EC extract strongly phosphorylated p38 mitogen-activated protein kinase (MAPK) and the subsequent downstream molecule, cAMP response element-binding protein (CREB). To examine the involvement of p38 MAPK pathway in EC extract-induced migration, SB203580, a specific inhibitor of p38 MAPK, was used. The results showed that EC extract-induced migration was abrogated by SB203580 pretreatment. In addition, SB203580 also blocked p38 MAPK and CREB activation. **Conclusion:** Taken together, these data suggest that EC extract promotes migration in human keratinocytes via p38 MAPK and CREB phosphorylation.

Key words: *Eucheuma cottonii*, keratinocytes, migration, p38 mitogen-activated protein kinase, wound healing

SUMMARY

- *Eucheuma cottonii* (EC) promotes keratinocyte migration
- p38 mitogen-activated protein kinase and cAMP response element-binding protein are involved in EC extract-induced keratinocyte migration
- EC extract could be considered as a new treatment option for wound healing.



Abbreviations used: CREB: cAMP response element-binding protein; EC: *Eucheuma cottonii*; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase.

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INTRODUCTION

Recently, natural products from marine organisms have been under examination and are reported to have various applications in many fields.[1,2] In particular, seaweeds are a newly rising reserve for food and medicine. Among various seaweeds, *Eucheuma cottonii* (EC) is a rapidly growing red seaweed in Southeast Asia. EC contains many useful substances including antioxidants, polyphenols, and phytochemicals.[3] Thus, EC has been reported to possess antioxidant, anticoagulant, antitumor, and anti-inflammatory effects.[4-6] However, the effects of ethanolic extracts from EC on keratinocyte migration have not been reported.

The skin epidermis is composed of many cell types. About 80% of keratinocytes are composed of epidermal cells. Keratinocytes execute many functions in regeneration, homeostasis, and wound healing of the skin.[7] Wound healing is essential for skin maintenance because it decreases many problems, including bacterial infection, scar formation, harmful environment stimulation, and water loss.[8,9] In response to injury, keratinocytes participate in re-epithelialization through various activities, including cell migration.[10,11] These processes fill the wound area and restore the skin barrier. Thus, increasing keratinocyte migration has great

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potential to accelerate wound healing. Therefore, identifying new products that induce keratinocyte migration is a subject of increasing attention.

In wounded skin, keratinocyte migration is controlled by cellular signaling networks. The extracellular signal-activated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and Akt pathways are reported to play crucial roles in keratinocyte migration.[12,13] ERK has been shown to regulate migration in a variety of cells.[14-16] Furthermore, ERK pathway inhibitors have been reported to decrease cell migration in the endothelial cells and fibroblasts.[17,18] On the other hand, skin injury can activate ERK, and activated ERK facilitates keratinocyte migration.[19] The Akt pathway has also been shown to control keratinocyte migration. Activated Akt induces migration in epithelial cells and promotes wound healing.[20] Skin injury also activates the p38 MAPK in keratinocytes, which is related to keratinocyte migration.[21,22] Therefore, the relationship of the ERK, p38 MAPK, and Akt pathways in EC extract-regulated keratinocyte migration was investigated.

In this study, we investigated the influence of EC extract on the regulation of keratinocyte migration. Moreover, mechanisms underlying induction of keratinocyte migration by the EC extract were examined. Our results show that the EC extract exerts strong effects as a wound-healing agent.

MATERIALS AND METHODS

Materials

LY294002 and SB203580 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies that recognize phospho-specific Akt (Ser473), total Akt, phospho-specific p38 MAPK (T180/Y182), total p38 MAPK, phospho-specific ERK1/2 (Thr202/Thr204), total ERK1/2, phospho-specific cAMP response element-binding protein (CREB) (Ser133), and total CREB (86B10) were all purchased from Cell Signaling Technology, Inc.

Preparation of red seaweed samples

Red seaweed, EC, was obtained from seaweed farmers in Bontomatene, Selayar Island, Indonesia. The EC was put in a cooler containing ice and sea water. EC was then washed thrice with running water and stored in a refrigerator (−20°C). The frozen sample was then dried in a freeze dryer at −60°C. Next, the sample was crushed in a mortar, blended, and sifted using a 40-mesh size sieve.

Red seaweed extraction

In total, 3.183 kg of EC was dissolved in 25 L of 70% ethanol and was then allowed to stand for 24 h with occasional stirring. Thereafter, the sample was filtered through filter paper to obtain the extracts and pulp. The pulp was again soaked with the same solvent for 72 h. The filtrate was then collected and again filtered through filter paper. The solvent was then evaporated with a rotary evaporator to obtain the EC extract (5.4 g).

Cell culture

HaCaT human keratinocytes[23] were cultured as previously described.[24]

Cell viability

After treatment with EC extract (0–200 μg/mL) for 24 h, cell viability was assessed as previously described.[24]

Migration assay

A migration assay was performed as previously described.[24]

Western blotting

The protein samples were prepared and western blot analysis were performed as previously described.[24]

Statistics

The Student's *t*-test was used for statistical analysis.

RESULTS

Effects of *Eucheuma cottonii* extract on cell viability

First, we examined the cytotoxicity of EC extract in HaCaT cells. EC extract showed no cytotoxicity at concentrations of 1–100 μg/mL [Figure 1]. However, 200 μg/mL of EC extract decreased the cell viability significantly. Since the EC extract showed no significant cytotoxicity at concentrations of 1–100 μg/mL, this concentration range was used for the following experiments.

Effects of *Eucheuma cottonii* extract on keratinocyte migration

To examine the influence of EC extract on the migration of HaCaT cells, scratch migration assay was performed. We added the EC extract to confluent cells that were subjected to a scratch wound. After 24 h of EC extract treatment, we observed cell migration via microscopy. As shown in Figure 2, the EC extract promoted cell migration in a concentration-dependent manner.

Eucheuma cottonii extract-induced signal transduction

The ERK, p38 MAPK, and Akt pathways have been shown to regulate keratinocyte migration. Therefore, we examined whether EC extract influences the signaling pathways in HaCaT cells. As shown in Figure 3a, the EC extract showed no effects on ERK activation, whereas Akt was slightly phosphorylated. In contrast, the EC extract induced phosphorylation of p38 MAPK after 2–360 min [Figure 3b]. Furthermore, it is reported that CREB is downstream of the p38 MAPK pathway in the migration process.[25] Thus, we also checked CREB phosphorylation. In agreement with p38 MAPK activation, the EC extract also induced CREB phosphorylation.

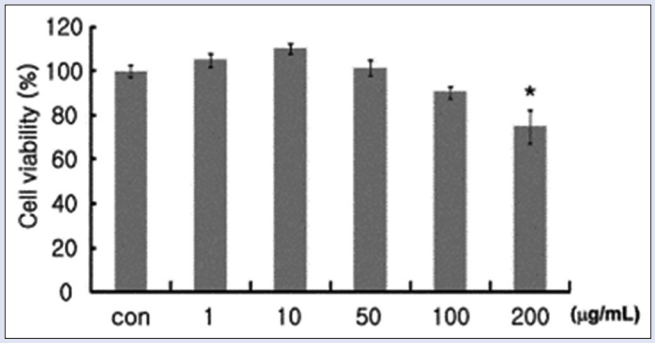

Figure 1: Effects of *Eucheuma cottonii* extract on HaCaT cell viability. HaCaT cells (4 × 10^4 cells/well) were seeded into a 24-well plate and serum starved for 24 h. The cells were then treated with *Eucheuma cottonii* extract (0, 1, 10, 50, 100 and 200 μg/mL) for another 24 h. Cell viability was determined using a crystal violet assay. Data represent the mean ± standard deviation of triplicate assays expressed as percentages of the control. *$P < 0.01$ compared to the untreated control

Effects of LY294002 and SB203580 on *Eucheuma cottonii* extract-mediated keratinocyte migration

Because Akt and p38 MAPK were activated by the EC extract, we next tested the effects of LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor, and SB203580, a p38 MAPK inhibitor, on EC extracted-mediated keratinocyte migration. Scratched cells were cultured with EC extract for 24 h with or without LY294002 and SB203580. As shown in Figure 4, the EC extract closed the scratched area more compared to the untreated control. However, EC extract-induced keratinocyte migration was significantly abrogated by SB203580. In contrast, LY294002 slightly recovered the EC extract-induced migration. Therefore, these observations indicated that EC extract-induced keratinocyte migration resulted from p38 MAPK activation.

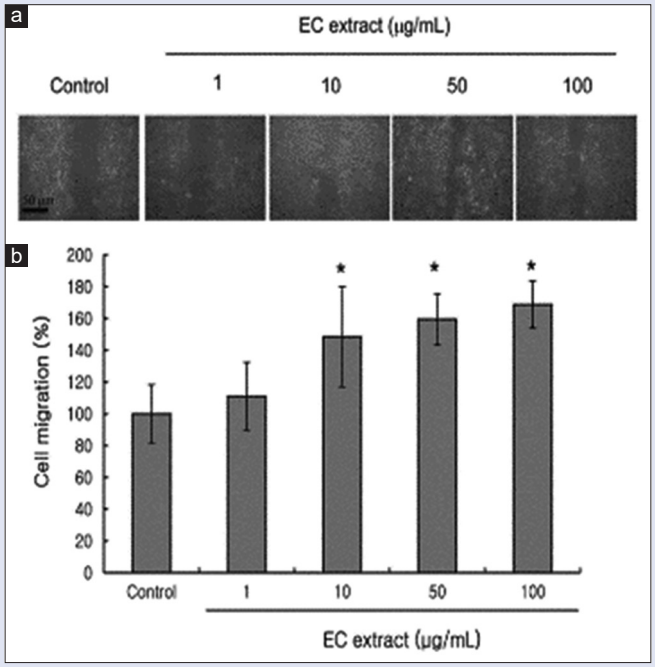

Figure 2: Effects of *Eucheuma cottonii* extract on migration in HaCaT cells. HaCaT cells (5×10^5 cells/well) were seeded into a 6-well plate and serum starved for 24 h. Thereafter, wounds were made in the cultures as described in "Materials and Methods." HaCaT cells were then treated with *Eucheuma cottonii* extract (1–100 μg/mL) for another 24 h. (a) A digital video camera was used to capture phase contrast photographs of the wound widths. (b) The cell migration rate is quantified in the graph. Data represent the mean ± standard deviation of wound widths in 10 randomly chosen fields along the wound and are expressed as percentages of the control

Effects of SB203580 on the *Eucheuma cottonii* extract-induced signaling pathway

To verify the relationship between the p38 MAPK pathway and EC extract-induced keratinocyte migration, we cultured the cells with EC extract and SB203580. The results showed that EC extract-induced p38 MAPK activation was blocked by SB203580 [Figure 5a]. Furthermore, CREB phosphorylation was also inhibited by SB203580 [Figure 5b]. These results indicate that EC extract promotes keratinocyte migration via the p38 MAPK and CREB pathway.

DISCUSSION

Acute or chronic wounds are major health problems. Therefore, wound healing should be continuously monitored in specific populations, including infected patients, elderly individuals, and especially individuals with diabetes. Delayed wound healing can result in complications for infection and worsened scarring, leading to considerably increased morbidity and cost of continued medical care. As mentioned above, wound healing is controlled by various biological processes.[8,26] Once skin injury occurs, keratinocytes migrate to the wound site. This process is important because it triggers subsequent re-epithelialization and induces cell proliferation and therefore recovers the wound space.[10] Therefore, rapid keratinocyte migration is crucial to determine the efficiency of the wound-healing process. Here, we performed the scratch migration assay to investigate the effects of the EC extract on keratinocyte migration.

Signal transductions play an important role in the regulation of keratinocyte migration. As mentioned above, the ERK pathway is activated upon injury or stretching of the epidermis[27] and the ERK-activating peptide promoted wound healing through keratinocyte migration.[19] In addition, adiponectin, a key mediator in diabetes pathogenesis, induced keratinocyte migration via the ERK pathway.[28] However, the EC extract showed no effect on ERK [Figure 3a]. This result indicates that ERK did not play a role in EC extract-induced migration. Activation of the Akt pathway accelerates keratinocyte migration. In a previous study, we reported that LGI3 promotes keratinocyte migration through the Akt pathway.[24] Akt leads to β-catenin accumulation, which promotes cell migration. Moreover, phosphorylated Akt controls the downstream molecules, Rac1 and Rho1, which regulate cell migration.[29] We hypothesized that the EC extract could also induce keratinocyte migration through the Akt pathway. However, our data showed that the EC extract had little effect on Akt [Figure 3a]. To investigate the possible relationship of Akt and EC extract-induced migration, the cells were pretreated with LY294002, a selective PI3K inhibitor, and performed

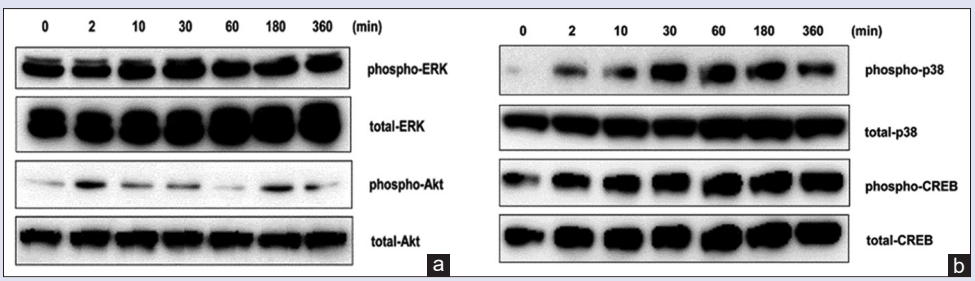

Figure 3: Effects of *Eucheuma cottonii* extract on migration-related signaling pathways. After serum starvation, HaCaT cells were treated with *Eucheuma cottonii* extract (50 μg/mL) at the indicated time periods and harvested. Protein samples were then examined by western blotting with antibodies specific for phospho-ERK, phospho-Akt (a), phospho-p38 mitogen-activated protein kinase, and phospho-cAMP response element-binding protein (b). Equal protein loading was confirmed by total ERK, Akt, p38 mitogen-activated protein, and cAMP response element-binding protein expression levels

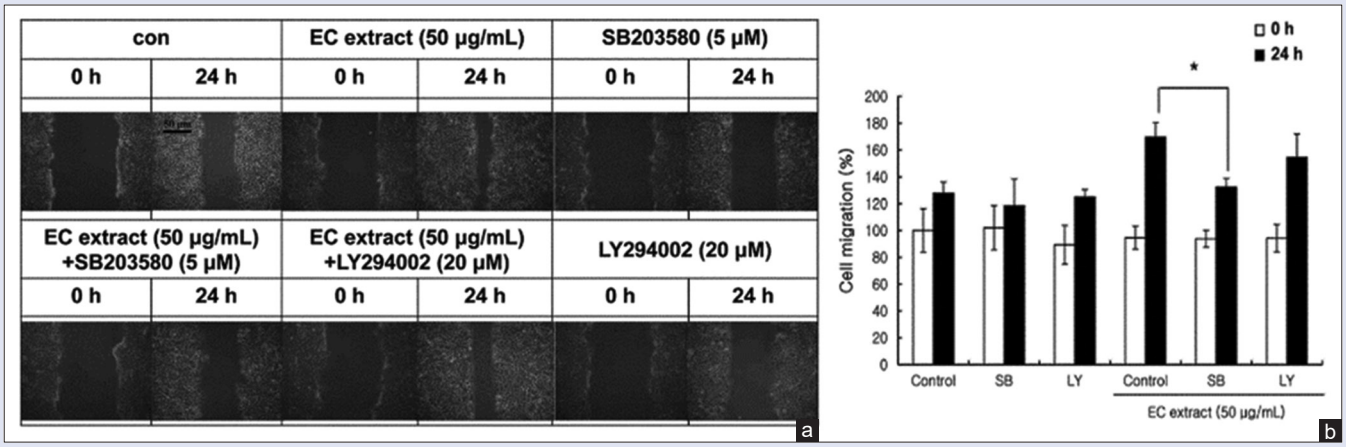

Figure 4: Effects of LY294002 and SB203580 on *Eucheuma cottonii* extract-mediated keratinocyte migration. HaCaT cells (5×10^5 cells/well) were seeded into a 6-well plate and serum starved for 24 h. Thereafter, wounds were made in cultures as described in "Materials and Methods." HaCaT cells were pretreated with LY294002 (20 μM) and SB203580 (5 μM) for 30 min, followed by addition of the *Eucheuma cottonii* extract (50 μg/mL) and were then incubated for 24 h. (a) Phase contrast images of wound widths were captured using a digital video camera. (b) Quantification of the cell migration rate is shown in the graph. HaCaT cells were maintained for 0 h and 24 h. Data represent the mean ± standard deviation of wound widths in 10 randomly chosen fields expressed as percentages of the control. *$P < 0.01$ compared to the *Eucheuma cottonii* extract-treated control

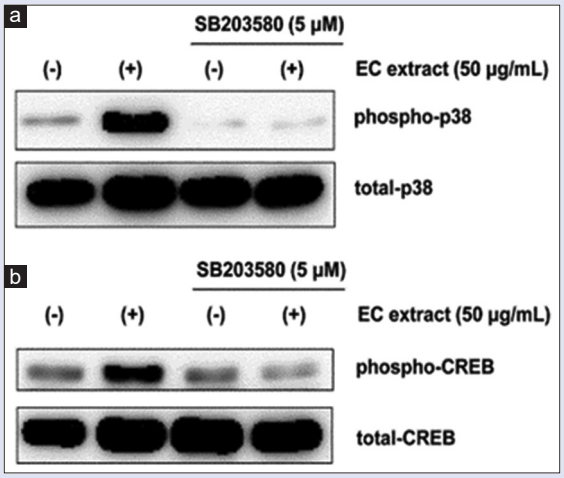

Figure 5: Effects of SB203580 on *Eucheuma cottonii* extract-mediated keratinocyte migration. After serum starvation, HaCaT cells were pretreated with SB203580 (5 μM) for 30 min, followed by the *Eucheuma cottonii* extract (50 μg/mL), and were then incubated for 24 h. Protein samples were analyzed by western blotting with antibodies specific for phospho-p38 mitogen-activated protein kinase (a) and phospho-cAMP response element-binding protein (b). Equal protein loading was confirmed by total p38 and cAMP response element-binding protein expression levels

scratch migration assay. In another study, LY294002 was shown to block insulin-induced keratinocyte migration.[30] However, in our study, LY294002 only blocked EC extract-induced keratinocyte migration slightly [Figure 4]. Thus, the EC extract had little influence on the Akt pathway.

There are several studies indicating that p38 MAPK is related to keratinocyte migration. Hypoxia was reported to regulate keratinocyte migration through p38 MAPK activation.[31] Moreover, the extract of *Centella asiatica*, a medicinal plant containing madecassoside and madecassic acid, was found to activate p38 MAPK that is involved in keratinocyte migration.[32] Our data also showed that the EC extract strongly induced p38 MAPK phosphorylation [Figure 3b]. Another study showed that SB203580 inhibited p38 MAPK-induced keratinocyte migration.[31] As shown in Figure 4, SB203580 downregulated EC extract-induced keratinocyte migration. After we identified that EC extract provokes keratinocyte migration via p38 MAPK, we attempted to examine the downstream pathway of p38 MAPK. Although the relationship between p38 MAPK and keratinocyte migration has been demonstrated, downstream molecules of p38 MAPK has not been studied in keratinocytes till date. Interleukin-6 induces fibroblast migration through p38 MAPK and its downstream molecule CREB.[25] Therefore, we hypothesized that EC extract induces keratinocyte migration through the p38 MAPK/CREB pathway. As shown in Figure 3b, EC-extract induced CREB activation. Moreover, EC extract-induced phosphorylation of CREB was blocked by SB203580 [Figure 5b]. These data demonstrated that CREB plays a key role in EC extract-induced keratinocyte migration.

In summary, our results indicated that EC extract promotes keratinocyte migration. We demonstrated that p38 MAPK and CREB are involved in EC extract-induced keratinocyte migration. Thus, EC extract could be considered as a new treatment option for wound healing.

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Conflicts of interest

There are no conflicts of interest.

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