Acemannan Induces Expression of Keratinocyte Differentiation Markers and Promotes Cell Migration in HaCaT Cell

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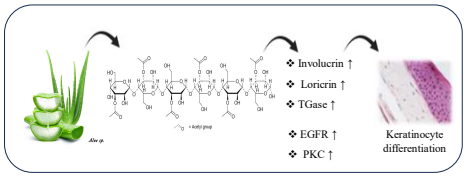
Abstract

Aloe vera (L.), a popular medicinal plant that is widely used in the cosmetic, pharmaceutical, and food industries. The pharmacological effects of the polysaccharide derived from Aloe vera have been reported for digestive-related disease protection, antimicrobial, and prebiotic activities. ACE is a bioactive polysaccharide isolated from Aloe vera using a proprietary fractionation and state-of-the-art purification technology. Here, we used HaCaT cells model to uncover the potential biological function of MannanPro in keratinocytes. Using a cell counting method, we found that ACE induces cell growth in a concentration-dependent manner. Moreover, a higher incorporation of BrdU was detected in MannanPro-treated cells than in vehicle-treated cells, indicating that ACE promotes cell proliferation. We further examined the influence of ACE on cell migration by wound healing assay which was conducted in the presence of mitomycin C to exclude interference with ACE-induced cell growth and found that MannanPro can promote cell migration in a concentration-dependent manner. To understand the effects on cell differentiation, cells were treated with ACE as well as the differentiation-inducing agent (PMA). Immunoblotting experiments showed an increase in the expression of differentiation markers including involucrin, loricrin, and TGase 1 in both time- and concentration-dependent manners in the presence of ACE. Similar to PMA, ACE also activated PKC and EGFR, which play pivotal roles in keratinocyte differentiation. The action mechanism of ACE regulating the proliferation and differentiation of keratinocytes by activating EGFR and PKC will be further explored in our future study.

Introduction

Aloe species (family Asphodelaceae) has been used as medicinal plants for centuries. *A. vera* has been reported to have pharmacological activities including digestive disease protection, antidiabetic, antimicrobial, prebiotic activity, anticancer, cardioprotective effect, bone protection, and skin protection. These pharmacological activities are mainly due to the rich content of bioactive phytochemicals in *A. vera*. Emodin has been reported to be associated with hepatitis, genotoxicity, nephrotoxicity, and phototoxicity. Acemannan (ACE), as one of the main bioactive polysaccharides of *A. vera*, has been reported of great value in applications in medical and industrial fields for the prevention or treatment of oral diseases, and beneficial antimicrobial and prebiotic activity.



The pharmacological activity exhibited by ACE appears to have a high potential for medical and healthcare applications. However, there are only a few literature review reports on the pharmacological advancement of ACE, and the structure-action relationship of ACE has not been fully understood. In this study, we used HaCaT cells as a model to explore the potential biological function of ACE in keratinocytes. We explored the possible effects of ACE on the growth, migration, and UVB irradiation-induced death of HaCaT cells. In addition, the effect of ACE on the expression of inflammatory genes was also analyzed. The current data help us to further decipher the mechanism by which ACE regulates cell physiology of keratinocytes.

Materials and Methods

Cell culture, antibodies, reagents, and test compounds

Human keratinocyte cell line, HaCaT, was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 10% penicillin-streptomycin solution and incubated at 37 ℃ with 5% CO_2. Primary antibodies against β-actin, Involucrin, Keratin 10 (K10), TGase 1, and PKC b II were purchased from Santa Cruz Biotechnology, Inc. (USA), p63-a, phospho-EGFR Y1068, phospho-PKC b II S660, and peroxidase-conjugated anti-rabbit/mouse IgG secondary antibody were from Cell Signaling Technology, Inc, USA), EGFR from EMD Millipore Corp, and loricrin from GeneTex, USA). Phorbol-12-myristate-13-acetate (PMA), lipopolysaccharides (LPS), epidermal growth factor (EGF), and poly (I:C) were from Sigma-Aldrich (USA). The ACE was purified by Dazzeon Biotech Co., Limited using proprietary, patented fractionation technology. ACE was dissolved in sterilized H_2O (50 mg/ml) or 0.2% ethanol and stored at 4℃ and stored at room temperature.

MTT assay of cell viability

The cells were seeded in a 24-well plate in 500 ml DMEM at 37 ℃ with 5% CO_2. When the cell confluence reached 60%, the cells were treated with vehicle and different concentrations of ACE for the indicated time period. Then 1 mg/ml MTT solution [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; (Sigma-Aldrich, USA) was added into the medium for 2 h to form crystals by mitochondrial dehydrogenases of living cells. The medium was removed and replaced with 150 ml DMSO to dissolve the purple crystal. The cell viability was determined by the OD value (OD_{550nm}-OD_{630nm}) of the DMSO solution containing the crystal product.

Cell migration assay

A 2-well silicone culture insert was placed in each well of a 24-well plate, and then cells were seeded ($2x10^4$ cells) in the wells of the silicone culture insert overnight. Silicone culture insert were removed and fresh DMEM containing 2 mg/ml mitomycin C was added. Cells were treated with various concentrations of ACE and cell migration was observed at the indicated time points with a microscope and recorded by taking photos. The percentage of gap recovery area was analyzed and quantified using Image J software.

Cell proliferation assay

Cells ($2x10^4$) were seeded on a 12-well plate in DMEM without FBS and incubated for 24 h in a CO_2 incubator. Remove the medium and add fresh DMEM with 10% FBS in the presence or absence of ACE (0.5, 1, and 3 mg/ml). The cell proliferation was determined by counting the cell number every 24 h with trypan blue staining. For BrdU incorporation assay, cells ($1x10^4$) were seeded on a black 96-well plate with a clear and flat bottom in DMEM without FBS and incubated for 24 h. The medium was replenished with fresh DMEM containing 10% FBS in the presence or absence of ACE (1, and 3 mg/ml) for 46 h. And then BrdU labeling solution was added for additional 2 h incubation. Cells were fixed, incubated with anti-BrdU-peroxidase for 90 min, and washed before conducting substrate reaction according to the manufacturer's protocol (Cell proliferation ELISA, BrdU, Roche, Switzerland). Chemiluminescence was measured using a Synergy H1 microplate reader (BioTeK, Winooski, VT, USA).

Flow cytometry analysis of cell viability

Cells were harvested and re-suspended in the staining buffer containing Annexin V-FITC/PI according to the manufacturer's protocol (BioLegend, USA), After the 20-min incubation at 37 ℃, the cells were measured by flow cytometry (FACSCalibur, BD, USA). FlowJo software was used for data analysis.

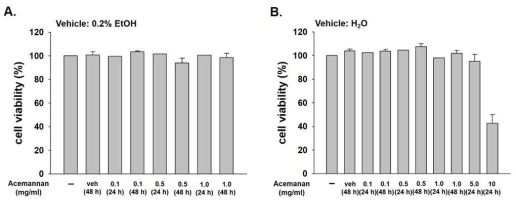
Western blot

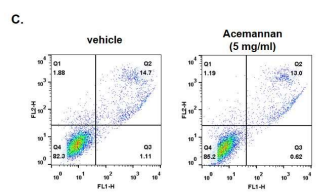
Cells were lysed using RIPA buffer. After homogenization, the cell extract was diluted in 5X sample buffer, heated for 10-15 min at 95℃, and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins on the gel were transferred to a polyvinylidene difluoride membrane (PVDF, GE Healthcare Life Sciences, USA) and then blocked for 30 min with TBS-T containing 5% skim milk. The membrane was rinsed three times consecutively with TBS-T buffer, followed by incubation with 1:1000-2000 dilutions of primary antibodies in TBS-T buffer for 4-8 h. The membrane was washed three times with TBS-T buffer for 15 min and then incubated for 1 h with horseradish peroxidase-conjugated anti-goat IgG (1:10000) or anti-rabbit/mouse IgG secondary antibody (1:10000) in TBS-T buffer. Development was carried out using an enhanced chemiluminescence solution (Western Lightning Plus-ECL, PerkinElmer, Inc. USA). The protein signals were acquired using a ChemiDoc™ MP Image System (Bio-Rad), and analyzed by using Image Lab software (Bio-Rad).

Results

HaCaT cells were treated cells with different concentrations of ACE dissolved in 2% ethanol (**Fig. 1A**) and H_2O (**Fig. 1B**), and then MTT assays were performed to analyze cell viability. The results showed that ACE at concentrations up to 1 mg/ml in both vehicles did not cause cytotoxicity after 48 h incubation. Cells treated with 5 mg/ml ACE dissolved in H_2O for 24 and 48 h also did not cause cytotoxicity as indexed by MTT assay (**Fig. 1B**) or by Annexin V/PI staining (**Fig. 1C**). When the ACE concentration was 10 mg/ml, the cell viability was reduced to about 40% after 24 h of treatment (**Fig. 1B**). To avoid interference resulting from cell death, ACE dissolved in H_2O at concentrations not higher than 5 mg/ml was used for analysis in subsequent experiments.

Figure 1. Cytotoxicity of ACE in HaCaT cells

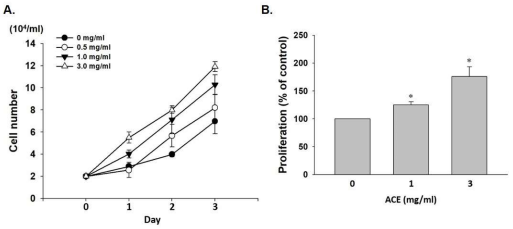




HaCaT Cells ($2x10^4$) seeded on a 24-well plate and incubated overnight at 37 ℃ with 5% CO_2, were treat with various concentrations of ACE or vehicle followed by incubation for 24 or 48 h. MTT assay was conducted to determine the effect of ACE on cell viability. (C) The cell viability was also determined by flow cytometry with Annexin V-FITC/PI staining after the treatment with 5 mg/ml ACE for 48 h.

We examined whether ACE affects cell growth. After 24 h of starvation, the cells were replaced with DMEM containing 10% FBS and added with or without ACE at different concentrations. HaCaT cell growth was promoted by ACE in a concentration-dependent manner (**Fig. 2A**). The data from BrdU assay also showed that ACE had a higher BrdU uptake, indicating that ACE promotes the DNA synthesis of cells in the S-phase of the cell cycle (**Fig. 2B**).

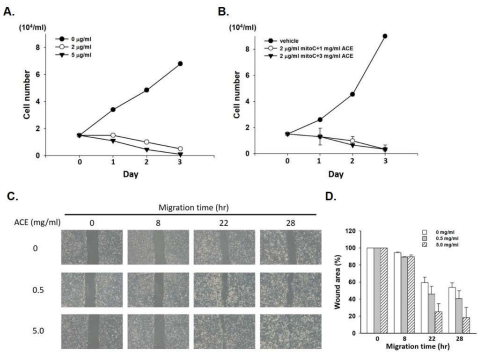
Figure 2. ACE promotes HaCaT cell proliferation



ACE enhances HaCaT cell migration

Since ACE can increase cell growth as shown in Fig. 2, to avoid cell growth interfering with cell migration results, we treated cells with mitomycin C first, to determine proper concentration of mitomycin C to inhibit cell growth without inducing cell death. As shown in **Fig. 3A**, mitomycin C at 2 mg/ml and 5 mg/ml indeed blocked cell growth and reduced cell number. The reduction of cell number occurred at 48 h and 24 h by mitomycin C treatment at 2 mg/ml and 5 mg/ml, respectively. This finding indicates mitomycin C at 2 mg/ml might be proper in the migration assay within 24 h. To further confirm this, we tested the effects of mitomycin C (2 mg/ml) together with ACE. We found ACE (1 and 3 mg/ml)-induced cell growth as seen in **Fig. 3A** was abrogated by mitomycin C (2 mg/ml) (**Fig. 3B**).

Figure 3. ACE accelerates HaCaT cell migration

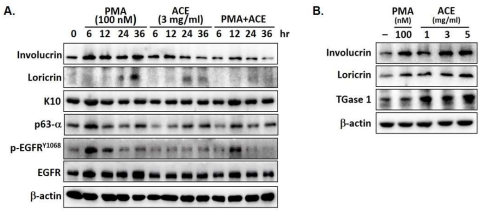


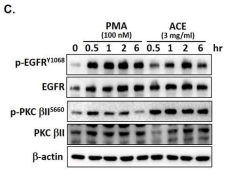
Subsequently, we also measured cell migration using mitomycin C (2 mg/ml) and ACE (0.5 and 5 mg/ml) co-treatment. As shown in **Fig. 3C and 3D**, cells migrated faster in the presence of ACE (5 mg/ml) than in vehicle treatment, reaffirming the effect of ACE on promoting cell migration.

ACE induces the expression of keratinocyte differentiation markers in HaCaT cells

To explore whether ACE affects the differentiation of HaCaT cells, we treated cells with different concentrations of ACE and analyzed at indicated time points for the expression levels of specific differentiation marker proteins. Cells treated with the differentiation-inducing reagent PMA (100 nM) were used as the standard response. Treatment with ACE (3 mg/ml) increased involucrin, loricrin, and TGase1 similar to PMA, but not K10 (**Fig. 4A, 4B**).

Figure 4. ACE induces keratinocyte differentiation and activates PKC and EGFR as PMA





(A) Cells were treated with PMA (100 nM) or ACE (3 mg/ml) for 6, 12, 24, and 36 h, respectively. (B) Cells were treated with PMA (100 nM) or ACE (1, 3, or 5 mg/ml) for 36 h. (C) Cells were treated with PMA (100 nM) or ACE (3 mg/ml) for 0.5, 1, 2, and 6 h, respectively. The cell lysates were collected and prepared for immunoblotting assays with primary antibodies against the target proteins.

We found both PMA and ACE can transiently downregulate p63-a and the onset was 12-24 h for PMA and 0-12 h for ACE. These results suggest that ACE may function to regulate HaCaT differentiation. Since EGFR and PKC are important regulators for keratinocyte differentiation, we deciphered the action mechanisms of ACE on both pathways. Similar to the effect of PMA, ACE can activate EGFR and PKC βII by promoting their phosphorylation (**Fig. 4C**). However, ACE-induced EGFR phosphorylation appeared to be weaker in response and shorter in duration than PMA. Of note, ACE induced more obvious activation of PKC βII than PMA. All these findings indicate ACE might induce keratinocyte differentiation via PKC activation.

Conclusions

- **In this study, we observed that ACE isolated from the *Aloe* vera promotes HaCaT cell proliferation and migration by inducing expression of differentiation markers such as involucrin, loricrin, and TGase1.**
- **ACE also activated EGFR and PKC by promoting their phosphorylation and increasing EGFR protein expression.**
- **Taken together, our data suggest that ACE could be one of the components contributing to the effects of *Aloe* extract and regulated proliferation and migration of HaCaT cells could possibly be mediated through activation of EGFR and PKC.**