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Short communication

Asiaticoside enhances normal human skin cell migration, attachment and growth *in vitro* wound healing model

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ABSTRACT

Wound healing proceeds through a complex collaborative process involving many types of cells. Keratinocytes and fibroblasts of epidermal and dermal layers of the skin play prominent roles in this process. Asiaticoside, an active component of *Centella asiatica*, is known for beneficial effects on keloid and hypertrophic scar. However, the effects of this compound on normal human skin cells are not well known. Using *in vitro* systems, we observed the effects of asiaticoside on normal human skin cell behaviors related to healing. In a wound closure seeding model, asiaticoside increased migration rates of skin cells. By observing the numbers of cells attached and the area occupied by the cells, we concluded that asiaticoside also enhanced the initial skin cell adhesion. In cell proliferation assays, asiaticoside induced an increase in the number of normal human dermal fibroblasts. In conclusion, asiaticoside promotes skin cell behaviors involved in wound healing; and as a bioactive component of an artificial skin, may have therapeutic value.

Introduction

Skin is the largest organ in the body comprising approximately 15% of body weight, with epidermis, dermis and the subcutaneous layer included. Epidermis, the outermost layer of the skin, maintains a vital barrier against external insults. Dermis, the layer between the epidermis and subcutaneous tissue, endows the skin with firmness, elasticity and strength, and also regulates body temperature through control of blood flow and sweating. Keratinocytes comprise 95% of epidermis, as the major cellular constituent, and fibroblasts predominate among cell types in the dermis (Zouboulis 2000; Braun-falco et al. 2000).

Wound healing spans several complicated phases, including inflammation, granulation and re-epithelialization (new tissue formation), contraction and regeneration of tissues (Singer and Clark 1999). Soon after wounding, cytokines attract inflammatory cells to remove damaged tissues and foreign substances. As inflammation subsides, fibroblasts and keratinocytes migrate into the wound area, adhere and proliferate to form new tissue. In this phase, changes in gene expression and phenotype guide the proliferation, migration and differentiation of keratinocytes and fibroblasts. New tissue formation begins with the migration of keratinocytes over injured dermis followed by capillary formation to support fibroblasts and macrophages as these cells replace the fibrin clot with granulation tissue. A second wave of keratinocytes will use the granulation tissue as a substrate (Wong et al. 2007; Gurtner et al. 2008). Fibroblasts also compensate for lost tissue and protect the wound area from intrusion by debris. During re-epithelialization, keratinocytes proliferate and differentiate to restore function to the epithelium as a barrier against external conditions (Gurtner et al. 2008). Wound repair concludes through contraction of the wound site and tissue remodeling through differentiation and programmed cell death. Cutaneous wound healing is important for both medical and esthetic reasons and a large number of bioactive compounds have been tested for capacity to promote this process (Gupta et al. 2005; Phan et al. 2001; Kim and Mendis 2006).

Centella asiatica has a long history of use in Asia for treating skin and vascular disease. Active components derived from the leaves of this small flowering plant, include asiaticoside, asiatic acid, madecassic acid and other compounds not yet identified. Among these components, asiaticoside displays the highest activity, as observed in the healing of gastric ulcer, leprosy and certain types of tuberculosis (Guo et al. 2004; Boiteau and Ratsimamanga 1956; Shukla et al. 1999). Asiaticoside may also inhibit proliferative activity related to keloid and hypertrophic scar. The effects of asiaticoside on skin disorders such as keloid and hypertrophic scar have been studied

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in vitro and *in vivo* (Xie et al. 2009; Tang et al. 2011; El-Hefnawi 1962); however, studies have not yet clearly defined the effects of asiaticoside on normal skin cells in wound healing. We studied the effects of asiaticoside on behaviors of normal human skin cell to evaluate asiaticoside as a natural pharmaceutical for wound healing.

Materials and methods

Cell culture conditions

Adult human dermal fibroblasts (aHDFs) were purchased from Lonza Group, Ltd. (Walkersville, MD, USA) and maintained in fibroblast basal medium-2 (FBM-2) supplemented with growth kit (10 ml of fetal bovine serum, 0.5 ml of insulin, 0.5 ml of gentamicin sulfate amphotericin-B (GA-1000) and 0.5 ml of r-human fibroblast growth factor-B, Lonza). Adult normal human epidermal keratinocytes (aNHEKs) were purchased from Lonza and maintained in keratinocyte basal media (KBM-Gold) supplemented with growth kit (2 ml of bovine pituitary extract, 0.5 ml of insulin, transferrin, hydrocortisone, GA-1000 and r-human epidermal growth factor, and 0.25 ml of epinephrine, Lonza). Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Asiaticoside treatment

Asiaticoside ($C_{48}H_{78}O_{19}$, molecular weight = 959.12, purity < 99%) determined by HPLC was purchased from Xi'an Bosheng Biomedical Technology (Xi'an, Shaanxi, China). To dissolve the water-insoluble asiaticoside, dimethyl sulfoxide (DMSO; Sigma–Aldrich Corporation, St. Louis, MO, USA) was used to prepare a stock solution of 200 mM asiaticoside for *in vitro* assays. The stock solution was diluted with serum free media without growth factors to concentrations of 0, 62.5, 125, 250, 500, and 1000 μM asiaticoside and cells were treated with equal volumes at each concentration. The same amount of DMSO was used to make various concentrations of asiaticoside using the stock solution and to avoid the effect of DMSO on skin cells behaviors.

Cell viability assay

The cytotoxicity of asiaticoside was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The aHDFs were plated at a density of 1×10^5 cells/well in 48-well plates and aNHEKs were plated at 1×10^5 cells/well in 24-well plates. Cells were incubated for 24 h and treated with various concentrations of asiaticoside (62.5 μM to 1 mM) as described above. After 24 h of asiaticoside treatment, cells were incubated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Amresco, Inc., Solon, OH, USA) for 4 h in the dark. The formazan salts formed were dissolved in DMSO and the optical density was measured at 570 nm.

Cell migration assay (wound-healing assay)

A wound closure seeding model was constructed using silicon culture inserts (Ibidi, LLC, Munchen, Germany) with two individual wells for cell seeding. Each insert was placed in a culture dish, and 8×10^3 cells of aHDF or 2×10^4 cells of aNHEK were plated in each well and grown to form a confluent and homogeneous layer. Twenty-four hours after cell seeding, the culture insert was removed and a cell-free area, the "wound" made by the culture insert, could be observed. The wound was approximately 500 μm wide. The cells were treated with 10 μg/ml of mitomycin C in serum free media without growth factors for 2 h to suppress proliferation. Healing of the wound by migrating cells after asiaticoside treatment was observed over time by light microscopy (IX-70, Olympus) and analyzed using Image J software (NIH, USA).

Cell attachment assay

Cell attachment was determined at 4–6 h after seeding, depending on the cell type, using the MTT assay. Briefly, fibroblasts were seeded at an initial density of 5×10^4 cells/well in 24-well plates with asiaticoside co-treatment and incubated for 4 h in a CO_2 atmosphere. Keratinocytes were plated at the initial density of 5×10^4 cells/well in 48-well plates with treatment of asiaticoside as described above, and then incubated for 6 h. Unattached cells were removed by gentle washing with phosphate buffered saline (PBS) and cell numbers attached were determined by MTT assay as described above.

For cell morphometric analysis, cells were plated at the initial density of 5×10^4 cells/well in 24-well plates with asiaticoside co-treatment and incubated for 4–6 h in a CO_2 atmosphere as described above. Cells were then fixed with ice-cold 70% ethanol for 30 min. Actin cytoskeleton was visualized by staining with Alexa (488)-conjugated phalloidin (1 U/sample, Invitrogen, Carlsbad, CA, USA) and cell nuclei were counterstained with propidium iodide (Sigma–Aldrich Corporation, Steinheim, Germany). Six random

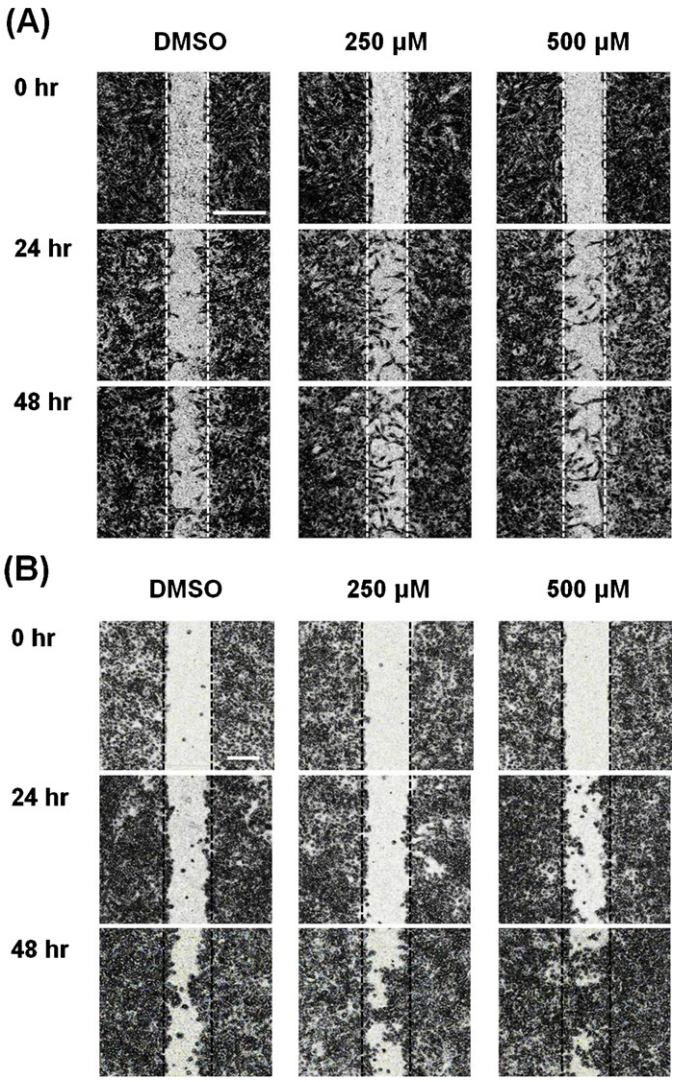

Fig. 1. Migration of fibroblasts (A) and keratinocytes (B) into the denuded area was observed by optical microscopy over time. Scale bar = 400 μm.