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Madecassoside suppresses migration of fibroblasts from keloids: involvement of p38 kinase and PI3K signaling pathways

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ABSTRACT

Keloid is a specific skin scar that expands beyond the boundaries of the original injury as it heals. The invasive nature of keloid and notable migratory activity of fibroblasts are a hallmark, which distinguishes keloids from other common scars. Madecassoside, a triterpenoid saponin occurring in *Centella asiatica* herbs, possesses unique pharmacological properties to enhance wound-healing and diminish keloid formation. However, the effects of madecassoside on the formation of keloid scars have been poorly understood. Here, we focused on the potential of madecassoside on the migration of keloid-derived fibroblasts (KFs) and its mechanism. Primary KF, originating from human earlobe keloids, were purified and cultured, and then treated with madecassoside (10, 30, and 100 μM). In both transwell migration assays and scratch-wound-closure assays, KF migration was considerably suppressed by madecassoside pretreatment. Furthermore, KFs treated with madecassoside showed decreased F-actin filaments, as revealed by fluorescein isothiocyanate (FITC)-phalloidin staining and confocal microscopy. By Western blot analysis, madecassoside was shown to remarkably attenuate the phosphorylation of cofilin, p38 MAPK and phosphatidylinositol-3-kinase (PI3K)/AKT signaling, but only exhibited a minor effect on MMP-13 and little effect on ERK1/2 phosphorylation. It was concluded that madecassoside could be of great use in the treatment and/or prevention of hypertrophic scars and keloids.

1. Introduction

Keloid, partially considered as a benign skin tumor, represents the most extreme example of cutaneous scarring that exclusively distresses humans as a pathological response to wound healing [1]. It is defined as one kind of scars that spread beyond the boundaries of the original wound without regressing spontaneously [2]. The proliferation and migration of fibroblasts, essential for resurfacing areas of skin loss, are recognized to be closely involved in keloid formation by synthesizing extracellular matrix (ECM). Although there are no known cases of keloids transforming into malignant tumors, accumulating data have shown that the invasion activity of keloid fibroblasts (KFs) is similar to some extent to that of malignant cells [3]. KFs show higher migration pattern towards the midline of scratch wound in comparison with normal fibroblasts [1]. Due to the recurrence property, the clinical cure of keloid scar is extremely difficult. Although many strategies are available for keloid scars, none are fully effective [4].

Centella asiatica (also known as gotu kola and Hydrocotyle asiatica) is a perennial, herbaceous creeper, found in

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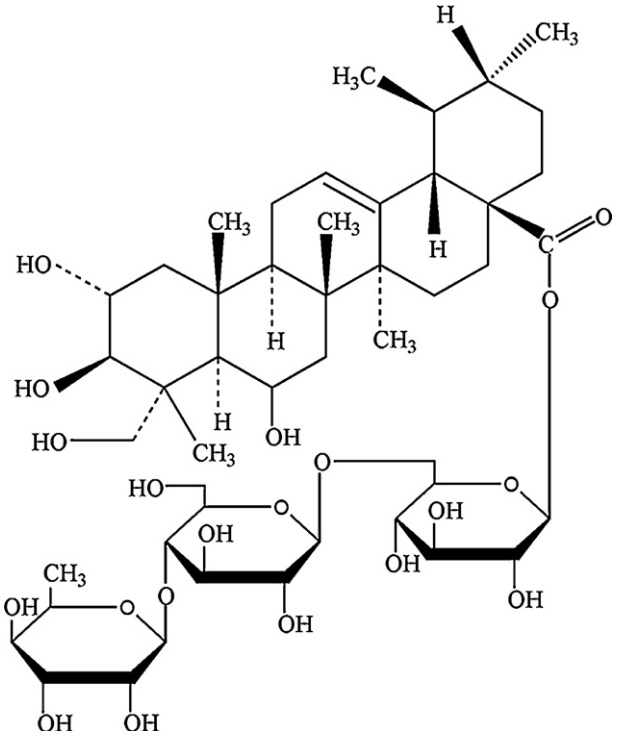

Fig. 1 – Chemical structure of madecassoside.

southeastern Asia and China, and has long been recommended for the treatment of keloids [5]. In one open clinical trial [6], 82% patients experienced relief of symptoms and disappearance of inflammation after treatment with *C. asiatica* herbs at doses of 60–150 mg daily. The herbs contain several active constituents, of which the most important are the triterpenoid saponins, including asiaticoside, centelloside, madecassoside, and asiatic acid [7]. Our previous study demonstrated that madecassoside (shown in Fig. 1), the highest content of triterpene constituent in *C. asiatica* herbs, induced apoptosis of KFs via a mitochondrial-dependent pathway [8]. Madecassoside was also reported to inhibit the proliferation of SVK-14 keratinocyte, an advantageous cell for studying skin psoriasis [9], and could suppressed LPS-induced TNF-alpha production in cardiomyocytes through inhibition of ERK, p38, and NF-kappa B activity [10]. The present study was performed to investigate the inhibitory effects of madecassoside on KF migration by scratch-wound-closure assays and transwell membrane assay in an attempt to get insight into the mechanisms for treating keloids.

2. Materials and methods

2.1. Chemicals and reagents

Madecassoside was isolated and purified by Dr. Gong Zhu-Nan (Nanjing Normal University, China) from *C. asiatica* extracts. In brief, the whole plants (2500 g) of *C. asiatica* were extracted by 25,000 mL of ethanol–water (75:25, v/v) solution. The solvent was removed from the extract under reduced pressure to obtain a semisolid mass (687.5 g). The residue was then dissolved with water and followed by petroleum ether, ethyl acetate, water-saturated *n*-BuOH extraction, respectively. The extract was concentrated under reduced pressure to dryness to obtain the *n*-BuOH extract (182.7 g). The *n*-BuOH extract was successively subjected to silica gel, ODS and Sephadex LH-20 column chromatography to give madecassoside (155 mg). The structure was established by comparison of its spectral data (UV, IR, MS, ^{1}H and ^{13}C NMR) with the literature data [11].The purity was determined to be above 98% by normalization of the peak areas detected by HPLC–ELSD. Tween 20, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), phenylmethylsulphonyl fluoride (PMSF), Type II collagenase, mitomycin c, and FITC-phalloidin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Newborn calf serum (NBCS) was purchased from PAA Laboratories (GmbH, Austria). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco by Invitrogen (Carlsbad, CA, USA). Transwell 8 μm pore size polycarbonate membrane was purchased from Corning Inc. (Acton, MA, USA). Matrigel was purchased from BD Bioscience (Bedford, MA, USA). MMP13, cofilin/p-cofilin, PI3K/p-PI3K, Akt/p-Akt, p38/p-p38, ERK/p-ERK and GAPDH antibodies were purchased from Nanjing Bioworld Biotech Co., Ltd. (Nanjing, China). The other chemicals and reagents used were of analytical grade.

2.2. Primary culture and treatment of human KFs

Primary KF cultures were established as described previously [12]. Earlobe keloid tissues were acquired from three patients undergoing surgical excision after obtaining informed consent. After rinsed in sterile phosphate-buffered saline (PBS) containing antibiotics, samples of keloid tissues were cut into pieces and incubated in DMEM containing 0.25% trypsin for 8 h at 4 °C. Then, after separation of the epidermis from the dermis, the minced dermis was subsequently digested in 1% collagenase II for 2 h at 37 °C. Afterwards, cells were pelleted and cultured in low-glucose DMEM supplemented with 10% (vol/vol) heat-inactivated NBCS, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C with 5% CO_2. KFs between passages 3–6 were used in the experiments.

2.3. In vitro scratch-wound-closure assay

In vitro scratch-wound-closure assays were performed as previously described [13]. KFs were seeded into 24-well dishes at a density of 10^5 cells per well. The dishes were cultured as confluent monolayers, and then treated with 10 mg/ml mitomycin c for 2 h before scratch-wounding with a standard 200 mL pipette tip. The wounded culture wells were washed twice with PBS to remove non-adherent cells, and treated with madecassoside (10, 30, 100 μM). Pictures were taken at 24 h later.

2.4. Transmembrane cell migration assay

For detection of fibroblast migration in the Transmembrane, invasion assays were carried out in 24-well format cell culture inserts with 8 μm pores, and the upper face of polycarbonate