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Dracorhodin perchlorate regulates fibroblast proliferation to promote rat's wound healing

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ABSTRACT

In recent years, plant-derived extracts are increasing interest from researchers worldwide due to good efficacy and lower side effects. Among the different plant extracts, Dracorhodin perchlorate (DP) is originated from *Dragon's blood* which has long been used as a natural medicine with various pharmacological activities. In the present study, we have explored the potential regulation of DP on fibroblast proliferation which promotes wound healing both *in vitro* and *in vivo*. DP at treatment of 12–24 h significantly induced fibroblast proliferation which is associated with increasing level of phosphorylated-extracellular signal-regulated kinase (ERK). Moreover, if ERK is halted with siRNA, DP cannot induce fibroblast proliferation. *In vivo*, DP ointment treatment at low- (2.5 μg/mL), medium- (5 μg/mL) and high- (10 μg/mL) doses, rat wounds healed more rapidly compared with the control group. After DP treatment for 7 days, Serpin family H member 1 (SERPINH1) staining confirmed enhanced fibroblast proliferation in the wound tissue. Finally, phosphorylated-ERK in the wound tissue remarkably increased with DP ointment treatment. Therefore, DP may be developed into a potential lead compounds for the treatment of wounds in clinical trials in the near future.

1. Introduction

With the rapid development of medical field research, more and more natural plants are used to study in treating clinical ailments. The most well-known of them is artemisinin, extracted from the stem of Artemisia annua, which has saved millions of malaria patients' lives.[1] In addition, ginseng extracts are widely used for various treatment of ailments including cancer.[2] Berberine can inhibit bacterial growth.[3] Morphine has analgesic effects.[4] *Dragon's blood* is a resin obtained from the fruit of a number of palmae plants predominantly distributed throughout Asia and Indonesia.[5] *Dragon's blood* is regarded as a natural medicine for improving blood circulation and reducing pain.[6] Research also showed that *Dragon's blood* possessed several pharmacological effects, including immunoregulatory,[7] anti-inflammation,[8] antidiarrheic,[9] antibacterial,[10] antiviral,[11] antioxidant,[12] and anticancer,[13] and so on. Interestingly, *Dragon's blood* can stimulate epithelial regeneration and wound healing by promoting fibroblasts proliferation and collagen synthesis.[14] *Dragon's blood* extracts comprise of many active compounds, mainly flavonoids and terpene resin acid. As the extract of the *Dragon's Blood*, *Dracorhodin* always exists in the form of a particular salt, which is called Dracorhodin perchlorate (DP). Recent research has demonstrated that DP promotes vascular endothelial cell proliferation and angiogenesis.[15] However, research on whether DP promote wound healing has not been reported. The purpose of this study was to determine the effects of DP on wound healing using both *in vitro* and *in vivo* models.

Skin damage initiates a series of biological reactions to achieve self-healing, including hemostasis, granulation tissue regeneration, and tissue reconstruction.[16] Various kinds of cells including fibroblasts, endothelial cells, keratinocytes, macrophages and platelets play key roles together in wound healing process. This process involves cell proliferation and migration, collagen production, and vascular formation. Fibroblasts are the most vital cells for

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extracellular matrix production and remodeling, which are beneficial for forming wound granulation tissues in wound healing.[17,18] At the same time, wound healing process is regulated by a large number of cytokines and growth factors, which are important regulators to elicit the cell growth, proliferation, migration, differentiation and adhesion, ECM deposition and proteinase activation. Moreover, significant study has demonstrated that cell proliferation is mediated by ERK signaling pathway in the process of wound healing.[19,20] We thus hypothesized that DP may activate cellular functions through ERK pathways, and the activation of ERK signaling pathway may alleviate the proliferation and migration of fibroblasts.

SERPINH1 (serpin peptidase inhibitor, clade H, member 1, also known as HSP47), a 47-kDa heat-shock protein, is a collagen-specific molecular chaperone localized in the endoplasmic reticulum research showed that SERPINH1-positive cells were regarded as fibroblasts in skin specimens.[21] Therefore, we selected the SERPINH1 antibody as a marker of fibroblast in skin tissues samples. This study aimed to identify the mechanisms whether DP induces proliferation and migration of fibroblasts during wound healing. Our results demonstrated that DP may be considered as potential treatments for wound healing and provided a basis for further clinical experiments.

2. Materials and methods

2.1. Cell viability and cell proliferation assays

DP was purchased from Shanghai yuanye Bio-Technology Co., Ltd (R20J6F1, Shanghai, China). NIH/3T3 fibroblasts were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Fibroblasts at passage 5–9 were used experimentally. Trypsin (0.25%, Gibco, Germany) was used to digest the fibroblasts until the cells were all resuspended. Fibroblasts were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated for 12 h in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, USA) containing 10% fetal bovine serum (FBS) (complete medium) (Gibco, Germany) at 37 °C in a humidified, 5% CO_2 atmosphere. After 12 h incubation, the complete medium was removed from each well and replaced with DMEM in absence of FBS (starvation medium) prior to a further 3 h incubation under identical growth conditions. After starvation for 3 h, complete medium containing DP at concentrations of 0 (control), 0.625, 1.25, 2.5, 5, 10, 20, and 40 μg/mL (n = 5 wells in triplicate plates) was added into every well respectively with 24 h treatment. In addition, 50 ng/mL epidermal growth factor (EGF) was regarded as the positive control group. At present, it has been reported that EGF gel promoted wound healing in rat model.[22] Many studies have reported that EGF significantly stimulated fibroblast proliferation, which is beneficial for wound healing.[23] After DP treatment, the cell counting kit-8 (CCK-8) assay was used to assess cell viability. Viable cells were measured at an absorbance (Abs) of 450 nm using enzyme-linked immune detector (Gene, China). Effective concentrations of DP were identified using the above experiment. NIH/3T3 cells were also treated with 0 (control), 1.25, 2.5, and 5 μg/mL DP for 0, 6, 12, 24 and 36 h separately and cell proliferation was determined using the CCK-8 as described previously. Cell viability was determined as follows:

$$\text{Cell viability}(\%) = \frac{\text{Abs of DP treated group}}{\text{Abs of control group}} \times 100\%$$

2.2. Western blot analysis

Phosphorylation of ERK in NIH/3T3 fibroblasts was determined after treatment with 2.5 μg/mL DP for 0, 15, 30, 60, and 120 min. At each time point, the total protein in cells was harvested and the proteins concentrations was detected dividely by using the Enhanced BCA Protein Assay Kit. The rest of the cell suspension was then aspirated and mixed with the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer in a ratio of 1: 4. Equal amounts of proteins were separated by SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated in tris-buffered saline (TBS) containing 5% skimmed milk and 0.1% Tween-20 for 60 min, and blotted with primary antibodies at 4 °C overnight.

The following primary antibodies were used: anti-phospho-ERK1/2 (1:1000, No. 4370, Cell Signaling Technology, MA, USA), anti-ERK1/2 (1:1000, No. 4695, Cell Signaling Technology), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 1:1000, No. 97166, Cell Signaling Technology). The membranes were incubated for 2 h with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2000, Cell Signaling Technology). Reaction products were visualized by detection of chemiluminescence using an ECL Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA). Quantification of relative band densities was performed by scanning densitometry using Image J software (National Institute of Health, Bethesda, MD, USA). Gray intensity of p-ERK/ERK was calculated by the following formula:

$$\text{Gray intensity of p}-\text{ERK/ERK} = \frac{\text{Gray intensity of p}-\text{ERK}}{\text{Gray intensity of ERK}}$$

2.3. RNA interference experiments

To further verify whether DP promoted fibroblast proliferation via the ERK pathway, siRNA for ERK (No. 6560, Cell Signaling Technology, USA) was selected to block ERK signal (forward sequence: CCUCCAACCUGCUCAUCAA; reverse sequence: UUGAUGAGCAGGUUGGAGG). NIH/3T3 fibroblasts were transfected with ERK siRNA using Lipofectamine2000 (Lipo2000, Invitrogen, Japan) and OPTI-MEM (Invitrogen, Japan) according to the manufacturer's protocol. The final concentration of siRNA (ERK) was 100 nM, siRNA control (No. 6568, Cell Signaling Technology, USA) and Lipo2000 were used as control groups. The transfected cells were used experimentally after 48 h. All cells were treated with or without 2.5 μg/mL DP. The experiments were divided into control groups (siRNA control using Lipo2000, with or without DP treatment), Lipo2000 control groups (only Lipo2000, with or without DP treatment) and siRNA ERK groups (siRNA ERK using Lipo2000, with or without DP treatment). The ERK expression was detected after 120 min drug treatment and cell viability was detected after 24 h drug treatment.

PD98059 (9900S, Cell Signaling Technology, USA) is regarded as an ERK signal inhibitor and can reduce the level of ERK phosphorylation. The four experimental groups included[1] control group (without DP and inhibitor),[2] inhibitor group (PD98059 only),[3] drug-treated group (2.5 μg/mL DP only), and[4] drug-inhibitor group (2.5 μg/mL DP + PD98059). The inhibitor was diluted with complete medium and exposed to cells for 24 h. And then every group was treated with or without DP for 120 min. Densities of p-ERK and ERK were examined by Image J software. The density ratio of p-ERK/ERK was calculated and cell viability was assessed by CCK-8 in all groups.

2.4. In vivo rat wound models to determine the effect of DP in wound healing

The design of the current experiment had restrictively followed the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, International. After getting the

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