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The effects of glycyrrhizic acid and glabridin in the regulation of CXCL5 inflammation gene on acceleration of wound healing

Hong Yung Yip, Melissa Su Wei Poh, Yoke Yin Chia*

School of Biosciences, Division of Medicine, Pharmacy and Health Sciences, Taylor's University, No. 1 Jalan Taylor's, 47500, Subang Jaya, Selangor Darul Ehsan, Malaysia

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ABSTRACT

Objective: To evaluate the anti-inflammatory property of both glycyrrhizic acid (GA) and glabridin in reducing inflammation to accelerate wound regeneration on 3T3-L1 and NIH-3T3 fibroblast cell lines.

Methods: Cell proliferation and viability assay (MTT assay), scratch wound healing assays, and quantitative real-time PCR were conducted to investigate the effects on cell proliferation, cell migration, and expression of CXC chemokine ligand 5 inflammation gene respectively.

Results: Results showed that at a low concentration of 1×10^{-8} mol/L, glabridin down regulated cell proliferation in NIH-3T3 significantly, suggesting its involvement in ERK1/2 signaling pathway. GA and glabridin significantly accelerated cell migration through wound healing in both 3T3-L1 and NIH-3T3 and significantly down regulated the expression of CXC chemokine ligand 5 in 3T3-L1 at concentration 1×10^{-8} mol/L, indicating the possible involvement of nuclear factor-κB and cyclooxygenase 2 transcriptions modulation.

Conclusions: Both GA and glabridin can serve as potential treatment for chronic inflammatory disease, and glabridin as an oncogenic inhibitor due to its anti-proliferative property.

1. Introduction

Wound healing is a complex biological event due to the interplay of various tissue structures and many resident and infiltrating cell types that serve as both inflammatory and immunological effector cells. It consists of four phases: hemostasis, inflammation (0–3 days), migration and proliferation (3–12 days), and tissue remodeling (maturation) (3–6 months) that influence the anabolic and catabolic reactions of tissue repair. Profound study especially on chemokines which involved in the specific trafficking and recruitment of leukocytes during inflammation phase is essential to illustrate the dynamic correlation between inflammation, cell proliferation and migration [1].

Leukocyte subsets such as neutrophils which act as immunological effector cells migrate immediately to the wound surface to form a dense barrier against infecting pathogens, which often associated intimately with the onset of acute inflammation. However, prolonged inflammation will lead to undesirable pathogenesis [2]. Chemokines of the CXC family, particularly CXC chemokine ligand 5 (CXCL5) has been reported as key regulatory links between inflammation and angiogenesis corresponding to CXC chemokine receptor 2 (CXCR2) [3,4]. Major efforts have focused in finding efficient and novel therapeutic agents. The use of antiseptics and antibiotics are the current wound healing treatments that work in preventing and ceasing of infection but do not directly involve in supporting the wound healing process.

Prior study showed that liquorice (*Glycyrrhiza glabra*) was a potent healing agent in open skin wounds in rabbits [5]. These plant-derived compounds: glycyrrhizic acid (GA) and glabridin from liquorice extract contain anti-oxidant and anti-inflammatory properties that could be potential therapeutic candidate to accelerate wound healing. It is hypothesized that anti-inflammatory compounds could down regulate

*Corresponding author: Yoke Yin Chia, School of Biosciences, Division of Medicine, Pharmacy and Health Sciences, Taylor's University, No. 1 Jalan Taylor's, 47500, Subang Jaya, Selangor Darul Ehsan, Malaysia.
Tel: +603 5629 5650
Fax: +603 5629 5455
E-mail: AdelineYokeYin.Chia@taylors.edu.my

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inflammation phases during wound healing and thus, improve wound closure. Present study aimed to evaluate the effects of GA and glabridin on wound healing by accessing the cell proliferation, cell migration and CXCL5 gene expression in 3T3-L1 and NIH-3T3 mouse fibroblast cell lines.

2. Materials and methods

2.1. Cell culture and maintenance

NIH-3T3 mouse fibroblast and 3T3-L1 pre-adipocyte cell lines were adopted due to their angiogenesis nature. They were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, USA) supplemented with 10% fetal calf serum. Phosphate-buffered saline (PBS; Sigma–Aldrich, USA) was used to rinse and wash the cells prior to trypsinization with trypsin (Sigma–Aldrich, USA) to detach the cells during sub-culturing, passaging and seeding.

2.2. Cell proliferation and viability assay (MTT assay)

Both NIH-3T3 and 3T3-L1 cell lines were harvested at 80%–90% confluence and seeded into 96 well plates at density 1.5×10^4 cells. Twenty-four hours post seeding, 150 μL of culture medium was replaced with equal volume of GA and glabridin respectively at concentrations ranging from 1×10^{-4} mol/L to 1×10^{-10} mol/L. After 24 h of incubation, MTT solution (10 μL of 5 μg/mL MTT in sterile PBS) was added and subsequently incubated for another 3.5 h. The supernatant was decanted and 100 μL of dimethyl sulfoxide was added to each well. The absorbance at 595 nm was measured using a micro plate reader. The assay was carried out in triplicates [6]. Results were analyzed statistically using statistical software, IBM SPSS Statistics version 20 via independent *t*-test with control: DMEM medium with 0.1% absolute ethanol. Results were marked significant at $P < 0.05$ and $P < 0.01$.

2.3. Scratch wound healing assay

NIH-3T3 and 3T3-L1 fibroblast cell lines were seeded into 6 well plates at density of 2.0×10^5 cells. Pipette tip (size: 10–200 μL) was used to create the wound after 24 h of cell seeding. Each well was first washed with sterile PBS and subsequently 2 mL per well of GA and glabridin were added separately into each well with concentrations ranging from 1×10^{-5} mol/L to 1×10^{-9} mol/L with DMEM medium with 0.1% absolute ethanol as negative control. Images of wound closure were captured and the measurement of each wound gap was taken at a treatment interval of 6, 12 and 24 h in five replicates. The percentage of wound closure was calculated based on the formula below [7]:

$$[(\text{Initial wound size at 0 treatment hour} - \text{Specific interval of wound size})/\text{Initial wound size}] \times 100\%$$

Results obtained were analyzed statistically using statistical software, IBM SPSS Statistics version 20 via independent *t*-test and marked significant at $P < 0.05$.

2.4. RT-qPCR quantification of total CXCL5 gene expression

2.4.1. Seeding and treatment of cell culture

Both NIH-3T3 and 3T3-L1 cell lines were seeded at density of 1×10^6 into culture dishes. After 24 h of incubation, cells were then washed with sterile PBS and treated with 3 mL volume of GA and glabridin at concentration that induced the best migration rate. DMEM medium with 0.1% absolute ethanol was added as control and treatments were done in triplicates.

2.4.2. Total mRNA extraction from cell lines

Total mRNA extraction for both control and treated cell lines were done using FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit. The purity and quantity of total RNA extracted was checked using Beckman Coulter NanoDrop Spectrophotometer (USA) at wavelength 280 nm and 260 nm. The integrity of the total RNA extracted was also being assessed by denaturation at 72 °C for 10 min followed by visualization using 2% agarose gel electrophoresis run at 80 V for approximately 40 min.

2.4.3. cDNA synthesis

The extracted RNA samples were incubated with RNase-Free DNase (Promega RQ1 RNase-free DNase) reaction mixture at 37 °C for 30 min. A volume of 1 μL of RQ1 DNase Stop Solution was added to stop the reaction followed by 10 min incubation at 65 °C. The treated total RNA was then used to synthesize cDNA using qPCRBIO cDNA Synthesis Kit (PCR Biosystems, United Kingdom). The RNA samples were incubated at 42 °C for 30 min followed by 85 °C for 10 min to denature the RTase.

2.4.4. Primer design for mouse total CXCL5 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene

Gene specific forward and reverse primers were designed for mouse total CXCL5 and GAPDH gene (Table 1) by Primer3 BioTools and cross-checked via National Centre for Biotechnology Information website using the standard nucleotide Basic Local Alignment Searching Tool program.

Expression of the respective genes was quantified using the optimized RT-qPCR parameters and protocols (Table 2) by 2 × qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, United Kingdom) in 10 μL reaction mixtures. All RT-qPCR and analysis were carried out on Eppendorf Mastercycler® ep realplex PCR thermal cycler (Germany).

The comparison of mouse total CXCL5 expression between control, GA-treated and glabridin-treated cell samples were performed using the comparative CT (ΔΔCT) method [8]. The

Table 1
Characteristics of primers for total CXCL5 and GAPDH amplification.

Primer	Sequence (5′->3′)	Length (bp)	T_m (°C)	Amplicon size (bp)
Mouse CXCL5 forward primer	TTCCTCAGTCATAGCCGCAA	20	56.3	168
Mouse CXCL5 reverse primer	GGATCCAGACAGACCTCCTTC	21	56.6	168
Mouse GAPDH forward primer	GTTTCCTCGTCCCGTAGACA	20	56.4	200
Mouse GAPDH reverse primer	CCTTGACTGTGCCGTTGAAT	20	55.7	200