Glycyrrhizin Ameliorates Imiquimod-Induced Psoriasis-like Skin Lesions in BALB/c Mice and Inhibits TNF-α-Induced ICAM-1 Expression via NF-κB/MAPK in HaCaT Cells

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Key Words
Glycyrrhizin • ICAM-1 • NF-κB • MAPKs • Imiquimod • Inflammation • Keratinocytes • Psoriasis

Abstract
Background/Aim: Glycyrrhizin (GL) is an important derivative of certain herbal medicines used in Asian countries. Currently, GL is used to treat hepatitis and allergic disease worldwide because of its anti-viral and anti-allergy effects. In addition to these prominent functions, GL likely regulates cellular functions such as tumor cell growth and cellular immunity. However, how GL affects the keratinocyte inflammation response remains poorly understood. The current paper investigates the effect of GL on psoriasis and explores the mechanisms involved. Methods: We used an in vitro cell model of tumor necrosis factor (TNF)-α-induced keratinocyte inflammation and the topical application of imiquimod (IMQ) using an animal model (mouse skin) of IMQ-induced psoriasis-like inflammation (IPI) to investigate the effect of GL on skin inflammation. Cell viability was analyzed using the Cell Counting Kit-8 (CCK8). Carboxyfluorescein succinimidyl ester (CFSE) labeling was used to trace monocyte adherence to keratinocytes. A Western blot analysis was used to detect the expression of intercellular adhesion molecule 1 (ICAM-1) and the activation of the nuclear factor (NF)-κB/mitogen-activated protein kinase (MAPK) signaling pathway. A modified version of the Psoriasis Area Severity Index (PASI) was used to monitor disease severity. Hematoxylin and eosin (H&E) staining was used to observe pathological changes. An immunohistochemistry (IHC) analysis was used to detect ICAM-1 expression in mouse skin. Results: GL treatment significantly

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reduced the levels of ICAM-1 in TNF-α-stimulated HaCaT cells, inhibited subsequent monocyte adhesion to keratinocytes, and suppressed the nuclear translation and phosphorylation of p65 following the degradation of inhibitor κB (IκB). GL treatment blocked the phosphorylation of extracellular signal-regulated kinase (ERK)/p38 MAPK. GL effectively delayed the onset of IPI in mice and ameliorated ongoing IPI, thereby reducing ICAM-1 expression in epidermal tissues.

**Conclusions:** These results demonstrate that GL treatment ameliorates skin inflammation by inhibiting ICAM-1 expression via interference with the ERK/p38 MAPK and NF-κB signaling pathways in keratinocytes. Therefore, GL can be used as an anti-psoriasis drug.

### Introduction

Psoriasis is a common chronic inflammatory skin disorder that primarily affects the epidermis [1]. Keratinocytes, the principal cell type of the epidermis, play an important role in the pathogenesis of inflammatory skin disease. A consensus now exists that the crosstalk between keratinocytes and immunocytes regulates skin inflammation in psoriasis [2]. Upon stimulation due to inflammatory cytokines (e.g. tumour necrosis factor alpha; TNF-α), epidermal keratinocytes express intercellular adhesion molecule-1 (ICAM-1, CD54). The up-regulation of ICAM-1 on the surface of keratinocytes increases lymphocyte infiltration into inflamed areas [3, 4]. This infiltration is one of the most critical steps involved in the development of psoriasis. Therefore, the modulation of ICAM-1 expression provides a target for the development of therapeutic agents against psoriasis.

Glycyrrhizin (GL) is a glycoconjugated triterpene (Fig. 1) isolated from the root of an oriental herbal medicine, the licorice plant (*Glycyrrhiza glabra*) [5]. GL has been used as a traditional Chinese medicine for many centuries to treat allergic disease [6]. In 1946, Revers [7] reported the anti-ulcer effects of licorice. Since then, GL has been used as an anti-ulcer drug in Europe. Furthermore, GL has been used for more than 20 years to treat patients with chronic hepatitis given its anti-virus effects [8, 9]. Recently, GL has been found to treat SARS-associated coronavirus [10]. In addition, GL might regulate cellular functions such as anti-tumor/cell growth activities, antioxidation [11] and free radical-scavenging activities [12]; it might also show anti-angiogenic effects [13] and lead to immune responses [14]. GL and its derivatives have been used recently to treat inflammatory skin diseases with low toxicity. Nevertheless, the molecular details regarding the effects of GL on psoriasis are poorly understood.

In this paper, we investigate the effect of GL on psoriasis. We treated human keratinocytes with TNF-α to elicit an inflammatory response. We observed that GL significantly suppressed the TNF-α-induced protein expression of ICAM-1 and inhibited TNF-α-induced monocyte adhesion to HaCaT cells. In addition, GL significantly inhibited the TNF-α-induced activation of the NF-κB and extracellular signal-regulated kinase (ERK)/p38 mitogen-activated protein kinase (MAPK) signaling pathways. These findings prompted us to test whether GL could be used to treat psoriasis. We used an *in vivo* mouse model of imiquimod (IMQ)-induced psoriasis-like inflammation (IPI) to investigate the effect of GL on psoriasis. We found that the GL treatment effectively reduced the onset of IPI lesions in mice; furthermore, the administration of GL significantly decreased the level of inflammation in IPI-inflicted mice. We also investigated the molecular mechanisms that underlie the GL-mediated effects of IPI and found that GL attenuated ICAM-1 expression in the lesions of IMQ-treated mice. Therefore, this study revealed a novel anti-inflammation function of GL with regard to psoriasis by modulating ICAM-1 expression via the NF-κB and ERK/p38 MAPK pathways in keratinocytes. Our study addresses how GL regulates skin inflammation and suggests that a new type of drug should be developed to inhibit ICAM-1 expression to treat inflammatory skin disease.
Materials and Methods

Cell cultures
The human keratinocyte cell line, HaCaT, and the human monocytic leukemia cell line, THP-1, were stored in our lab. HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, USA), and the THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.4 mM L-glutamine (Corning Cellgro, USA). All cells were maintained in a humidified incubator at 37°C with 5% CO₂. TNF-α (10 ng/ml, Peprotech, USA) was used for stimulation.

Cell viability assays
To investigate the toxicity of GL, cell viability was assessed using the Cell Counting Kit-8 (CCK8) assay (Dojindo Laboratory, Japan) according to the manufacturer's instructions. Briefly, HaCaT cells were seeded into 96-well plates (1×10⁶ cells per well) and treated with various concentrations of GL for 24 h. Next, 10 μl of CCK8 was added to each well, and the cells were incubated for 2 h at 37°C. Absorbance was assessed at 450 nm with a microplate reader (Bio-Tek, USA). The mean optical density (OD) of the three wells in each group was used to calculate the percentage of cell proliferation as follows:

Cell proliferation (%) = (OD treatment group - OD blank)/(OD control group - OD blank) × 100

In vitro cell adhesion assay
HaCaT cell were seeded into 24-well plates (1×10⁵ cells per well) and treated with the indicated conditions. Equal volumes of carboxyfluorescein succinimidyl ester (CFSE; 10 μM, AnaSpec, USA) and THP-1 (2×10⁶/ ml) were mixed quickly. After incubated for 7 minutes at room temperature, the staining was stopped with FBS by adding 10% FBS RPMI-1640. Then, the THP-1 cells were washed with 10% FBS RPMI-1640 twice, and resuspended with 10% FBS DMEM. CFSE-labeled THP-1 (1×10⁶ per well) cells was co-cultured with HaCaT cells under the indicated conditions for 1 h, following with three washes with phosphate-buffered saline (PBS). Fluorescence was observed and captured using an Olympus inverted microscope with a fluorescence illumination source. The photographs shown are representative of each experiment.

Immunoblotting assay

For the total protein extraction, HaCaT cells were lysed with a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS] and 1% neutrophil
proteinase 4 [NP4]) mixed with protease and phosphatase inhibitors (Roche, Germany). A Cytoplasmic and Nuclear Protein Extraction Kit (Boster, China) was used to achieve nuclear-cytoplasmic separation. Antibodies against p-P65, P65, IKB-α, p-ERK, ERK, c-Jun N-terminal kinase (JNK), p-JNK, p-P38, P38, ICAM-1 (1:1,000 dilution, Cell Signaling Technology, USA), Lamin B1 and β-tublin (1:5,000 dilution, Epitomics, USA) were used for immunoblotting according to the manufacturers’ protocols.

**Experimental animals**

Female BALB/c mice (8-10 weeks old) were obtained from the Animal Experiment Center at Sun Yat-Sen University (Guangzhou, China). All of the mice used for this research were housed and bred in a specific pathogen-free colony at this center. All animal experiments were performed in accordance with the National Institutes of Health Guidelines on Laboratory Research and approved by the Animal Care Committee at Sun Yat-Sen University.

**IPI induction and treatment**

The mice were administered a daily topical dose of 62.5 mg of 5% IMQ (Aldara; 3M Pharmaceuticals, UK) to a 3 cm × 4 cm shaved area on their backs for 7 consecutive days. The severity of their IPI was monitored and graded using a modified human scoring system, the Psoriasis Area and Severity Index (PASI), which consists of measures for skin erythema, scaling and thickness. Each parameter was scored independently on a scale from 0 to 4, where 0 = no clinical signs; 1 = slight clinical signs; 2 = moderate clinical signs; 3 = marked clinical signs; and 4 = very marked clinical signs. The cumulative score denotes the severity of inflammation. To prevent IPI development, GL was prepared at 20 mg/ml in PBS and injected intraperitoneally (IP) at a dose of 50 mg/kg on day 0, continuing through day 7. At the end of the experiment, all of the mice were sacrificed, and samples of their skin were collected for additional experiments.

**Histopathological and immunohistochemical (IHC) examinations**

The skin samples from back lesions were collected in 10% formalin and embedded in paraffin. For the histopathological examination, 4-μm sections were stained with hematoxylin and eosin (HE) and observed under a light microscope (Olympus, Tokyo, Japan). For the IHC examination, 4-μm sections were dewaxed and rehydrated followed by endogenous peroxidase quenching, antigen retrieval (saline sodium citrate, microwaving) and non-specific binding site blockade. Anti-mouse ICAM-1 antibody (Bioss, China) was used at a 1:50 dilution. All procedures were conducted in accordance with the instructions for the IHC kits bought at Zhongshan Jinqiao (China). This company serves as an agent for foreign biological companies such as Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Statistical analyses**

Data from at least triplicate sample determinations were expressed as the mean ± the standard error. Differences between the control and experimental conditions were determined using Student’s t-tests; p-values < 0.05 were considered significant.

**Results**

**GL inhibited TNF-α-induced ICAM-1 expression and monocyte adhesion to HaCaT cells**

To study how GL influences keratinocyte function, we first investigated whether isolated GL affected HaCaT cell viability using the CCK8 assay in HaCaT cells. Under culture conditions (Fig. 2), GL showed a non-toxic range at doses of 2-200 μg/ml; however, it was toxic to keratinocytes at higher doses (> 300 μg/ml). Therefore, we used a GL dose of 2-200 μg/ml for the following studies with cultured HaCaT cells.

Mounting evidence suggests that the up-regulation of adhesion molecules on the surface of epidermal keratinocytes plays an essential role in skin inflammation [15, 16]. In particular, ICAM-1 is a marker of inflammatory skin diseases. In addition, the pharmacological inhibition of ICAM-1 is used to treat inflammatory skin disease [17]. Therefore, we investigated the effect of GL on TNF-α-induced ICAM-1 expression in HaCaT cells. We initiated an inflammatory response by exposing keratinocytes to TNF-α for 12 h following 12 h of pretreatment with
GL. Then, ICAM-1 protein expression was measured by a Western blot analysis. As shown in Fig. 3A and B, TNF-α induced ICAM-1 expression, whereas GL significantly suppressed TNF-α-induced ICAM-1 expression; the effect became stronger as the dose increased. In addition, we explored the effect of GL on the adhesion of monocytes to keratinocytes. As shown in Fig. 3C and D, GL significantly inhibited TNF-α-induced monocyte adhesion to HaCaT cells in a dose-dependent manner. This finding suggests that GL directly affects the inflammatory process in keratinocytes.
Xiong et al.: Glycyrrhizin Ameliorates Skin Inflammation In Vitro and In Vivo

The GL suppression of NF-κB activation in HaCaT cells in response to TNF-α

To further investigate the anti-inflammatory effects of GL, we studied the effects of GL on the signaling pathways induced by TNF-α in detail. NF-κB signal modulation is a prominent therapeutic target for inflammatory diseases because aberrantly activated NF-κB signaling contributes to most human skin inflammation disorders [6, 18]. TNF-α can activate the NF-κB signaling pathway to induce ICAM-1 expression [17, 19]. To elucidate the mechanism of GL in the suppression of the inflammation induced by TNF-α, we examined the effects of GL treatment on the NF-κB signaling pathway in human keratinocyte cytoplasmic extracts. Nuclear extracts from TNF-α-stimulated HaCaT cells were assayed using a Western blot analysis. In resting HaCaT cells, p65 was found in the cytoplasm and showed a low basal...
level in the nucleus. When the cells were exposed to TNF-α, p65 was phosphorylated, then translocated from the cytoplasm to the nucleus. GL treatment significantly inhibited this process (Fig. 4A and B). Similarly, we found that pretreatment with GL effectively inhibited the degradation of the inhibitory protein IκB in the cytoplasm (Fig. 4C). These results suggest that GL exerts an anti-inflammatory effect by inhibiting the nuclear translocation and phosphorylation of p65 following the inhibition of IκB degradation in keratinocytes.

The effect of GL on MAPK pathway activation in TNF-α-stimulated HaCaT cells

TNF-α is a pleiotropic cytokine that activates various signaling pathways. MAPK is a signaling pathway in keratinocyte inflammation [20]. We examined the effect of GL on TNF-α-induced MAPK activation. TNF-α exposure quickly activated ERK, JNK and p38 in HaCaT cells (see Fig. 5). Interestingly, the pretreatment of keratinocyte cells for 12 h with various

Fig. 5. The effect of GL on the TNF-α-induced activation of MAPKs in HaCaT cells. HaCaT cells were pretreated with GL for 12 h and then stimulated with TNF-α at 10 ng/ml for 20 min. The total cell lysates were subjected to a Western blot analysis using specific antibodies for phospho-ERK (Thr202/Tyr204), total ERK, phospho-p38 (Thr180/Tyr182), total p38, phospho-JNK (Thr183/Tyr185), and total JNK. (A) Representative image. (B, C, and D) The densitometry analyses of the immunoblotting results. The means ± SDs of 3 individual experiments are plotted (*p < 0.05, **p < 0.01 and ***p < 0.001 compared with TNF-α treatment alone).
concentrations of GL selectively attenuated the activation of ERK and p38 MAPK in a dose-dependent manner; however, pretreatment had a minimal effect on JNK phosphorylation (Fig. 5A). Taken together, our results suggest that GL suppressed keratinocyte inflammation by selectively inhibiting the phosphorylation of the ERK/p38 MAPK signal cascades.

Fig. 6. GL treatment ameliorated IPI. (A) Phenotypical presentation (left) and corresponding histological analyses (H&E staining) of mouse back skin (right) are shown. IPI was elicited in mice via a topical IMQ application for 7 days. In each group, the mice were injected with either PBS or GL. The photographs were taken on day 7. Bar = 100 μm. (B) Clinical scores for erythema, (C) skin thickness, and (D) scaling on the indicated days (score range: 0 to 4). (E) The cumulative scores. The standards are described in the methods section. The data are presented as the means ± SDs (n = 3-4 per experiment). *p < 0.05 versus PBS-injected mice following IMQ application. (F) IHC staining for the ICAM-1 in mouse back skin are shown. Skin was harvested from PBS-treated (left) and GL-treated (right) IPI mice. Bar = 250 μm.
GL ameliorates the severity of IPI

The finding that GL regulated the keratinocyte inflammation reaction prompted us to investigate how GL treatment influences the development of inflammatory skin diseases such as psoriasis. We used IPI, a new murine model of human psoriasis to address this question.

We identified a nontoxic dose (20 mg/kg) of GL in mice. Compared with sham cream-treated mice, GL-treated mice did not show obviously decreased weights (data not shown). As shown in Fig. 6B, C, and D, on days 3-5 after the IMQ challenge, the back skin of IMQ-treated mice began to display erythema (day 3), thickening (day 4), and scales (day 5). From days 3-5 onward, inflammation was visible, and it increased in severity up to day 7. These findings are in agreement with the literature [21]. Similar observations were made with regard to the GL-treated IPI model mice. However, the whole process was delayed at least 1 day compared with the mock-treated IPI model mice. In addition, the clinical scores of the GL-treated mice were much lower than those of the mock-treated mice (Fig. 6E). The histopathological results (Fig. 6A) from the IMQ-treated mouse skin showed increased epidermal hyperplasia, elongated “rete-like” ridges, acanthosis in the epidermis and perivascular infiltration of the inflammatory cells in the upper dermis similar to the phenotypes common in human psoriatic skin. GL attenuated most of these changes; however, not much improvement was observed with regard to hyperkeratosis. These results suggest that the administration of GL effectively delays the onset of IPI in mice and ameliorates IPI progression, which indicates that it can be used to treat psoriasis.

The effect of GL on ICAM-1 expression in skin

The aforementioned in vitro finding suggests that GL is an effective agent in the regulation of the keratinocyte inflammation reaction via the suppression of ICAM-1 expression. Thus, we focused our analysis on the ICAM-1 expression in the lesions of IPI-afflicted mice. Interestingly, ICAM-1 expression was notably reduced in GL-treated IPI mice compared with mock-treated IPI mice (Fig. 6F). These results might provide additional evidence suggesting that GL inhibits ICAM-1 expression in the epidermis during the inflammatory response. Therefore, GL treatment effectively ameliorated IPI and was associated with reduced epidermal ICAM-1 expression.

Discussion

Natural plant ingredients are often used as novel therapeutic agents to control inflammation with few side effects. However, few studies have sought to identify and characterize these natural plant compounds, thereby hindering their clinical use. GL, a triterpene glycoside isolated from licorice root, is a promising physiochemical used to regulate the inflammation reaction. GL inhibits the inflammatory response in several cell types, including mammary epithelial cells [22], RAW264.7 cells [23], and human umbilical vein endothelial cells [14]. However, the underlying mechanisms of inhibition, especially those with regard to skin inflammation, remain poorly defined. The present study marks the first in vitro and in vivo investigations of the effects of GL on skin inflammation. We found that GL showed slight side effects on the keratinocytes in vitro at effective doses; furthermore, and in vivo GL administration produced minimal toxicity. At appropriate doses, GL can suppress the TNF-α-induced up-regulation of ICAM-1 in a dose-dependent manner. In addition, GL can significantly inhibit monocyte. Similarly, GL can suppress Jurat T cell adhesion at 200μg/ml although no effect at lower dose (data not shown).

Psoriasis is a classic type I autoimmune skin disease with activated immune pathways. As the major cell type involved in skin immunity, epidermal keratinocytes can direct the migration of the immunocytes that are recruited to the skin by secreting cytokines, chemokines or adhesion molecules in an antigen-specific manner, thereby leading to increased
Xiong et al.: Glycyrrhizin Ameliorates Skin Inflammation In Vitro and In Vivo

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2015;35:1335-1346
DOI: 10.1159/000373955
Published online: February 12, 2015
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www.karger.com/cpb

Cytokine production [24]. ICAM-1, which can be strongly induced on various cell types by proinflammatory cytokines such as TNF-α, plays a key role in immunocyte recruitment to the skin, and it likely contributes to psoriasis. We treated human keratinocytes with TNF-α to mimic this process in vitro to investigate the effect of GL on epidermal inflammation. The phenotype identified showed that GL suppressed TNF-α-induced ICAM-1 expression and THP adhesion, which suggests that GL directly affects keratinocytes to suppress inflammation in skin disease.

NF-κB is a key regulatory element in inflammatory pathways, and it is a crucial mediator in the pathogenesis of psoriasis [25, 26]. NF-κB links alter keratinocytes and immune cell states through its effects on cellular proliferation, differentiation and apoptosis as well as cytokine and chemokine production [27]. When keratinocytes are stimulated with TNF-α, the NF-κB signaling is activated, which leads to the over-expression and release of a wide range of proinflammatory mediators such as cytokines, chemokines and enzymes [28]. Because we demonstrated that GL effectively suppresses the expression of adhesion mediators such as ICAM-1, we performed additional experiments to detect the effects of GL treatment on NF-κB signaling. We found that GL treatment altered NF-κB signaling by significantly dampening IκB degradation in the cytoplasm upon TNF-α stimulation and by inhibiting p65 phosphorylation and translocation into the nucleus.

Based on the facts that TNF-α is a multifunctional cytokine and the inflammatory response to TNF-α is initiated and maintained by the activation of various signaling pathways, we investigated the effect of GL on the MAPK signaling pathway as well as whether NF-κB signaling and MAPK signaling can be activated by TNF-α and is responsible for the expression of various proinflammatory genes including ICAM-1 [29]. We found that GL decreased the TNF-α-induced activation of ERK and p38 MAPK in a dose-dependent manner, although it had minimal effects on JNK levels. Different MAPKs might play different roles in the TNF-α-induced expression of proinflammatory genes in keratinocytes. For example, TNF-α-induced matrix metalloproteinase 9 (MMP9) expression in keratinocytes is related to the activation of JNK and not p38 [30]. Previous research has also shown that the inhibition of ERK and p38 can suppress the TNF-α-induced expression of ICAM-1 at both mRNA and protein levels, whereas JNK inhibition does not [31]. Our results suggest that GL attenuated but did not abrogate MAPK signaling. Thus, the protective effect of GL was exerted through its selective inhibition of the ERK and p38 MAPK signaling pathways.

Our in vitro findings suggest that GL should be used to treat inflammatory skin disease. In fact, GL treatment delayed the onset of IPI and ameliorated ongoing IPI in mice, and these improvements were associated with decreased ICAM-1 expression. Repeated topical application of IMQ to murine skin provoked inflammatory lesions [21]. In this model, IMQ likely exerts its effects through the adenosine receptor on keratinocytes [32], thereby leading to the secretion of the proinflammatory cytokines that induce inflammatory leukocyte recruitment and acanthosis [29, 33]. Importantly, the IPI model recapitulates the hallmarks of human psoriasis, including erythema, hyperkeratosis, scaling, neutrophil microabscesses in the epidermis, and the infiltration of γδ T cells and Th17 cells [34]. Using this model, we investigated the effects of GL on psoriasis development. We demonstrated that GL administration delayed the onset of IPI-induced lesions. In addition, GL treatment attenuated the psoriatic phenotype. Furthermore, we provided evidence that GL attenuates ICAM-1 expression in the epidermis. Although additional research is required to determine the precise molecular underpinnings of the immune signaling in the skin modified by GL in vivo, the present findings indicate that GL can alleviate IPI conditions, thereby suggesting new avenues for the treatment of inflammatory diseases of the skin and perhaps elsewhere.

In conclusion, the current study demonstrated the anti-inflammation effects of GL on keratinocytes in vitro. This immunosuppressive mechanism is involved the inhibition of the TNF-α-induced activation of NF-κB and the phosphorylation of ERK/p38 MAPK to activate the protein kinase cascade in keratinocytes. In addition, GL administration effectively delays the onset of IPI and can ameliorate IPI; therefore, it might be used to treat psoriasis.
Acknowledgments

Grants from the Chinese Society of Dermatology (Emsai-SNMC China Skin Grant, 2013) and the Natural Science Foundation of Guangdong Province (Project No. S2013010011656) supported this work.

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