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Glycyrrhizin ameliorates atopic dermatitis-like symptoms through inhibition of HMGB1

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ABSTRACT

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease prevalent worldwide. This study investigated the effects of glycyrrhizin, an extract of licorice root, on the well-established model of 2,4-dinitrochlorobenzene-induced AD-like symptoms in mice. The severity of dermatitis, histopathological changes, serum IgE levels, changes in expression of high-mobility group box 1 (HMGB1), the receptor for advanced glycation end products (RAGE), nuclear factor (NF)-κB and inflammatory cytokines were evaluated. Treatment with glycyrrhizin inhibited the HMGB1 signaling cascade and ameliorated the symptoms of AD. Furthermore, in an in vitro study, the expression of RAGE was detected in a mouse mast cell line, P815 cells, and rmHMGB1 was found to be a potent inducer of mast cell activation by increasing Ca^{2+} influx, upregulating the CD117 and activating NF-κB signaling; these effects were also inhibited by glycyrrhizin. These findings implicate HMGB1 in the pathogenesis of AD and suggest that GL could be an effective therapeutic approach for cutaneous inflammation.

1. Introduction

Atopic dermatitis (AD) is a chronic, recurrent inflammatory skin disease, whose incidence is increasing worldwide [1]. Patients experience severe itching, which seriously affects quality of life and can bring sustained financial burden to the family. The exact pathogenesis of AD remains unknown, but immune abnormalities involving the release of inflammatory mediators play an important role [2]. At present, the administration of antihistamines and as well as the use of topical glucocorticoids, calcineurin inhibitors and humectants are the preferred treatment for AD. However, the treatment for refractory AD remains unsatisfactory. Although the systemic use of corticosteroids is effective in treating allergic diseases, its efficacy in treating AD is limited due to its adverse effects [3]. Therefore, safe and effective therapies are urgently needed.

Glycyrrhizin (GL) is the major bioactive ingredient in licorice root and has been shown to have cytoprotective, anti-inflammatory, antiviral and immune regulatory effects [4]. Clinical pharmacology studies of the safety of GL have found that GL treatment is well-tolerated [5,6]. In recent years, the anti-inflammatory effect of GL as a high-mobility group box 1 (HMGB1) inhibitor has gained attention [7]. HMGB1 is a highly conserved, non-histone chromatin protein in the nucleus. Its main function is to stabilize the structure of nucleosomes and regulate gene transcription. It can be released from cells, either actively or passively, to act as a pro-inflammatory cytokine via binding to its various receptors, such as the receptor for advanced glycation end products (RAGE), leading to the activation of nuclear factor (NF)-κB and upregulation of several inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 [8,9]. GL binds directly to HMGBl (helix 1 and 2 in the HMG boxes) to inhibit its cytokine activity, which plays a therapeutic role in inflammatory diseases [10].

It was reported that 2,4-dinitrochlorobenzene (DNCB), an electrophilic and cytotoxic benzene derivative, could induce stable clinical AD-like symptoms in mice including pruritic, eczematous skin lesions and elevated serum immunoglobulin E (IgE) [11,12]. Previous studies have reported that DNCB-induced hypersensitivity dermatitis was predominantly the result of T-cell mediated immune responses [13,14], whereas the participation of HMGB1 and RAGE in dermatitis was less well-understood. Here, we investigated the possible role of HMGB1 in skin inflammation in a DNCB-induced mouse model of AD; inhibition of HMGB1 by GL was performed. The effects of GL were compared with those of dexamethasone (Dex), a medication commonly used to effectively treat allergic disorders. Furthermore, the effects of HMGB1 and GL on mast cell activation were studied.

2. Materials and methods

2.1. Animals and treatment

Female BALB/c mice (6- to 8-week old, weighing 18–21 g) were

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purchased from LCB (Liaoning, China). They were housed under specific pathogen-free conditions at 22 ± 2 °C with a 12-h light-dark cycle. All experiments with mice were performed in accordance with the national guidelines and approved by the animal care committee of Shengjing Hospital of China Medical University (Approval No. 2016PS001K, Shenyang, China).

After 1 week of acclimation, the mice were divided into four groups (n = 9 per group): Control, DNCB, DNCB + GL and DNCB + Dex. Animal studies were performed according to approved protocols [11,12]. Briefly, a day after complete dorsal hair removal, 200 μl of 1% DNCB (Sigma, MO, USA) dissolved in an acetone: olive oil mixture (3:1 v/v) was dropped on the dorsal skin and 10 μl on each ear. Four days later, 0.2% DNCB was used three times a week for 3 weeks (days 0–20). For the control, only the acetone: olive oil mixture (3:1 v/v) was applied. GL (1%, 50 mg/kg per mouse per day, Solarbio, Beijing, China) dissolved in phosphate-buffered saline (PBS), Dex (0.02%, 1 mg/kg per mouse per day) or PBS was injected intraperitoneally seven times a week for 3 weeks (days 0–20).

2.2. Evaluation of dermatitis severity

Dermatitis score was assigned during different periods according to defined macroscopic diagnostic criteria in a blinded fashion as follows: grade 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for the manifestation of erythema/hemorrhage, dryness/scaling, edema and excoriation. The dermatitis score was the sum of individual scores [15].

2.3. IgE assay

At the end of the study (day 21), the mice were sacrificed, and their blood and dorsal skin tissues were harvested. The serum IgE concentration was measured using an enzyme-linked immunosorbent assay kit (CUSABIO, Wuhan, China) according to the manufacturer's instructions.

2.4. Cell culture

P815 cells, a mouse mastocytoma cell line, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, AusGeneX, QLD, Australia) at a density of 4×10^5 cells per ml in a total volume of 5 ml in each 25 cm^2 T-flask (Corning, New York, USA) and maintained in a 5% CO_2 incubator at 37 °C. When the cells had attained 80% confluence, they were resuspended in fresh medium and seeded in 96- or 12-well plates for the experiments.

2.5. Histopathology and immunofluorescence

Skin tissue was 4% paraformaldehyde fixed and paraffin embedded. Sections of 3.5 μm thickness were stained with hematoxylin and eosin (HE) or toluidine blue (TB). For immunofluorescence, skin tissue sections were pretreated using heat-mediated antigen retrieval with Tris/EDTA buffer (pH 9.0) for the anti-HMGB1 antibody (1:400, Abcam, Cambridge, UK) or sodium citrate buffer (pH 6.0) for the anti-RAGE antibody (1:400, Abcam). Sections were incubated with the primary antibody overnight at 4 °C. An Alexa Fluor 594-conjugated anti-rabbit immunoglobulin G (IgG) H&L (1:200, Abcam) was used as the secondary antibody. 4,6-Diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei.

P815 cells were fixed with 4% paraformaldehyde; in double immunofluorescence experiments, cells were co-stained with antibody against RAGE (1:200) along with an anti-mast cell tryptase (MC tryptase) antibody (1:100, Abcam). Secondary antibodies were Alexa Fluor 594-conjugated anti-rabbit IgG H&L and Alexa Fluor 488-conjugated anti-mouse IgG H&L (1:200, Abcam).

2.6. Degranulation

To assess the effects of HMGB1 on mast cell degranulation, the activity of β-hexosaminidase was measured as previously described [16]. Briefly, P815 cells were resuspended in Tyrode's buffer (Sigma) at 1×10^5 cells per 100 μl and plated into 96-well plates, preloaded with different concentrations of recombinant mouse HMGB1 (rmHMGB1, ng/ml, Sion Biological, Beijing, China) with or without GL (100 μg/ml), and then incubated for 30 min. The absorbance of the culture supernatant and the frozen-thawed cell lysate was measured at 405 nm using a microplate reader (Bio-Tek, VT, USA).

2.7. Flow cytometry

To determine the effects of HMGB1 on Ca^{2+} influx in P815 cells, the cells (1×10^6) were pre-treated with different concentrations of rmHMGB1 (ng/ml) with or without GL (100 μg/ml) for 30 min. The cells were washed, resuspended in 200 μl PBS and loaded with the fluorescent Ca^{2+} indicator, Fluo-4 AM (5 μM/ml, Life Technologies, Maryland, USA), for 30 min, then the mean fluorescence intensity was measured using a FACSCalibur flow cytometer (BD, New Jersey, USA).

In addition, P815 cells (1×10^5) were pre-treated with different concentrations of rmHMGB1 (ng/ml) with or without GL (100 μg/ml) for 24 h. The cells were washed and loaded with PE-conjugated anti-mouse CD117 antibody (0.6 μg/ml, BioLegend, California, USA) for 30 min. Surface expression of CD117 was measured using the FACSCalibur flow cytometer.

2.8. Western blot

P815 cells (1×10^5) were pre-treated with rmHMGB1 (20 ng/ml) with or without GL (100 μg/ml) for 24 h; cells were washed and collected.

Skin tissue and cell samples were homogenized with lysis buffer; lysate protein concentrations were measured using the bicinchoninic acid method. Western blot analyses were performed according to a described protocol [17]. Primary antibodies were antibodies against HMGB1 (1:10000), RAGE (1:1000), MC tryptase (1:500), NF-κB (1:1000), phospho-NF-κB (1:500, pNF-κB, Abcam), pERK (1:500), PI3Kp110 (1:1000), PI3Kp85 (1:500), pAKT (1:1000), TNF-α (1:500) and IL-6 (1:1000) (CST, Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (1:5000, GAPDH, Proteintech, CHI, USA) was used as an internal control. Bands intensities were acquired using a chemiluminescence imaging system C300 (Azure Biosystems, CA, USA) with enhanced chemiluminescence solution (ThermoScientific, MA, USA). The blot was quantified with densitometric analysis using ImageJ Launcher (NIH, Maryland, USA).

2.9. RT-PCR

P815 cells (1×10^5) were pretreated with rmHMGB1 (20 ng/ml) with or without GL (100 μg/ml) for 24 h and then collected. Total RNA was extracted using a Column Trizol Total RNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. cDNA was synthesized from the isolated total RNA using a RevertAid Premium Reverse Transcriptase kit (ThermoScientific) following the manufacturer's instructions, then amplified by polymerase chain reaction with 2× SG Fast qPCR Master Mix (Roche, Basel, Switzerland) in a total reaction volume of 20 μl using the Roche Lightcycler 480 (Roche). The primers for quantitative real-time detection designed with Primer Premier 5.0 (Primer Biosoft, CAN) are listed in Table 1. Relative gene expression levels were normalized to the internal control (GAPDH) based on the $2^{-\Delta\Delta Ct}$ method.