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Effects and mechanisms of Geniposide on rats with adjuvant arthritis

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ABSTRACT

Geniposide (GE), an iridoid glycoside compound, is the major active ingredient of *Gardenia jasminoides* Ellis (GJ) fruit which has anti-inflammatory and other important therapeutic activities. The aim of this study was to investigate the effects of GE on adjuvant arthritis (AA) rats and its possible mechanisms. AA was induced by injecting with Freund's complete adjuvant (FCA). Male SD rats were subjected to treatment with GE at 30, 60 and 120 mg/kg from days 18 to 24 after immunization. Lymphocyte proliferation was assessed by MTT. Interleukin (IL)-6, IL-17, IL-4 and transforming growth factor-beta 1 (TGF-β_1) were determined by ELISA. c-Jun N-terminal kinase (JNK) and phospho-JNK (p-JNK) were detected by Western blot. GE (60, 120 mg/kg) significantly relieved the secondary hind paw swelling and arthritis index, along with decreased Th17-cells cytokines and increased Treg-cell cytokines in mesenteric lymph node lymphocytes (MLNL) and peripheral blood lymphocytes (PBL) of AA rats. In addition, GE decreased the expression of p-JNK in MLNL and PBL of AA rats. In vivo study, it was also observed that GE attenuated histopathologic changes of MLN in AA rats. Collectively, GE might exert its anti-inflammatory and immunoregulatory effects through inducing Th17 cell immune tolerance and enhancing Treg cell-mediated activities by down-regulating the expression of p-JNK. The mechanisms of GE on JNK signaling in MLNL and PBL may play critical roles in the pathogenesis of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory and systemic autoimmune disease, which is characterized by synovial inflammation and joint destruction [1]. It is generally believed that the autoimmune response is associated with a variety of immune cells, especially $CD4^+$ T helper (Th) cells [2]. $CD4^+$ T cells were classically thought to differentiate into two subgroups: Th1 cells and Th2 cells. It has been reported that the imbalance of Th1/Th2 was closely related to RA [3]. However, recent studies have shown that $CD4^+$ T cells could be divided into four subsets, such as pro-inflammatory Th1 cells, anti-inflammatory Th2 cells, and newly defined Th17 cells and regulatory T (Treg) cells. The imbalance of Th17/Treg played an even greater role in the development and pathogenesis of RA [4,5]. The recently identified Th17 cells were $CD4^+$ T cells characterized by the secretion of IL-17. In addition to their key effector cytokine IL-17A (IL-17) which could participate in tissue inflammation and joint destruction of RA by inducing many pro-inflammatory cytokines and chemokines [6,7], they also produced other inflammatory cytokines, including IL-17F, IL-6 and tumor necrosis factor (TNF)-α [8,9]. Furthermore, Th17 cells were crucial for defending against extracellular bacteria and mediating chronic inflammation, autoimmune disease, malignant tumors, and so on [10]. Treg cells, characterized by the expression of the forkhead–winged-helix transcription factor (Foxp3), could suppress immune responses to exert anti-inflammatory effect and maintain unresponsiveness to self-antigens by means of cell–cell contact inhibition and secreting anti-inflammatory cytokines such as IL-10 and TGF-β_1 [11]. A previous study has shown that the imbalance of Th17/Treg was existed in the peripheral blood of patients with RA and related to pathogenetic condition [5]. However, how this imbalance is existed in patients with RA has not been reported.

AA was chosen as an experimental model for RA, which shares some features with human RA in a number of pathological, histological and immunological aspects. AA was induced by injecting with FCA that containing heat-killed *Mycobacterium tuberculosis* (MT) by a mechanism involving heat shock proteins (HSPs).

Geniposide (GE), an iridoid glycoside compound, is the main bioactive components (structure, see Fig. 1) of *Gardenia jasminoides* Ellis (GJ) fruit which commonly was used as a traditional medicine in many Asian countries for its antiphlogistic and antipyretic effects [12]. It has been shown that GE exhibited an anti-inflammatory property by down-regulating the expression of Toll-like receptor 4 (TLR4) up-regulated by lipopolysaccharide (LPS) in the primary mouse macrophages and mouse models [13]. In addition, recent reports have demonstrated that GE exerted anti-inflammatory activity in the Carrageenan-induced rat paw edema model and displayed inhibitory effects on acetic acid-induced vascular permeability changes [14]. However, the anti-inflammatory effect and the mechanisms of GE on AA rats remain unclear. GE was extracted and purified from GJ by means of solvent extraction and column chromatography. The structure was identified by physicochemical properties and spectroscopic analysis, and the content was determined by Ultra-Performance Liquid Chromatography (UPLC).

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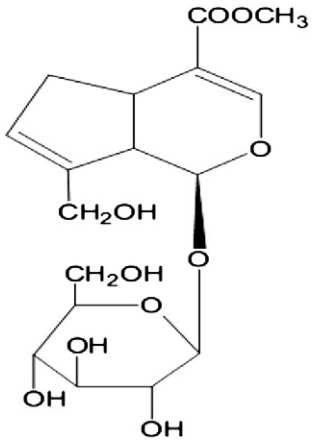

Fig. 1. Structure of Geniposide (formula $C_{17}H_{24}O_{11}$, molecular weight 404.36).

Total glucosides of paeony (TGP), as the positive control drug, were used for the treatment of RA.

The c-Jun N-terminal kinases (JNKs), which belong to the mitogen-activated protein kinase (MAPK) family, play vital roles in the production of cytokines and the degradation of extracellular matrix by regulating matrix metalloproteinase (MMP) gene expression in fibroblast-like synoviocytes (FLSs) and mediating joint destruction in AA rats [15,16]. The JNK signal pathway could be activated by cytokines, growth factors, stresses, and so on. Many studies suggested that the JNK signal pathway plays a pivotal role in cell differentiation, apoptosis, initiation and progress of considerable human diseases as well as in animal models, including ischemia/reperfusion (I/R), RA, diabetes mellitus, and tumor [17,18]. Experience gathered has confirmed that IL-17 could synergize with local inflammatory mediator IL-6 to induce the expression of IL-6 by a transcriptional mechanism that involves JNK signal pathway, and blockage of JNK pathway with JNK inhibitor II could partially inhibit the synergistic effect of IL-17 and IL-6 on the expression of IL-6 [19]. Additionally, it has been shown that the transcription factor Eomesodermin (Eomes), whose expression could substantially suppress Th17 cell induction in primary T cells, was suppressed by TGF-β_1 via the JNK-c-Jun signal pathway [20]. We therefore wondered whether the balance of Th17/Treg could be associated with the JNK signal pathway. In this study, we discussed the role of GE in rectifying Th17/Treg balance by inhibiting excessive phosphorylation of JNK. Here, we evaluated the effects and mechanisms of GE on immune balance of Th17/Treg at levels of organ, molecule, and protein, respectively. Our results showed that GE could inhibit the progressive inflammation by inducing Th17 cells tolerance and enhancing Treg-driving effects, suggesting that GE has a significant therapeutic potential in treating autoimmune and other chronic inflammatory disorders. This may provide useful information for both clinical therapy and basic scientific research in RA.

2. Materials and methods

2.1. Animals

Sprague–Dawley (SD) rats (♂, 180 ± 20 g. Grade II, Certificate No. 011) were purchased from the Animal Department of Anhui University of Chinese Medicine (Hefei, Anhui Province, China). All animals were housed under specific pathogen-free conditions with a 12-hour light/dark cycle in a temperature-controlled room at 23 °C (±1) and allowed food and water ad libitum in each group of no more than six. All experiments using rats were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of Anhui University of Chinese Medicine.

2.2. Materials

Mouse anti-JNK monoclonal antibody (D-2, sc-7354) and mouse anti-p-JNK monoclonal antibody (G-7, sc-6254) were obtained from Santa Cruz Biotechnology, Inc. Mouse anti-beta actin monoclonal antibody (TA-09) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (2B-2305) were purchased from ZSGB-BIO. IL-6, IL-17, IL-4 and TGF-β_1 ELISA kits were supplied from Elabscience Biotechnology Co., Ltd. The following reagents were offered commercially: Concanavalin A (ConA, stored at −20 °C), lectin from *Phaseolus vulgaris*-phytohemagglutinin (PHA-P, stored at 2–8 °C) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) from Sigma Chemical Co. (St. Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM) from Thermo Scientific Co. (USA). Other chemicals used in these experiments were analytical grade from commercial sources.

2.3. Drugs

GE is a yellow powder with >94.6% purity (determined by UPLC, ACQuity H-CLASS, Waters Co., USA), which was provided by the Chemistry Lab of Anhui University of Chinese Medicine (Hefei, Anhui Province, China). Standard of TGP (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). GE and TGP were suspended in water before use.

2.4. Induction of AA

AA was induced as previously described [21]. Briefly, rats were immunized on day 0 by a single intradermal injection into the left hind paw with 100 μL of FCA for each rat.

2.5. Treatment of AA

Before the onset of arthritis, rats were divided into six groups randomly, in which the AA rats were given intragastrically GE (30, 60, 120 mg/kg) and TGP (50 mg/kg) from days 18 to 24 after immunizations. While in groups of normal and AA model, rats were given an equal volume of water at the same time.

2.6. Arthritis assessment

Rats were assessed daily for signs of arthritis by two independent observers who were blinded to the experimental design. Non-injected hind paw volume was determined with YLS-7A volume meter (Shandong Academy of Medical Sciences Equipment Station, China). The severity of arthritis in each paw was graded on a scale of 0–4: 0, no swelling; 1, swelling of finger joints; 2, swelling of phalanx joint and digits; 3, severe swelling of the entire paws; 4, deformity or ankylosis. The maximum arthritis score was 12 including three secondary arthritis paws for each rat [22,23].

2.7. Lymphocyte proliferation assay by MTT

MLNs and peripheral blood were removed in sterile condition. MLNL and PBL were isolated by routine method. Then, the cells were cultured in triplicate in a concentration of 1×10^{10} cell/L in 100 μL DMEM containing 10% FBS. First, the cells were stimulated with 5 mg/L ConA for 48 h at 37 °C and 5% of CO_2. A 10 μL sample of MTT (5 g/L) was added before the end of stimulation for 4 h and then the cultures were stimulated for 4 h continuously. After incubation, the cultures were centrifuged (760 ×g, 10 min) and the supernatants were discarded. A 150 μL of dimethylsulfoxide (DMSO) was added to each well and the absorbance (A) was examined at 490 nm using a MSS ELISA Microwell Reader (Thermo Scientific Co., USA).