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Quercetin attenuates collagen-induced arthritis by restoration of Th17/Treg balance and activation of Heme Oxygenase 1-mediated anti-inflammatory effect

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ABSTRACT

Quercetin (QU) has been shown obvious anti-arthritic property in pre-clinical studies or clinical studies. Howbeit, the underlying mechanism of it is still not revealed distinctly and should be gotten further insight into. QU at a dosage of 150 mg/kg was administered orally in collagen-induced arthritis rats and then the clinical symptoms were monitored. The protein levels of Th17/Treg-related cytokines were determined by ELISA, and the mRNA levels of cytokines and transcription factors associated with the Th17 and Treg phenotypes were evaluated by real-time PCR, the proportions of Th17 and Treg cells were assessed by flow cytometry. The results showed that QU administration yielded an obvious mitigation of arthritic manifestations including high arthritic scores and paw edema, which was accompanied with decrement of Th17-related cytokines (IL-17A and IL-21) and increment of Treg-related cytokines (IL-10 and TGF-β). QU decreased the percentage of Th17 cells, while increased the percentage of Treg cells. In addition, the activation of NLRP3 inflammasome which plays a crucial role in the development of RA was determined and found that the protein expressions of NLRP3, Caspase-1 and IL-1β were diminished by QU significantly. Moreover, the protein levels of inflammatory mediators which were recognized as chief culprits in inflammatory reaction were assayed. The contents of inflammatory mediators inclusive of TNF-α, IL-1β, IL-6, PGE2, COX-2 and iNOS were down-regulated markedly by QU. But the inhibitory effect of QU on inflammatory mediators was nearly abolished by Heme Oxygenase 1 (HO-1) siRNA. Taken together, QU attenuates CIA *via* modulating the Th17/Treg balance, inhibiting NLRP3 inflammasome activation as well as activating HO-1-mediated anti-inflammatory response.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease which presents the grievous inflammation of synovial membrane and severe cartilage destruction and bone erosion [1]. To date, the epidemiologic survey displayed 1% of the world population is affected with RA [2]. Although the exact pathophysiology of RA has remained elusive, a growing body of evidence has identified the immune imbalance and inflammation clearly contributed to the rheumatic symptoms, especially the joint damage [3,4].

A complex immune mediated response with the participation of many cell types including CD4 + T cells was existed in RA [5]. Under arthritic condition, naïve CD4 + T cells transform into different kinds of effective T-helper cells especially for IL-17-producing CD4 + T cells (Th17 cells) and Foxp3 + regulatory T cells (Treg cells) which secret their own sets of cytokine profiles that mediate different functions. It has been reported that Th17/Treg cell imbalance is found to be the culprit in the early pathogenetic phases of RA [6]. Significant increase of Th17 cells differentiation has been found in RA patients, along with up-regulation of IL-17 and IL-23 levels [7]. The overexpression of Th17 cytokines in turn exacerbates arthritis via promoting the infiltration of the immune cells, secretion of vascular endothelial growth factor,

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growth of the blood vessel and the production of chemokines, pro-inflammatory cytokines and inflammatory mediators [8,9]. And IL-17 neutralization has been proven to be highly effective in relieving RA [10]. In contrast, Treg cytokines have been revealed to be involved in antagonism of Th17 cell functions to restrain the development of RA. Furthermore, Treg cells orchestrate the entire immune response and have much to do with preservation of peripheral immune tolerance through adjusting the function of the effector T cells [11]. Therefore, restoration of Th17/Treg balance would be helpful in suppressing RA.

In addition, studies have shown that inflammatory response is mechanistically linked to physiological and pathological processes of arthritis as well. A growing body of literatures has been reported that inflammatory cytokines, important mediators relating to host inflammatory processes, contribute the process of joint swollen and bone destruction in RA. Moreover, pyrin domain-containing 3 (NLRP3) inflammasome, the upstream factors of IL-1β, is proven to be the new and effective target of the pharmacological treatment for RA. The protein expression of NLRP3 is augmented in the early onset of arthritis and the synovial NLRP3 content is closely related to the clinical severity of arthritis and radiological scores [12]. Moreover, inflammatory mediators including iNOS and COX-2 are also involved in arthritis, and the therapies targeting them have demonstrated an amelioration of disease progression. COX-2 is able to regulate downstream target molecules to trigger inflammation via a series of signaling pathways. Besides, PGE2, a metabolite of arachidonicacid produced from the reactions catalyzed by COX-2, is also a pivotal regulatory factor originated from inflammatory cells [13].

Heme Oxygenase 1 (HO-1), a rate-limiting catabolic enzyme that catalyses the degradation of heme to free iron, carbon monoxide and biliiverdin in mammalian cells, plays a crucial role in inflammatory response and might be a pivotal therapeutic target for RA [14]. It is well documented that HO-1 induction inhibits collagen-induced arthritis (CIA) and decreases the expression of matrix metalloproteinase 1/3 in synoviocytes [15]. Moreover, HO-1 also showed a strong anti-inflammatory efficacy. Up-regulated HO-1 significantly inhibited the secretion of pro-inflammatory cytokines and release of chemokines monocyte chemoattractant protein-1 and macrophage inflammatory protein-1β [16,17].

Quercetin (QU) which widely exists in vegetables, fruits and beverage such as onion, apples and red wine has been proven to exert anti-arthritic activity [18], but the underlying mechanism remains elusive. In this study, we aimed at gaining biologic evidence that QU restores the Th17/Treg balance, inhibits NLRP3 inflammasome activation, prompts HO-1-mediated reduction of inflammatory cytokine and mediators (Fig. 1).

2. Materials and methods

2.1. Animals

Female Wistar rats (150–170 g) were purchased from Darso Biological Technology Co. Ltd. (Chengdu, China). Animals were kept in a specific pathogen-free condition and fed with a standard chow diet and water ad libitum. They were housed at room temperature (22 ± 2 °C) under a 12 h light/dark cycle and all procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, The United States). All efforts were made to minimize animals' suffering and to reduce the number of animals used.

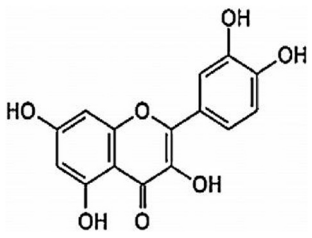

Fig. 1. Chemical structure of quercetin.

2.2. Chemicals and reagents

QU (purity > 98%), Chicken Type II collagen (CII), Cobalt protoporphyrin IX (CoPP, the inducer of HO-1), Lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, USA). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Becton Drive Co. (New Jersey, USA). ELISA kits for anti-collagen type II (CII) IgG, anti-CII IgG1 and anti-CII IgG2a were purchased from Chondrex Inc. (Redmond, USA). IL-17A, IL-10, TNF-α, IL-1β and IL-6 were purchased from NeoBioscience Technology (Shenzhen, China). TGF-β, IL-21 and PGE2 were purchased from Cusabio Biotech (Hubei, China). Rat-FITC-anti-CD4, rat-APC-anti-CD25, rat-PE-anti-IL-17A, rat-PE-anti-Foxp3, fixation/permeabilization concentrate and diluent were purchased from eBioscience (San Diego, USA). PMA/Ionomycin mixture and BFA/Monensin mixture were purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). Rat tissue lymphocyte separation medium were purchased from Tianjin Hao Yang Biological Technology Co., Ltd. (Tianjin, China). Ssofast EvaGreen Supermix and iscript cDNA synthesis kit used in real-time polymerase chain reaction (RT-PCR) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Mouse anti-HO-1 antibody, rabbit anti-iNOS antibody, mouse anti-COX-2 antibody and mouse anti-IL-1 β were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-NLRP3 antibody and rabbit anti-Caspase-1 antibody were purchased from Abcam (Cambridge, MA, USA). Mouse anti-GAPDH antibody was purchased from Kangchen Biotech (Shanghai, China). Lipofectamine™ 2000 was purchased from Invitrogen Co. (Invitrogen, Carlsbad, USA). Other chemical products used were of the analytical grade available.

2.3. Induction and evaluation of CIA in the rats and QU treatment

Female Wistar rats were immunized on day 0, at the base of the tail, with an intradermal injection of 200 μg CII emulsified in CFA. On day 7, a booster immunization was conducted with 100 μg CII dissolved in IFA at the base of the tail of rats, avoiding the primary injection sites [19]. On day 14, corresponding to the onset of arthritis, all CIA rats were randomly divided into 2 groups: Model and QU (150 mg/kg) group. The rats in QU group were treated orally with QU once daily for 2 weeks. In Normal (without immunization) and Model group, only the vehicle for QU, carboxymethyl cellulose (CMC) at 0.5%, was given. The dose of QU was chosen according to the previous experiments, which had proven that QU (150 mg/kg) dramatically ameliorated the level of C-reactive protein, monocyte chemotactic protein-1 and IL-1β and inhibited NF-κB activation [20]. Moreover, Huang et al. reported that QU administration (100 or 200 mg/kg) eliminated paw swelling by dwindling histological signs of acute inflammation and diminishing several makers of inflammation [21]. The arthritis index (AI) scores and swelling of paws were determined at the indicated times. Paw volumes were assessed by plethysmometry and AI scores for the progression of CIA were graded on a scale of 0–4. 0 = no change, 1 = red or slight swelling, 2 = mild swelling, 3 = pronounced swelling, 4 = the limb deformity and inability to use. The AI score for each rat was defined as the sum of four affected paws, with the highest score of 16 for each rat [22].

2.4. Cell culture and treatment

Fibroblast like synoviocytes (FLS) were isolated from the synovial membranes of the knee joints of CIA rats and digested by sequential incubation with collagenase Type II (4 mg/ml) (Gibco BRL, Grand Island, NY, USA) and trypsin (2.5 mg/ml). Briefly, aseptically minced tissues were digested in Dulbecco's modified Eagle's medium (DMEM)