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Sinomenine suppresses collagen-induced arthritis by reciprocal modulation of regulatory T cells and Th17 cells in gut-associated lymphoid tissues

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ABSTRACT

Sinomenine (SIN) has long been used as a therapeutic agent of rheumatoid arthritis (RA) in China. However, the discrepancy between low oral bioavailability and higher minimal effective concentration made its action mode mysterious. The present study aimed to gain insight into the mechanisms by which SIN suppressed collagen-induced arthritis (CIA) in rats in view of Th17 and regulatory T (Treg) cell balance. SIN was orally administered, and the clinical symptoms of CIA rats were monitored; inflammatory cytokines levels in serum were measured by ELISA; pharmacokinetic studies were performed in normal and CIA rats; Th17 and Treg cell frequencies were analyzed by flow cytometry. The data showed that SIN treatment resulted in a dramatic decrease of arthritis scores and paw volume of CIA rats, which was accompanied by down-regulation of IL-17A and up-regulation of IL-10 in rat serum. The frequency of Treg cells was increased and the frequency of Th17 cells was decreased in the gut lymphoid tissues of SIN-treated rats. Immunohistochemistry assay demonstrated that more α4β7-positive cells were detained in joint tissues after SIN treatment. Moreover, the anti-arthritis efficacy of SIN disappeared when it was given by intraperitoneal injection, further confirming the action of SIN was gut-dependent. In conclusion, SIN exerts anti-RA action probably through modulating the frequencies of Treg cells and Th17 cells in intestinal lymph nodes and yielding a trafficking of lymphocytes (especially Treg cells) from gut to joint.

1. Introduction

Sinomenine (SIN) is an alkaloid constituent occurring in *Sinomenium acutum* Rehd. et Wils. and other medicinal plants. In China, it has been used for the treatment of rheumatoid arthritis (RA) for several decades. The satisfactory therapeutic efficacy and fewer side effects of SIN in patients have been confirmed in open clinical trials (Huang et al., 2007; Shi et al., 1985). When orally administered at the doses of 100 and 300 mg/kg, SIN could attenuate adjuvant-induced arthritis (AIA) in rat and collagen-induced arthritis (CIA) in mouse through suppressing the production of autoantibody and modulating Th1/Th2 response (Feng et al., 2007; Mu et al., 2013). *In vitro* mechanistic studies showed that SIN could suppress the activation of T lymphocytes, induce the apoptosis of macrophage, reduce antigen-induced activation of basophils, and hinder migration of synoviocytes at concentrations over 0.5 mM (He et al., 2005; Huang et al., 2008; Liu et al., 1994; Ou et al., 2011). Overall, the minimal effective concentration of SIN against immunity or inflammation effector cells is quite high. In contrast, pharmacokinetic studies manifested that the oral bioavailability of SIN was low. When it was orally administered at a dose of 90 mg/kg in rats, the C_{max} was about 13.9 μg/mL (≈42 μM) (Liu et al., 2005), which was obviously lower than the *in vitro* minimal effective concentration as mentioned above. How SIN exerts anti-arthritic effects needs to be clarified.

RA is a chronic autoimmune disease that primarily targets the synovium, cartilage, and bone of multiple joints. As the concept of an imbalance between pro-inflammatory and anti-inflammatory forces exists for several decades, RA is formerly considered as a disorder driven by T helper 1 (Th1) cells through producing inflammatory cytokines, such as interferon-γ (IFN-γ). Recently, research interest has expanded to the balance between IL-17-producing T cell (Th17) and IL-10-producing regulatory T cell (Treg).

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Rebuilding the balance of Th17/Treg cells through either boosting the number of Treg cells or reducing the number of Th17 cells is likely to be one of the most effective strategies for RA intervention (Kohm et al., 2002; Park et al., 2014a). Interestingly, a large number of compounds from medicinal plants have manifested beneficial regulatory effects on the Th17/Treg balance, such as epigallocatechin-3-gallate (EGCG) (Wang et al., 2012), grape seed proanthocyanidin extract (GSPE) (Park et al., 2011), halofuginone (Park et al., 2014b), and curcumin (Park et al., 2013).

Considering the possible residence of SIN in intestines after oral administration and the central roles of Th17/Treg cell imbalance in RA pathogenesis, the present study explores the anti-arthritis mechanism of SIN with a focus on the regulation of Th17/Treg balance, especially in the intestinal tract.

2. Materials and methods

2.1. Animals

Specific pathogen-free Wistar female rats (weighed 130–150 g) were obtained from B&K Universal Group Limited (Shanghai, China). They were maintained at a constant temperature and humidity, with a 12-h light–dark cycle. Water and a nutritionally adequate diet were provided *ad libitum*. All rats were observed daily for general health and clinical signs of disease. At the end of the study, rats were euthanized by CO_2 asphyxiation, followed by exsanguination, and tissues were collected postmortem. Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations at China Pharmaceutical University. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Drugs, reagents and antibodies

Sinomenine (SIN, purity > 98%) was purchased from Nanjing Zelang Pharmaceutical Technology Co., Ltd. (Nanjing, China). Leflunomide (LEF) was purchased from Suzhou Changzheng Xinkai Pharmaceutical Co., Ltd. (Suzhou, China). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were obtained from Becton Drive Co., Ltd. (New Jersey, USA). Chicken type II collagen (CII) was purchased from Sigma–Aldrich (St. Louis, USA). Rat peripheral blood mononuclear cell (PBMC) separation medium and rat organ tissue lymphocyte separation medium were purchased from Tianjin Hao Yang Biological Technology Co., Ltd. (Tianjin, China). RPMI-1640 medium, trypsin, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, USA). Newborn calf serum (NCS) was purchased from PAA Laboratories GmbH. (Morningside, Australia). FITC-anti-CD4, APC-anti-CD25, PE-anti-Foxp3, PE-anti-IL-17A, fixation/permeabilization concentrate and diluent solutions, and IgG2a K isotype control PE were purchased from eBioscience (San Diego, USA). PMA/Ionomycin mixture and BFA/Monensin mixture were purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). Rat IL-1β, TNF-α, IL-6, IFN-γ, IL-4, IL-10 and IL-17A ELISA kits were purchased from Dakewe Biotech Co., Ltd. (Shenzhen, China). TRIzol reagent was purchased from Invitrogen (Carlsbad, USA). HiScript™ Q RT SuperMix and AceQ™ qPCR SYBR® Green Master Mix were purchased from Vazyme Biotech Co., Ltd. (Piscataway, USA). Abs for Foxp3 and RORγt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs for integrin alpha4 beta7 (α4β7) and CC-chemokine receptor 2 (CCR2) were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The other chemicals and reagents used were of analytical grade.

2.3. Induction and assessment of CIA and administration of SIN

To induce CIA, 200 μg of chicken type II collagen (CII) and CFA were immunized intradermally into the base of rat tail on day 0. On day 7, rats were boosted with an injection of 200 μg CII dissolved in IFA. The clinical symptoms were scored three times per week from the first immunization, with each paw assigned a clinical score as follows: 0 = no swelling or erythema, 1 = slight swelling and/or erythema, 2 = low to moderate edema, 3 = pronounced edema with limited joint usage, and 4 = excess edema with joint rigidity. The volumes of hind paws were determined by the paw volume plethysmometer. According to the articular index scores and paw volumes, rats were divided into the following five groups on day 14: normal group (without immunization), model group (immunization and treatment with vehicle), SIN (60, 120 mg/kg) groups and LEF (2 mg/kg) group. In treated groups, CIA rats were orally administered SIN or LEF for consecutive 2 weeks from day 14 to 28; normal and model rats were orally given an equal volume of vehicle in the same schedule. In the experiment of intraperitoneal injection, SIN (20 mg/kg) was injected from day 14 to 28.

2.4. Histopathology

Rat joints for histological analysis were removed and immediately fixed in 4% paraformaldehyde. Fixed samples were decalcified in EDTA bone decalcifier, and embedded in paraffin. Joint tissues were sectioned at 7 μm thickness, dewaxed using xylene, dehydrated through a gradient of alcohol, and stained with hematoxylin and eosin (H&E). The stained sections were evaluated to assess joint inflammation on a scale of 0–3 under blinded conditions, inflammatory cell infiltration, synovial hyperplasia and congestion, fibrous tissue hyperplasia, and bone erosion and regeneration.

2.5. Cytokine measurement

On day 28, serum samples were collected. Serum concentrations of various cytokines were detected using rat ELISA kits following the manufacturer's instructions.

2.6. Pharmacokinetic analysis of SIN

On day 20 after the first immunization, the age-matched CIA rats and normal rats were fasted overnight and orally given SIN at a dose of 120 mg/kg. Blood samples (approximately 200 μL) were collected into heparinized tubes *via* the oculi chorioideae vein before and 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720, and 1440 min after dosing. The plasma samples were obtained by centrifugation at 5000 rpm for 10 min and stored at −80 °C for further analysis.

The plasma concentration of SIN was analyzed by a UPLC procedure according to the previous study (Chen et al., 2012; Ma et al., 2013). Briefly, 100 μL plasma samples were spiked with 100 μL of internal standard (100 ng/mL tetrandrine, v/v). Then, 200 μL acetonitrile was added, and the samples were vortex-mixed. After centrifugation at 15,000 rpm for 10 min, the supernatant layer was transferred to a clean tube and evaporated to dryness at 37 °C under a gentle stream of nitrogen. The dried residue was then reconstituted in 200 μL mobile phase and centrifuged (1000 rpm for 10 min). The supernatant was transferred to 2 mL glass vials and an aliquot of 5 μL was injected for UPLC–MS/MS analysis.

A Waters ACQUITY™ triple-quadrupole tandem mass spectrometer (Waters Corp., Milford, MA, USA) was connected to the UPLC system *via* an electrospray ionization (ESI) interface. An ACQUITY UPLC™ BEH C18 (2.1 × 100 mm I.D., 1.7 μm, Waters, Milford, USA) column was used for all the analyses. The mobile phase composed of A (0.1% formic acid, v/v) and B (acetonitrile) with a