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Clematichinenoside AR induces immunosuppression involving Treg cells in Peyer's patches of rats with adjuvant induced arthritis

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ABSTRACT

Ethnopharmacological relevance: Clematichinenoside AR (AR) has been defined as a major active ingredient of triterpenoid saponins extracted from *Clematidis Radix et Rhizoma*, which is a traditional Chinese herbal medicine that has long been used in the treatment of rheumatoid arthritis (RA). To further explore the mechanism of AR in the treatment of RA, we investigated whether its immunomodulatory effects are related to Treg-mediated suppression derived from Peyer's patches (PPs) in adjuvant induced arthritis (AIA) rat model.

Materials and methods: AR (8, 16, 32 mg/kg) was orally administered daily from Day 18 to Day 31 after immunization. The effect of AR on AIA rats was evaluated by hind paw swelling and histopathological examination. Percentages of $CD4^+CD25^+Foxp3^+$ T regulatory cells were determined by flow cytometry. Levels of IL-10, TGF-β_1, IL-17A and TNF-α were measured by ELISA. Expressions of Foxp3 and RORγ in synovium were detected using immunohistochemical analysis.

Results: AR treatment significantly reduced paw swelling of AIA rats, and histopathological analysis confirmed it could suppress severity of established arthritis. AR treatment upregulated the percentages of $CD4^+CD25^+Foxp3^+$ Treg cells among CD4+ T cells in PPs lymphocytes, and increased the levels of IL-10 and TGF-β_1 secreted from ConA-activated PPs lymphocytes, whereas decreased the levels of IL-17 A and TNF-α. Similar tendency of circulating $CD4^+CD25^+Foxp3^+$ Treg cells percentages and serum cytokine levels were observed. Moreover, AR decreased the expression levels of Foxp3 and RORγ in joint synovial membrane.

Conclusions: In conclusion, these results suggested AR has a potent protective effect on the progression of AIA, probably by augmenting $CD4^+CD25^+Foxp3^+$ Treg cells in PPs to induce immunosuppression, and modulating the balance between Treg cells and Th17 cells systemically. These findings may help to develop AR as a potent immunosuppressive agent for the treatment of RA.

1. Introduction

Clematis chinensis Osbeck, a member of *Ranunculaceae* family, is a semi-evergreen perennial deciduous vine and mainly distributed in People's Republic of China. The dried roots and rhizomes of *Clematis chinensis* Osbeck, *Clematis hexapetala* Pall., and *C. mandshurica* Rupr., collectively termed *Radix Clematidis et Rhizoma* (Weilingxian, clematis root) with the effects of dispelling wind, eliminating dampness and activating meridians to stop pain, have long been used in traditional Chinese herbal medicine for the treatment of rheumatic arthralgia, limbs numbness, tendons spasm, discomforts of flexion and extension. According to the traditional Chinese medicinal view, the nature and flavor of *Radix Clematidis* are pungent, salty and warm, and channel tropism shows that *Radix Clematidis* acts on the urinary bladder meridian (State Pharmacopoeia Committee of People's Republic of China, 2010). Accumulative evidence has demonstrated that saponins extracted from *Clematidis Radix* possess anti-inflamatory (Fu et al., 2010; Shi et al., 2006) and chondroprotective (Hsieh et al., 2011;

Abbreviations: AIA, adjuvant-induced arthritis; AR, clematichinenoside AR; Foxp3, forkhead box P3; GALT, gut-associated lymphoid tissues; IL-10, interleukin-10; IL-17 A, interleukin-17 A; PPs, Peyer's patches; RA, rheumatoid arthritis; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Treg cells, regulatory T cells

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Wu et al., 2013, 2010) properties, and clematichinenoside AR (AR) has been isolated and identified as the major active component of triterpenoid saponins (Liu et al., 2009; Ma et al.,2009; Zhou et al., 2012).

Our previous studies indicated that intragastric administration of AR markedly improved the hind paw swollen ratio and histopathological changes in collagen-induced arthritis (CIA) rats, decreased the levels of IL-1β and TNF-α in serum, and down-regulated the expression levels of NF-κB p65 subunits, TNF-α and COX-2 in synovial membrane tissues (Peng et al., 2012). Moreover, AR treatment significantly reduced the expression levels of TNF-α, PI3K and p-Akt in synovium of the CIA rats (Han et al., 2013). These studies demonstrated that AR is beneficial in preventing experimental arthritis, due to its anti-inflammatory and immunosuppressive properties. In addition, AR has been shown to have a favorable membrane permeability within the whole segment of small intestine, and be able to diffuse passively into the intestinal mucosa (Wang et al., 2012). AR was mainly metabolized by intestinal microflora, eight metabolites of which have been identified and might contribute synergistically to the total pharmacological activities of AR (Li et al., 2013). Therefore, we hypothesized that the mechanism underlying the anti-arthritis effect of AR may be related to the regulation of enteric mucosal immune system when orally administrated.

Peyer's Patches (PPs), located within the small intestinal epithelium, are highly organized lymphoid follicles of the gut-associated lymphoid tissues (GALT), which is a well-developed immune network. PPs serve as essential physiological inductive sites of oral tolerance, mucosal immunity and systemic immunity. Moreover, PPs are hotbeds for Regulatory T cells (Treg cells) generation (Ahlers and Belyakov, 2010; Nagatani et al., 2004). Treg cells are the kind of cells that acquire their regulatory potential during differentiation in the thymus and the population induced from naive T cells in the periphery under various tolerogenic conditions, which are termed as naturally occurring Tregs and inducible Tregs, respectively. Although the phrase 'Treg cells' encompasses different cell subtypes, those expressing the specific transcription factor forkhead box P3 (Foxp3) named $CD4^+CD25^+Foxp3^+$ Treg cells have received considerable attention due to their prominent immunosuppressive properties. $CD4^+CD25^+Foxp3^+$ Treg cells are responsible for the induction of mucosal immune suppression, maintaining immune system homeostasis and negative regulation of systemic immunity. The expansion of naturally occurring and inducible $CD4^+CD25^+Foxp3^+$ Treg cells considerably induced therapeutic benifits in experimental polyarthritis models and RA patients (Huang et al., 2012; Kong et al., 2012; Moon et al., 2013). It has been established that *de novo* induction of $CD4^+CD25^+Foxp3^+$ Treg cells in peripheral sites outside the thymus preferentially occurs in GALT (Ahlers and Belyakov, 2010), and PPs have been demonstrated to be the key site for the generation of interleukin-10 (IL-10) producing $CD4^+CD25^+Foxp3^+$ Treg cells and transforming growth factor-β (TGF-β)$^+$ Treg cells (Takayama et al., 2007; Zhong et al., 2010).

There is a constant migration of lymphocytes from PPs or other mucosal inductive sites to the mucosal regulatory network, and to the peripheral circulation where they control the systemic immune homeostasis (Park et al., 2009; Simecka, 1998; Wang et al., 2009). Once the function of GALT lymphocytes was induced to change, the function of systemic immune cells might alter subsequently (Biondo et al., 2008; Wieten et al., 2010). Therefore, modulation of PPs lymphocytes activity might influence the host's ability against immunologic responses, and upregulation of Treg cells derived from PPs has been confirmed to be beneficial for controlling the severity of experimental arthritis (Park et al., 2008; Zhou et al., 2006). Based on the characteristics of intestinal absorption and metabolism, the anti-arthritis effect of AR was supposed to be involved in the enteric mucosal immune system, and this hypothesis was tested in this study using the adjuvant-induced arthritis (AIA) rat model.

RA is characterized by T cells imbalance, among which Treg and Th17 subsets play the antagonistic roles in the initiation and development of autoimmune arthritis, and modulation of the balance between Treg and Th17 subsets is crucial for the treatment of RA (Moon et al., 2013; Seki et al., 2008). So far, the effects of AR on mucosal and peripheral immunity associated with Treg cells have not been investigated. This study would emphasize on the effects of AR via oral administration on PPs-associated and systemic immune function based on the regulation of $CD4^+CD25^+Foxp3^+$ Treg cells. In addition, the function of Th17 cells was also studied on account of the immunomodulatory effect of AR. Taken together, the aim of the study is to determine the potential of AR as a therapeutic agent for RA treatment.

2. Materials and methods

2.1. Drugs and reagents

AR was provided by Jiangsu Chia-tai Tianqing Pharmaceutical Co., Ltd. (Nanjing, China), and was suspended in distilled water at required concentrations. Tripterygium glycosides (TG) used as a positive control was purchased from Jiangsu Meitong Pharmaceutical Co., Ltd. (Taizhou, China), and was dissolved in 0.5% carboxymethylcellulose sodium (CMC-Na) solution.

The reagents included complete Freund's adjuvant (CFA) (Chondrex, USA), Rat organs and tissues lymphoprep (Hao Yang Biological Manufacture, CN), RPMI-1640 medium (Gibco, USA), Concanavalin A (ConA) (Sigma-Aldrich, USA), enzyme-linked immunosorbent assay (ELISA) kits for TGF-β_1 and IL-10 (ExCell Biology, CN), ELISA kits for TNF-α and IL-17 A, anti-Mouse/Rat Foxp3 Staining Set APC, anti-Rat CD4 FITC, anti-Rat CD25 PE, flow cytometry staining buffer and erythrocyte lysis buffer (eBioscience, USA), anti-Foxp3 antibody and anti-ROR gamma antibody (Abcam, USA). Other chemicals and reagents used in these experiments were analytical grade from commercial sources.

2.2. Animals

Male Sprague-Dawley rats weighing 110–130 g were purchased from Shanghai Super-B&K Laboratory Animal Co., Ltd. (Shanghai, China). Rats were adapted for a week and housed under standard laboratory condition of constant temperature (23 ± 2 °C) with a 12-h alternate light/dark cycle. Rats had free access to commercial standard diet and tap water ad libitum. The experimental use of animals and procedures were performed in accordance with current ethical regulations for animal care and use at China Pharmaceutical University.

2.3. Induction, treatment and evaluation of AIA

SD rats were immunized by intradermal inoculation into the plantar surfaces of right hind paws with 0.1 ml of CFA containing 10 mg/ml of heat-killed *M. tuberculosis H37 RA*. The day of inoculation was regarded as day 0. On day 18 after immunization, rats were randomly allocated into six groups, among which AR (8, 16, 32 mg/kg) and TG (18 mg/kg) were administered intragastrically once daily for 2 weeks. Normal and AIA model group rats were given distilled water in parallel.

The volumes of non-inoculated hind paws were measured with a YLS-7C plethysmometer (Jinan Yiyan Technology Development Co., Ltd., China). Paw volume (ml) was measured on days 0, 18, 21, 25, 28, and 32 after immunization. Results were expressed as the volume of increase with respect to day 0 volume.