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Anacardic acid suppresses fibroblast-like synoviocyte proliferation and invasion and ameliorates collagen-induced arthritis in a mouse model

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ABSTRACT

Anacardic acid, which is abundant in nutshell of *Anacardium occidentale*, has multiple pharmacological activities. In this study, we examined the therapeutic potential of anacardic acid in treating rheumatoid arthritis (RA). We explored the effects of anacardic acid on collagen-induced arthritis (CIA) in mice and on the proliferation and invasion of RA fibroblast-like synoviocytes (RA-FLSs). The underlying molecular mechanism was investigated. Anacardic acid treatment markedly suppressed paw swelling, joint destruction, and arthritis scores in CIA mice. The serum levels of tumor necrosis factor alpha (TNF- α) and interleutkin-1beta (IL- 1β) were significantly lowered by anacardic acid. *In vitro* assays demonstrated that anacardic acid impaired the proliferation and invasion abilities of RA-FLSs in the presence of TNF- α or IL- 1β. Western blot analysis revealed the reduction of Akt protein expression and phoshporylation in RA-FLSs by anacardic acid. However, the mRNA level of Akt remained unchanged. Anacardic acid treatment significantly increased the expression of miR-633 in RA-FLSs. Akt was identified as a novel target of miR-633. Overexpression of miR-633 significantly inhibited the proliferation and invasion of RA-FLSs, which was rescued by enforced expression of Akt. Depletion of miR-633 prevented anacardic acid-mediated suppression of proliferation and invasion of RA-FLSs, which was accompanied by increased expression of Akt protein. In conclusion, anacardic acid may serve as a promising agent in the treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint inflammation, cartilage destruction, and bone erosion [1]. Inflammatory cytokines especially tumor necrosis factor alpha (TNF-α) and interleutkin-1beta (IL-1β) play an essential role in the development of synovial inflammation in RA [2]. Hyperplasia of fibroblast-like synoviocytes (FLSs) is causally linked to the pathogenesis of RA [3]. RA-FLSs exhibit tumor- like aggressive phenotype including overproliferation and invasion [4]. RA-FLSs can produce inflammatory mediators and matrix degrading enzymes, causing cartilage and bone destruction. Therefore, FLSs serve as an important therapeutic target for the treatment of RA.

microRNAs (miRs) are a large class of small non-coding regulatory RNAs and repress the expression of multiple genes via cleavage of target mRNAs or inhibition of protein translation [5]. miRs typically interact with the 3′-untranslated region (UTR) of target mRNAs. Various biological processes are regulated by miRs, such as proliferation, survival, differentiation, invasion, and metastasis [6,7]. Aberrantly expressed miRs have been found in patients with RA, suggesting their involvement in this disease [8]. It has been documented that miR-125b induces inflammation in RA by activation of NF-κB pathway [9]. Another study reported that miR-192 inhibits the proliferation of RA-FLSs by repressing caveolin 1 [10].

Anacardic acid, which is abundantly detected in nutshell of *Anacardium occidentale*, is a general term applied to a family of 6-alkyl salicyclic acids having varying saturation degrees in the 15-carbon alkyl chain [11]. Anacardic acid is attracting increasing attention due to its multiple pharmacological activities including anticancer [12], cardioprotective [13], and antibacterial [14] properties. Anacardic acid acts as an inhibitor of histone acetyltransferase and shows the ability to potentiate apoptosis in tumor cells [15].

Several signaling pathways such as NF-κB [15], MAPK [16], and Akt [17] are regulated by anacardic acid. Since these signaling pathways are involved in the pathogenesis of RA [18], we hypothesized that anacardic acid may have therapeutic effects on RA. In this study, we

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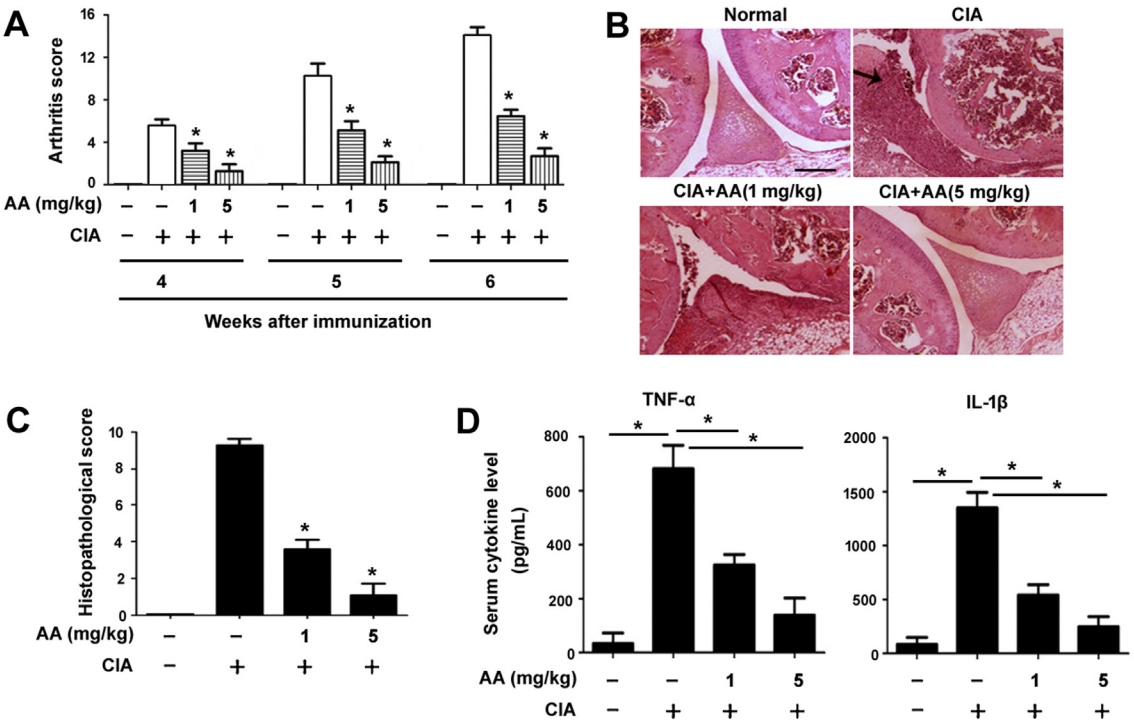

Fig. 1. Anacardic acid attenuates collagen-induced inflammatory arthritis in mice. (A) Assessment of the effect of anacardic acid on the severity of arthritis induced by collagen in mice. $^{*}P < 0.05$ *vs.* the CIA group. (B) Shown are representative photographs of knee joint sections stained with H&E. Arrow indicates cellular infiltration. Scale bar = 50 μm. (C) Histological scores were determined for each group. $^{*}P < 0.05$ *vs.* the CIA group. (D) Measurement of serum TNF-α and IL-1β levels. $^{*}P < 0.05$. AA = anacardic acid.

explored the effects of anacardic acid on collagen-induced arthritis (CIA) *in vivo* and on the biological behaviors of RA-FLSs *in vitro*. The molecular mechanism underlying in the action of anacardic acid was examined.

2. Materials and methods

2.1. Mice and CIA model

Animal studies were approved by the Institutional Animal Care and Use Committee of Zhengzhou University (Zhengzhou, China). Male DBA/1J mice (6 week old) were obtained from Laboratory Animal Resources, Chinese Academy of Sciences (Shanghai, China). For establishment of CIA model [19], mice were immunized with 100 μg chicken type II collagen (Sigma-Aldrich, St. Louis, MO, USA), which was emulsified with an equal volume (0.1 mL) of complete Freund's adjuvant supplemented with 4 mg/mL heat-killed mycobacterium (Chondrex, LLC, Seattle, WA, USA). A booster immunization was performed on day 21 with 100 μg chicken type II collagen emulsified with an equal volume of incomplete Freund's adjuvant. To determine therapeutic effects, anacardic acid (≥97% in purity; Tocris, Minneapolis, MN, USA) was administered via intraperitoneal injection at a dose of 1 or 5 mg/kg body weight twice per week for 3 weeks, starting from the day of booster immunization. Arthritis severity was evaluated twice per week in a blinded manner according to the following criteria [19]: 0 = no signs; 1 = mild swelling of the wrist or ankle; 2 = moderate swelling extending from the ankle to the tarsal bones; 3 = moderate swelling extending from the ankle to the metatarsal joints; 4 = severe swelling of the wrist and ankle including all digits. On day 42, animals were sacrificed and serum was collected. The hindlimbs were dissected and subjected to histological analysis.

2.2. Histological assessment

Ankle joints were routinely fixed, decalcified in 5% formic acid, and embedded in paraffin. Tissue sections were subjected to hematoxylin and eosin (H&E) staining and evaluated in a blind manner by experienced pathologists. The degree of synovitis, pannus formation, bone erosion, and cartilage destruction was scored according to the following criteria: 0, normal; 1, mild changes; 2, moderate changes, and 3, severe changes.

2.3. Serum cytokine levels quantified by ELISA

Serum samples were collected from experimental mice. The serum levels of TNF-α and IL-1β were determined using mouse specific ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

2.4. Cell culture and treatment

FLSs from RA patients were purchased from Cell Applications Inc. (San Diego, CA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). The medium was changed every 3 days. For anacardic acid treatment, RA-FLSs were incubated for 48 h with various concentrations of anacardic acid (5, 30, and 60 μM), in the presence of recombinant human TNF-α (10 ng/mL; R&D Systems) or IL-1β (10 ng/mL; R&D Systems) [20]. In some experiments, MK-2206 (1 μM; Selleck Chemicals, Houston, TX, USA) was added together with TNF-α (10 ng/mL) or IL-1β (10 ng/mL) to the culture medium and incubated for 48 h before measurement of cell proliferation and invasion.

2.5. EdU incorporation assay

RA-FLSs were seeded onto 24-well plates (2×10^4 cells/well) and