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Therapeutic potential of Oroxylin A in rheumatoid arthritis

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ABSTRACT

Excessive inflammation contributes greatly to the pathogenesis and development of rheumatoid arthritis (RA). Oroxylin A (OA) is a natural anti-inflammatory flavonoid compound. In this study, we investigated the effects of OA on collagen-induced arthritis (CIA) and human RA fibroblast-like synoviocytes (FLS). CIA was induced in DBA/1 mice and mice were intraperitoneally treated with OA (10 mg/kg) for 10 days. Arthritis severity was evaluated every day and the histopathologic examination of joints was done. Serum levels of anti-collagen II antibodies (anti-CII Abs) and cytokines were determined by ELISA. Frequency of regulatory T cells (Tregs) and Th17 cells in draining inguinal lymph nodes (ILN) was quantified by flow cytometry. FLS from patients with active RA were treated with varying doses of oroxylin A, followed by stimulation with tumor necrosis factor (TNF)-α (10 ng/mL). The production of cytokines was measured by ELISA. Signal transduction proteins were examined by western blot. OA significantly diminished the arthritis and histological damage. Serum anti-CII Abs, IL-1β, IL-6, TNFα, and IL-17 were significantly diminished by OA treatment. Analysis of CD4 + T cell populations in OA-treated mice showed an increase in Tregs and reduction in Th17 cells in the ILN. In vitro, OA decreased the secretion of IL-1β and IL-6 from TNFα-stimulated RA FLS in a dose-dependent manner. TNFα-induced p38 MAPK, ERK1/2 and NF-κB signaling pathways were suppressed by OA. Our results indicate that OA exerts an anti-inflammatory activity and may have therapeutic potential for human RA.

1. Introduction

Rheumatoid arthritis (RA) is an inflammatory disorder characterized by joint inflammation and destruction. Fibroblast-like synoviocytes (FLS) play a major role in the initiation and perpetuation of destructive arthritis [1]. The pathogenic potential of RA FLS originates from their ability to express immunomodulating cytokines and mediators as well as a wide variety of matrix-modeling enzymes [2]. RA-FLS, together with other immune cells, can disrupt immune homeostasis and create an inflammatory environment in the synovium, which eventually contributes to cartilage damage and joint destruction [2,3]. In addition, FLS can promote the development of RA by secreting multiple inflammatory cytokines, such as IL-6, IL-1β, TNFα and matrix metalloproteinases [4,5,6].

Radix Scutellariae is the dried root of the medicinal plant *Scutellariae baicalensis* Georgi, which exhibits a variety of therapeutic effects and has a long history of application in traditional formulations as well as in modern herbal medications [7]. Oroxylin A (OA), one of the main bioactive flavonoids extracted from Radix Scutellariae, exhibits great potential in the treatment of inflammatory diseases and cancers. OA rescues LPS-induced acute lung injury via regulation of NF-κB signaling pathway in rodents [8]. OA modulated NF-κB signaling pathway involved in inflammation-induced cancer initiation and progression and therefore could be a potential cancer chemoprevention agent for inflammation-related cancer [9]. Actually, the medicinal plant OA has been used widely in traditional Chinese medicine for anti-inflammation, anticancer, antiviral and antibacterial infections, reducing the total cholesterol level and decreasing blood pressures [10]. The aim of this study was to examine the in vivo and in vitro effects of OA on collagen-induced arthritis (CIA) and RA FLS and assess its feasibility as a potential candidate for the treatment of RA.

2. Materials and methods

2.1. Mice and CIA induction

Male DBA/1 mice were purchased from The Jackson Laboratories, Bar Harbor, USA. Mice were housed in specific pathogen-free conditions, and fed by standard mouse chow and water ad libitum. All experimental procedures were approved by the Animal Ethics Committee of our hospital and performed according to institutional guidelines for animal care.

CIA was induced in 8-week-old male DBA/1 mice as described previously [11]: 4 mg/mL of chicken type II collagen (CII) was dissolved in

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0.1 M acetic acid and emulsified in an equal volume of complete Freund's adjuvant (CFA). A total volume of 100 μL of emulsified CII/CFA per mouse was injected intradermally at the base of the tail (on day 0). On day 21 following the primary immunization, mice received a booster immunization with CII emulsified in incomplete Freund's adjuvant (IFA).

2.2. OA administration and assessment of arthritis

OA (Sigma-Aldrich, St Louis, MO, USA) was dissolved in sodium carboxymethylcellulose diluted in saline (CMC-Na, 5 g/L). Thirty mice with established CIA on day 6 after the booster immunization were randomly divided into 2 groups (n = 15/group: vehicle-treated CIA mice, OA-treated CIA mice) and were intraperitoneally administered OA (10 mg/kg) or vehicle (CMC-Na) daily for 10 days on the basis of published data [12].

Joint inflammation was assessed every day by scoring the four paws on a scale of 0–2, where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced edematous swelling [13,14]. In addition, the thickness of the arthritic hind paws was measured every day with microcalipers.

2.3. Histopathologic assessment of joints

Paws were removed, fixed in 4% formalin, decalcified with 10% EDTA. Fixed paws were embedded in paraffin, and sections (5 μM thick) were cut and stained with hematoxylin and eosin. Histopathologic damage in the ankle joints was assessed using a scale of 0–3, where 0 = normal, 1 = minimal synovitis without cartilage/bone erosion, 2 = synovitis with some marginal erosion but joint architecture maintained, and 3 = severe synovitis and erosion with loss of normal joint architecture. A value for each paw was obtained and yielded the maximum possible score of 12 [14].

2.4. ELISA for serum anti-mouse collagen II (anti-CII) Abs and cytokines

Anti-CII Abs ELISA kits were purchased from Chondrex (Redmond, WA). Sera from CII-immunized mice were obtained at the end of this experiment, and the levels of anti-CII Abs (total IgG, IgG1, IgG2a, and IgG2b) were measured according to the manufacturer's protocol [15]. Diluted serum samples were added to mouse CII-coated 96-well plates and incubated at 4 °C overnight. Bound IgG was detected by incubation with HRP-conjugated anti-mouse IgG, followed by OPD substrate. Serum cytokines were measured by specific enzyme-linked immunosorbent assay (ELISA) kits from R&D system (Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. Flow cytometry

Intracellular cytokine staining and flow cytometry were conducted as previously reported [16,17]. Single cell suspensions were isolated from draining inguinal lymph nodes (ILN), followed by stimulation with 20 ng/mL PMA (Sigma-Aldrich, St Louis, MO, USA) and 1 μg/mL ionomycin (Sigma-Aldrich, St Louis, MO, USA) in the presence of 10 μg/mL brefeldin A (Sigma-Aldrich, St Louis, MO, USA) for 4 h at 37 °C, 5% CO_2. Cells were stained with anti-CD4 antibody (BD Biosciences, San Diego, CA, USA), permeabilized with 0.5% saponin and stained with anti-IL-17 (MiltenyiBiotec, Bergisch Gladbach, Germany) antibodies. To analyze regulatory T cells (Tregs), single cell suspensions from ILN were stained with anti-CD4 (BD Biosciences, San Diego, CA, USA) antibodies, fixed, permeabilized and stained with anti-Foxp3 antibody (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Isotype controls were included in all experiments to adjust the background. Flow cytometer analysis was performed in a FACSCalibur instrument and analyzed with Cell Quest Pro software (BD Biosciences, San Diego, CA, USA).

2.6. Human RA FLS

Synovial biopsies were obtained from RA patients fulfilling the classifications [18]. Overall, biopsies from 10 different RA patients were used. Synovial biopsies were obtained surgically at the time of joint replacement surgery or joint synovectomy. FLS were obtained from synovial tissues after incubation in collagenase A (1 mg/mL) (Sigma-Aldrich) for 2 h. After filtration with a 70 μm cell strainer, cells were cultured in RPMI 1640 medium (Lonza, Belgium) supplemented with 10% of fetal bovine serum (Thermo Scientific, USA). Non-adherent cells were removed by washing with Dulbecco's phosphate buffered saline (DPBS) (Santa Cruz) at 24 h. Analysis by flow cytometry showed CD90 expression >95% of the isolated cells. Cells were used between passage 3 and 6. All patients gave their written consent. The study was approved by the local ethics committee in accordance with the Declaration of Helsinki.

2.7. Cell viability assay

Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RA FLS were seeded at 5×10^4 cells/well in 96-well plates, incubated overnight, and then exposed to OA at different concentrations (1 to 16 μM) for 24 h and then incubated with TNFα (R&D Systems, Minneapolis, USA, 10 ng/mL) for 12 h. At the end of treatment, 20 μL of 0.5% MTT was added to the medium and incubated for 4 h at 37 °C. The supernatant was removed and 0.1 mL DMSO was used to dissolve precipitate. At the end, absorbance was measured spectrophotometrically at 570 nm.

2.8. Detection of cytokines in the cell supernatants

RA FLS were stimulated with TNFα (10 ng/mL) for 12 h in the presence or absence of OA. The conditioned media were collected, and secreted IL-1β and IL-6 were measured by specific ELISA kits from R&D system (Minneapolis, MN, USA) according to the manufacturer's instructions.

2.9. Western blot

Equal amounts of protein samples (50 μg) were loaded on a 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST buffer for 2 h at room temperature, and subsequently incubated with primary antibodies at 4 °C overnight respectively. It was followed by incubation with respective HRP-conjugated secondary antibodies, and the bands were developed with ECL substrate and exposed to X-ray film, or scanned using Tannon 5200 (Tanon, Beijing, China) according to the manufacturer's instructions. Blots were analyzed for band intensities using Image J software. For quantification, the relative abundance of each protein was normalized to internal control GAPDH. The following primary antibodies were used: 1:1000 for phospho-p38 mitogen-activated protein kinase (MAPK), phospho-ERK and phospho-extracelluar signal-regulated kinase (ERK) and 1:5000 for GAPDH. The membranes were incubated with the secondary antibodies (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, all from Santa Cruz, CA, USA) for 1 h at room temperature.

2.10. Immunofluorescence and confocal microscopy of NF-κB p65

RA FLS were washed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 30 min at room temperature. After being permeabilized with 1% Triton X-100 for 10 min, the cells were blocked with PBS containing 5% bovine serum albumin for 30 min at room temperature and immunofluorescent staining was performed using a specific mouse polyclonal antibody against NF-κB p65 (dilution, 1:500) followed by Cy3-conjugated mouse anti-rabbit immunoglobulins. The slides were counterstained with Hoechst 33258. Finally, the cover slips were