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Lycium barbarum polysaccharide attenuates type II collagen-induced arthritis in mice

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ABSTRACT

No curative treatment is yet available for rheumatoid arthritis (RA), wherein chronic synovitis progresses to cartilage and bone destruction. Considering the recently recognized anti-inflammatory properties of *Lycium barbarum* polysaccharide (LBP; a derivative of the goji berry), we established the collagen type II-induced arthritis (CIA) mouse model to investigate the potential therapeutic effects and mechanisms of LBP. The CIA-induced changes and LBP-related effects were assessed by micro-computed tomography measurement of bone volume/tissue volume and by ELISA and western blotting detection of inflammatory mediators and matrix metalloproteinases (MMPs). The CIA mice showed substantial bone damage, bone loss, and increased concentrations of TNF-α, IL-6, IL-17, PGE2, MIP-1, anti-type II collagen IgG, MMP-1, and MMP-3. LBP treatments produced significant dose-dependent improvements in CIA-induced bone damage and bone loss, and significantly reduced CIA-stimulated expression of the inflammatory mediators and MMPs. Thus, LBP therapy can preserve bone integrity in CIA mice, possibly through down-regulation of inflammatory mediators.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis, characterized by infiltration of macrophages and lymphocytes into the synovium. The consequent local increase in secreted proinflammatory factors (i.e. cytokines, chemokines, and enzymes) further promotes an inflammatory synovial environment, stimulating potentially destructive signaling mechanisms that can compromise joint integrity [1]. This pathogenic process includes the generation of reactive oxygen species (ROS; e.g., superoxide, hydrogen peroxide, and hydroxyl radicals) by the accumulated macrophages, as well as by the neutrophils recruited to the site [2]. ROS-induced tissue damage involves physical breakdown of biomembranes through a destructive oxidation process known as lipid peroxidation [3]. Furthermore, the interaction of ROS and reactive nitrogen species (RNS; e.g. nitric oxide [NO]), representing the nitrosative stress pathway, is another pathogenic mechanism that has been implicated in RA-related joint damage [4].

Much of the current knowledge on the molecular pathogenesis of RA has been gained through studies using the mouse model of collagen type II-induced arthritis (CIA) [5,6]. These studies have focused on elucidating the roles of the proinflammatory cytokines that are up-regulated in RA patients, such as tumor necrosis factor (TNF)-α and various interleukins (ILs) [7]. Indeed, several TNF antagonists (e.g. infliximab, adalimumab, and etanercept) have been developed as effective RA treatments [8]. Ongoing studies of IL-6 blockade have provided promising initial results for this approach as a targeted molecular therapy of RA [9]. Finally, the demonstrated correlations between serum levels of cytokines and disease activity have allowed for improvements to be made in diagnosis and disease status monitoring in the clinical setting. Unfortunately, each of the currently available pharmacologic and biologic agents for RA is associated with numerous adverse effects and variable efficacies, and a safe curative treatment for RA remains to be discovered.

Researchers have recently turned their attentions towards plant-based bioactive derivatives with immune-modulation abilities. *Lycium barbarum*, also known as wolfberry, is a common Chinese Traditional Medicine and also an edible food; it has been used historically in the treatment and prevention of diseases such as insomnia, liver dysfunction, diabetes, and visual degeneration.

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One of its bioactive components is the *Lycium barbarum* polysaccharide (LBP), a polysaccharide-protein complex extracted from the goji berry[10] which has demonstrated anti-inflammatory properties capable of inhibiting chronic inflammation-related injury [11], as well as other promising therapeutic properties, including immune regulation enhancement[12,13], anti-tumor [14], anti-stress [15], anti-aging[16], and anti-oxidation [17]. Since RA is a chronic inflammatory polyarthritis, we hypothesized that LBP could modulate the inflammatory reaction to provide therapeutic protection. Thus, the present study was designed to determine the potential of LBP for use as a joint protective agent and to investigate its underlying mechanisms using the well-established CIA mouse model.

2. Materials and methods

2.1. Reagents and LBP powder composition analysis

All chemicals were of the highest purity and analytical grade. Bovine collagen type II (C-II), complete Freund's adjuvant (CFA) and cyclophosphamide (MTX) were purchased from Sigma Corp. (St. Louis, MO, USA). LBP (Versitech Ltd., Southern District, Hong Kong) was provided by the Hong Kong Institute of Biotechnology (Shatin, Hong Kong); neutral sugar composition analysis showed that the LBP power contained about 35% arabinose, 16% galactose, 10% rhamnose, and small portions of glucose, xylose, mannose and carotenoids, and phenol-sulfuric acid composition analysis showed that the purity of the LBP was approximately 76% (w/w carbohydrates).

2.2. Experimental animals

Male DBA/1 mice (8 weeks old, 18 ± 2 g) were obtained from the Shanghai Institute of Materia Medica at the Chinese Academy of Sciences (Production License No. SCXK [HU] 2008-0017). All mice were acclimated to the laboratory environment (20 ± 2 °C, 70 ± 10% humidity, 12/12 h light/dark cycle) for 2 weeks prior to experimentation. All animal protocols were designed according to the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the First Affiliated Hospital of the Third Military Medical University (Chongqing, China).

2.3. Establishment of the CIA mouse model

Forty mice were used to establish the CIA model by the previously described procedure [18]. Briefly, a 2 mg/mL solution of bovine C-II was dissolved in 10 mM acetic acid, mixed with an equal volume of CFA, and emulsified with a homogenizer. The emulsified solution (200 μL) was immediately injected intradermally into the tail base of each mouse (experimental day 1). A booster dose injection (prepared and delivered as described above) was given on day 21. Ten additional mice were injected with CFA alone and served as the unmodeled negative controls (group 1, control group).

To test the anti-arthritic effect of LBP, the CIA modeled mice were randomly divided into four equal-sized groups for immunization with the C-II routine described above (modeled controls; CIA group) or along with LPB treatment starting at day 24 (intraperitoneal injection, once daily for 10 days) at doses of 25 mg/kg (LBP 25 mg/kg, low-dose group), 50 mg/kg (LBP 50 mg/kg, mid-dose group) or 100 mg/kg (LBP 100 mg/kg, high-dose group). The experimental course ended on day 56 with all mice sacrificed by anesthesia overdose.

Before sacrifice, the severity of arthritis was assessed in each mouse by two investigators blinded to the group assignment and using the previously established CIA semi-quantitative scoring system[19] to rank inflammation in each of the four paws at specific time points, as follows: 0, no evidence of erythema or swelling; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling encompassing the ankle, foot, and digits. The sum of each posterior limb graded and calculated, and averaged between the two observers, was reported as the "mean clinical score".

2.4. Blood collection

A blood draw was performed on each mouse immediately after sacrifice; the samples (average volume: 400 μL) were placed on ice and immediately aliquoted for manual leukocyte counting and processing to obtain serum by separation-centrifugation ($600 \times g$, 4 °C, 15 min) in the presence or absence of 10 μg/mL indomethacin (to facilitate prostaglandin E2 (PGE2) estimation) for subsequent biochemical analysis.

2.5. Analysis of bone morphology by micro-computed tomography (CT)

The hind paws were removed, placed in an imaging tube, submerged in 10% neutral buffered formalin and subjected to 360° micro-CT scanning using an eXplore Locus SP specimen scanner (GE Healthcare Medical Equipment, Little Chalfont, UK) using the following imaging parameters: scanning time: 270 min; exposure time: 3 s; photo energy: 80 kVp; current: 80 μA; spatial resolution: 14 μm. The images were analyzed using the accompanying eXplore Reconstruction Utility and MicroView software packages. The bone mineral density was quantified using the Advanced Bone Analysis software.

2.6. Measurement of paw diameter

The CIA- and LBP-related changes in dorso-plantar paw diameter were quantitatively assessed by comparing micrometer screw gauge measurements taken on day 0 (one day before the CFA or CFA + C-II injections) to those taken on experimental days 4, 7, 11, 14, 18, 21 and 56 [20].

2.7. Enzyme-linked immunoassays (ELISAs) of proinflammatory factors

The concentrations of TNF-α, IL-6 and IL-17 in serum were assessed using targeted ELISA kits (R&D Systems, Minneapolis, MN, USA) and absorbance reading at 450 nm in an Asys Expert 96 microplate reader (Biochrom Ltd., Cambridge, UK). The levels of chemokines, such as MIP-1, were measured in the aqueous joint extracts as described previously [21]. In brief, joint tissues were mechanically homogenized (Beidi Instruments, Nanjing, China) on ice in 3 mL of lysis buffer and then centrifuged at $2000 \times g$ for 10 min. The supernatants were quantified by a protein assay kit (R&D Systems, Minneapolis, MN, USA).

2.8. Western blotting analysis of MMP-1 and -3

The total protein composition of bone tissue lysates from the hind paws was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred to a polyvinylidene fluoride membrane. After blocking with 5% skim milk for 4 h and washing with Tris-buffered saline (TBS; 3×5 min), the membrane was incubated overnight with rabbit anti-mouse MMP-1 or -3 polyclonal antibody (both at 1:200 dilutions; Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C, which was followed by a 2 h incubation with horseradish peroxidase-conjugated goat