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Ethyl pyruvate ameliorates inflammatory arthritis in mice

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ABSTRACT

Objectives: Ethyl pyruvate (EP) is the ethyl ester of pyruvate and has antioxidative and anti-inflammatory effects. This study aimed to evaluate the therapeutic effect of EP in inflammatory arthritis and to identify the underlying mechanisms.

Methods: Mice with collagen-induced arthritis (CIA) were treated with the vehicle control or EP at 20 mg/kg, and clinical and histological analyses were performed on the animals. The differentiation of murine CD4 + T cells into T helper 17 (Th17) cells in the presence of EP was investigated *in vitro*. The effects of EP on osteoclastogenesis were determined by staining for tartrate-resistant acid phosphatase, and measuring the mRNA levels of osteoclastogenesis-related genes. The expression of high-mobility group box 1 (HMGB1) was evaluated after EP therapy using immunohistochemical staining and Western blotting.

Results: EP significantly improved the clinical and histological features of arthritis in CIA mice. EP suppressed the differentiation of CD4 + T cells into Th17 cells, and inhibited the expression of RORγt. The generation of osteoclasts and osteoclastogenic markers from murine and human monocytes was significantly reduced in the presence of EP. The expression of HMGB1 in the synovium was significantly lower in CIA mice treated with EP, compared to control CIA mice. During osteoclastogenesis, HMGB1 release from monocytes was inhibited in the presence of EP.

Conclusions: EP attenuated synovial inflammation and bone destruction in the experimental arthritis model through suppression of IL-17 and HMGB-1. The data suggests that EP could be a novel therapeutic agent for the treatment of inflammatory arthritis, such as rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by synovial inflammation and bone erosion [1]. Interaction between immune cells and multiple inflammatory mediators contribute to the development and progression of chronic inflammation in the pathogenesis of RA [2]. Current treatments for RA are based on suppression of the complex immune system, leading to suppression of inflammatory arthritis. However, immune suppressive therapy lacks efficacy in a substantial number of patients, and is often accompanied by side effects, such as infection. More recent therapies target modulation of specific cells or cytokines, rather than global suppression of immunity [3]. Although this targeted therapy is highly effective for the control of inflammatory arthritis in patients with an inadequate response to conventional therapy, the increased risk of infection remains. Thus, there is a need to explore alternative therapies with an immune modulatory capacity and minimal side effects. For this reason, endogenous molecules with anti-inflammatory effects could be promising candidates.

Pyruvate, an important intermediate in energy metabolism, is a product of glycolysis and a substrate that initiates the tricarboxylic acid (TCA) cycle. Since pyruvate was proven to protect against reactive oxygen species (ROS)-induced cytotoxicity [4,5], numerous studies have been performed to evaluate the cytoprotective effects of pyruvate in hypoxic tissue injuries. Administration of pyruvate improved the survival rate in animal models of ischemia-reperfusion injury of kidney, heart, liver, and intestine [6–12]. Thus, pyruvate has been suggested to be an endogenous scavenger of ROS. Because oxidative stress is closely linked with the inflammatory response, pyruvate is expected to have a protective effect against excessive inflammation [13].

Despite its theoretical benefits, pyruvate has limited therapeutic potential due to its poor stability in aqueous solutions. In solution,

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pyruvate is spontaneously converted to parapyruvate [14], which has a different biological activity from pyruvate. Parapyruvate inhibits the TCA cycle by blocking the oxidative decarboxylation of α-ketoglutarate to succinate in the mitochondria [15]. To overcome the limitations of pyruvate as a therapeutic agent, ethyl pyruvate (EP), the ethyl ester of pyruvate, was developed as a more stable form with better bioavailability [9]. EP has been studied in numerous *in vivo* and *in vitro* models of various pathophysiologies, and has demonstrated better therapeutic efficacy than pyruvate [16].

Based on the anti-inflammatory role of EP in experimental models, we investigated the therapeutic effects of EP in mice with collagen-induced arthritis (CIA). The clinical and histological features of inflammatory arthritis were compared between CIA mice treated with EP or the vehicle control. *In vitro* experiments revealed the inhibition of inflammatory response and osteoclastogenesis by treatment with EP.

2. Methods

2.1. Mice

Six-week-old male DBA/1J mice were purchased from Charles River Laboratories (Yokohama, Japan) and maintained in the specific pathogen-free animal facilities of the Catholic University of Korea, South Korea. All experiments were approved by the Animal Research Ethics Committee at the Catholic University of Korea.

2.2. Induction of collagen-induced arthritis and treatment with EP

Bovine type II collagen (CII; Chondrex, Redmond, WA) was dissolved in 10 mM acetic acid. Male DBA/1J mice at 7 weeks of age were injected intradermally at the base of the tail with 100 μg of CII in complete Freund's adjuvant (Chondrex). Two weeks later, the mice were administered a booster of 100 μg of CII emulsified with incomplete Freund's adjuvant (Chondrex) in one hind footpad. The mice were intraperitoneally injected with either EP (20 mg/kg) or vehicle control (saline) three times per week beginning on day 14 after the primary immunization. The severity of arthritis was monitored for 8 weeks to investigate the effects of EP on CIA.

2.3. Assessment of arthritis

The development and severity of arthritis were examined three times per week for up to eight weeks after the primary immunization by three independent observers. The severity of inflammation was clinically graded on a scale from 0 to 4 as described previously [17]: 0 = no evidence of erythema and swelling; 1 = erythema and mild swelling confined to the mid-foot (tarsal) or ankle joint; 2 = erythema and mild swelling extending from the ankle to the mid-foot; 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 arthritic score was expressed as the sum of the scores from three limbs (excluding the boosted paw) with the highest possible score being 12 points. Regular monitoring was performed for 8 weeks to investigate the effects of EP on CIA.

2.4. Histological evaluation

Joint tissues of CIA mice were fixed in 10% paraformaldehyde and embedded in paraffin. Tissue sections of 7-μm thickness were prepared and stained with hematoxylin-eosin (H & E). For immunohistochemical staining, the sections were deparaffinized using xylene and were then dehydrated in a gradient of alcohol solutions. The endogenous peroxidase activity was stopped with 3% H_2O_2 in methanol. Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The tissue slices were first incubated with primary antibodies against high-mobility group box 1 (HMGB1) (Abcam, Cambridge, UK), and isotype control antibodies (Santa Cruz Biotechnology Inc.) overnight at 4 °C. The slices were then incubated with a biotinylated secondary antibody and a streptavidin–peroxidase complex for 1 h. The final colored product was developed using DAB chromogen (DAKO, Carpinteria, CA), and the sections were counterstained with hematoxylin. The images were captured using a DP71 digital camera (Olympus, Center Valley, PA) attached to an Olympus BX41 microscope.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Serum obtained from the orbital sinuses of CIA mice was analysed to measure the concentrations of immunoglobulin G (IgG). The concentration of IgG1 and IgG2a were determined using mouse IgG1 and IgG2a ELISA Quantitation Kits (Bethyl Laboratories, Montgomery, TX, USA), respectively. Absorbance values were determined with an ELISA microplate reader operating at 450 nm. For measurement of IL-17 and HMGB1, the culture supernatants were obtained under Th17 differentiation and osteoclastogenesis conditions, respectively. Antibodies against mouse IL-17 and biotinylated anti-mouse IL-17 antibodies (R & D Systems) were used as the capture and detection antibodies, and the ExtrAvidin-HRP (Sigma) was used for color development. The amount of IL-17 was determined from standard curves established with serial dilutions of recombinant IL-17 (R & D Systems). The concentration of HMGB1 was measured using a HMGB1 Detection kit (Chondrex) according to the manufacturer's instructions.

2.6. Flow cytometry

For intracellular detection of IL-17, CD4 + T cells cultured under Th17 promoting condition were incubated with 25 ng/mL phorbol 12-myristate 13-acetate (PMA), 250 ng/mL ionomycin (Sigma Aldrich, St Louis, MO), and monensin-containing GolgiStop (BD Biosciences) for 4 h. The cells were harvested and stained with PerCP-conjugated anti-CD4 antibodies (Biolegend, San Diego, CA). After fixation with fixation/permeabilization solution, the cells were stained with FITC-conjugated anti-IL-17 antibodies (eBioscience) or PE-Cy7-conjugated anti-interferon (IFN)-γ antibodies (BD biosciences). APC-conjugated anti-CD25 antibodies (eBioscience) and anti-FOXP3 antibodies (eBioscience) were used to determine the population of Treg cells. All analysis was performed using a BD LSRII fortessa (BD Biosciences) and FACS DIVA version 10.0 (BD Biosciences).

2.7. Th17 differentiation

CD4 + T cells were purified from mouse spleens using a MACS isolation kit (Miltenyi Biotec Inc. Bergisch Gladbach, Germany) with an autoMACS separator according to the manufacturer's instructions. To detect the expression of RAR-related orphan receptor gamma t (RORγt), naïve CD4 + T cells were isolated with a mouse naïve CD4 + T cell enrichment kit (eBioscience) [18]. Murine CD4 + T cells were cultured in RPMI1640 media (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS). CD4 + T cells and naïve CD4 + T cells were stimulated with plate-bound anti-CD3 (0.5 μg/mL) and anti-CD28 (1 μg/mL) (all from BD PharMingen, San Diego, CA). To evaluate the effect of EP on Th17 differentiation, activated CD4 + T cells were cultured in the absence or the presence of EP for 1 h. To establish Th17 differentiation, recombinant transforming growth factor-β (2 ng/mL) (PeproTech Rocky Hill, NJ), IL-6 (20 ng/mL), anti-interferon-γ (2 μg/mL), and anti-IL-4 (2 μg/mL) (R & D Systems, Minneapolis, MN) were added to the cultures.

2.8. Osteoclastogenesis assay

To induce osteoclastogenesis, we obtained bone marrow cells from mouse femurs and tibia and peripheral blood mononuclear cells from