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Emodin ameliorates ulcerative colitis by the flagellin-TLR5 dependent pathway in mice

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ABSTRACT

Emodin is an anthraquinone compound derived from *Rheum officinale Baill*. Several reports showed that emodin had efficacy on acute pancreatitis, keratitis, myocarditis, and rheumatoid arthritis. However, the potency of emodin on ulcerative colitis(UC) remains unclear. In this study, we investigated the effect of emodin on dextran sodium sulfate (DSS)-induced ulcerative colitis in mice. Our results showed that emodin significantly alleviated the symptoms of DSS-induced UC in mice, involving prevented the loss of body weight and colon shortening, decreased the disease activity index (DAI), intestinal damages and the count of white blood cells (WBC) in peripheral blood. In addition, emodin treatment decreased the level of anti-flagellin antibody in serum and significantly down-regulated the expression of TLR5 and NF-κB p65 in colon of the UC mice. Further study in vitro showed that emodin down-regulated the expression of TLR5 and MyD88, up-regulated the expression of IκB, inhibited the nuclear translocation of NF-κB p65 and decreased the release of IL-8 in flagellin-stimulated HT-29 cells. These results, for the first time, demonstrated that emodin had the therapeutic potential to ameliorate UC symptoms possibly via regulating the flagellin-TLR5 signaling pathway. Emodin may be a potential candidate ingredient for ulcerative colitis.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon, characterized by abdominal pain, diarrhea, and hematochezia [1]. In contrast with Crohn's disease, another kind of inflammatory bowel disease(IBD), inflammation of UC is limited to the colonic mucosa which starts in the rectum and extend continuously to proximal segments of the colon [2]. In recent years, the incidence of UC has been rising rapidly, especially in newly industrialised countries in South America, Eastern Europe, Asia, and Africa [3]. However, there is a lack of specific and safe therapies and medicine to cure.

The etiology of UC has not been completely understood, but intestinal microbiota is a crucial reason besides genetic, environmental, and immunological factors [4]. Flagellin, a primary structural component of bacterial flagella, is one of pathogen-associated molecular patterns (PAMPs) which can be recognized by Toll like receptor 5 (TLR5) and triggers signaling cascades to mediate the inflammatory response. Flagellin was an important antigen in IBD [5], and flagellin/TLR5 played a vital role in the development and progress of colitis [6]. Recent studies showed that TLR5 gene polymorphisms was significantly associated with UC [78], and the expression of TLR5 was up-regulated in UC patients [9]. Therefore, it is proposed that regulation of flagellin/TLR5 signaling pathway might be a therapeutic approach for UC.

Emodin is an important bioactive component of *Polygonaceae* plants, which possesses anti-inflammation [10], anti-cancer [11], anti-sepsis [12], antioxidant [13] and anti-fibrosis effects [14]. A recent investigation indicated that emodin inhibited activation of the NF-κB signaling pathway [15]. However, the effect of emodin on UC remains unknown. In the present study, we aimed to investigate whether emodin attenuated the DSS-induced colon injury and further elucidate the mechanism regulating flagellin-TLR5-NF-κB signaling pathway, to evaluate whether emodin can be a potential component for the treatment of UC.

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2. Materials and methods

2.1. Materials

Emodin was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). The purity of emodin is 96% at least(≥ 96%), measured by high-performance liquid chromatography (HPLC). Dextran sodium sulfate (DSS) was purchased from MP Biomedicals (California, USA). Dulbecco's Modified Eagle's Medium (DMEM/high glucose) and fetal bovine serum (FBS) were purchased from Gibco (California, USA). Anti-TLR5 antibody was obtained from Abcam (Cambridge, UK). Antibodies for MyD88, IκBα, NF-κB p65, β-actin and Immunohistochemistry Application Solution Kit Rabbit were purchased from CST Inc. (Massachusetts, USA). Goat anti-mouse IgG(H + L)-HRP and Goat anti-Rabbit IgG(H + L)-HRP were purchased from Tianjin Sungene Biotech Co., Ltd.(Tianjin, China). Cytometric Bead Array(CBA) was purchased from BD (New Jersey, USA). Anti-flagellin enzyme-linked immunosorbent assay (ELISA) kits was obtained from R&D Systems(USA). 3- (4,5- dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, USA).

2.2. Animals and treatment

Sixty male C57BL/6 mice (20–22 g) were purchased from Medical Experimental Animal Center of Guangdong province, Guangdong, China (License No: SCX Yue 2013–0002). They were maintained in a specific pathogen-free facility, in which Laboratory Animal Center of Guangzhou University of Chinese Medicine (License No: SCX Yue 2013-0085). All of mice were maintained under a standard temperature (20 ± 2 °C), humidity (55 ± 2%) and 12 h-light/dark cycle. Mice were allowed to acclimate to these conditions for at least 7 days before starting experiments. The experiments were approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine, Guangzhou, China. All animal treatments were strictly in accordance with International Ethics Guidelines and the National Institutes of Health Guidelines Concerning the Care and Use of Laboratory Animals.

Total 60 mice were divided into 6 groups randomly, 10 mice per group, including the control group, the model group, the mesalazine treatment group(0.8 g/kg bw) and the emodin treated groups(5,10 and 20 mg/kg bw). Five groups were given 3%DSS solution to drink freely for 7 days while the control group was given distilled water. Simultaneously, the control group and the model group were administrated with distilled water, and the other 3 groups were administration of corresponding medicine by oral gavage for 14 days continuously. The grouping and treatment with emodin in the DSS-induced UC mice are shown in Supplementary Fig. 1.

2.3. Assessment of inflammation and colitis severity

Disease activity index. The weight changes, water consumption, food consumption, morbidity, diarrhea, and rectal bleeding of mice were observed and recorded daily. According to the previously mentioned, the disease activity index (DAI) was determined as Cooper previously described [16] (as Supplementary Table 1). The DAI score was calculated as the average of weight loss score, diarrhea score, and fecal blood score.

The count of Leukocyte. At the 15th day of the experiment, the peripheral blood was collected to analyze the variety of cells number and percentage change by the automated hematology analyzer.

The level of anti-flagellin antibody in serum. The peripheral blood was collected, and rested for 20 min, centrifuged at 3000 rpm, 20 min. Then, collected the supernatant, and the level of anti-flagellin antibody in serum was measured by ELISA [17].

Histologic analysis of colon. The 15th day of the experiment, animals were anesthetized by pentobarbital sodium, and removed the colon. The colonic length was measured in centimeter. Then, washing the colon, and small segments of colon were fixed in 10% formaldehyde at room temperature overnight, embedded in paraffin, sectioned into 4 mm slices. Sections were stained with hematoxylin and eosin (H&E), and histological scores were blindly determined by two pathologists [18], as supplementary Table 2.

2.4. Immunocytochemistry (IHC)

The paraffin sections of colon tissue were dried in constant temperature oven at 60 °C for 1 h, incubated sections successively in three washes of TO for 5 min each, two washes of 100% ethanol for 10 min each, two washes of 95% ethanol for 10 min each, dH_2O for 5 min each. Then, placed the paraffin sections in microwave oven at medium-high heat for 8 min to antigen unmasking. 3% hydrogen peroxide was used to inhibit endogenous enzyme and blocking solution was used to block the non-specific antigen. After that, anti-TLR5 antibody or anti-NF-κB p65 antibody incubated at 4 °C overnight. Subsequently, incubated the second antibody, and DAB staining. Finally, dehydration, mounting, and observing the colon tissue under microscope.

2.5. Cell viability assay

HT-29 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. It were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C and 5%CO_2. Medium was changed every 2 days, and passaged when the purity is more than 80%. Experiments were performed using cells that had previously been passaged five to fifteen times.

HT-29 cells were homogenized in medium for 24 h, then washing with PBS. After that, the cells were divide into three groups, including the control group, the solvent control group, and different concentrations of emodin solution (2.5,5,10,20, 40 and 80 μM), and incubated for 44 h. Then, adding MTT to cells, incubating continuing for another 4 h. The supernatant was abandoned and was added to dimethylsulfoxide dissolve the formazan. Finally, optical density of cells at 490 nm were measured with multiscan spectrum.

2.6. Western blot analysis

The colon and cell protein sample were extracted with Protein Extraction Kit. BCA protein assay was conducted for protein concentration, and protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk at room temperature for 1 h, incubated respectively with primary antibody of TLR5, MyD88, IκB, NF-κB p65, and β-actin at 4 °C overnight, followed by a second horseradish peroxidase-conjugated antibody at room temperature for 2 h. Finally, the membranes were directly imaged with an enhanced chemiluminescence (ECL) reagent by fully automated chemiluminescence image analyzer (Tanno, China).

2.7. Immunofluorescence analysis

HT-29 cells were seeded in cell culture dish at 2×10^5 cells/mL for 1 mL, respectively treated with 5-ASA and different concentrations of emodin (20 μM, 40 μM, 80 μM) for 24 h and then stimulated with flagellin(500 mg/L) for 24 h. The control group received the same cell culture media without flagellin. Cells were washed with ice-cold PBS and then fixed with 4% paraformaldehyde for 20 min. After that, washed cells and incubated the samples for 20 min with 0.5% Triton X-100. Washing away the redundant Triton X-100, and incubated cells with 10% goat serum for 30 min to bloke non-specific antigen. After blocking, primary antibody (anti-NF-kB p65, 1:400) was added and