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The natural compound celastrol inhibits necroptosis and alleviates ulcerative colitis in mice

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ABSTRACT

Ulcerative colitis (UC) is a chronic intestinal inflammatory disease. Necroptosis plays an important role in the pathogenesis of UC. Celastrol, a triterpene from the root bark of the Chinese medicinal plant *Tripterygium wilfordii*, has been reported to have anti-oxidant and anti-inflammatory activities in colitis. It is not known, however, how celastrol exerts its beneficial effects. The aim of this study is to investigate the effects and possible mechanism of celastrol in UC. Colitis was induced in mice by administration of 5% dextran sulfate sodium (DSS) in drinking water for 4 days. Celastrol was administered intraperitoneally (1 mg/kg) for 7 days after colitis was induced. Our results showed that celastrol treatment ameliorated the severity of colitis, decreased the level of interleukin (IL)-1β, IL-6 and myeloperoxidase (MPO) and upregulated the level of E-cadherin in colitis mice. Moreover, the TUNEL staining and cleaved caspase-3 immunohistochemistry staining proved decreased necrotic cell death after celastrol treatment. On the mechanism, decreased level of necroptosis factors RIP3 and MLKL, and increased level of active caspase-8 were detected after celastrol treatment. Taken together, our results demonstrated that celastrol exerted beneficial effects in colitis treatment *via* suppressing the RIP3/MLKL necroptosis pathway.

1. Introduction

Inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation, resulting from a complex interplay of external factors, genetic susceptibility and immune system dysfunction. There are two main subtypes of IBD: Crohn's disease (CD) and ulcerative colitis (UC) with a combined prevalence of 150–250 per 100,000 population [1–2]. However, the exact etiology of UC remains unclear. It is believed that intestinal dysfunction may result from excessive translocation of commensal bacteria into the bowel wall [3]. Under normal conditions, the intestinal epithelium maintains intestinal homeostasis, which is breached under pathological conditions.

Necroptosis is a newly discovered pathway of regulated necrosis that requires the proteins RIP3 and MLKL which is induced by death receptors, interferons, toll-like receptors, intracellular RNA and DNA sensors, and probably other mediators. Recently, several studies have shown that cellular necroptosis was involved in the dysfunction of intestinal epithelium cells (IECs), which suggesting the possible role of necroptosis in the pathogenesis of IBD [4–5].

Celastrol, also known as Thunder of God Vine, is a triterpene from the root bark of the Chinese medicinal plant *Tripterygium wilfordii*. Celastrol possesses multiple biological and pharmacological activities, including immune modulation, anti-inflammatory, and anti-tumor activities [6–7]. The *in vivo* anti-inflammatory effects of this triterpene have been demonstrated in multiple animal models, including Alzheimer disease, asthma, systemic lupus erythematosus, and rheumatoid arthritis. Moreover, previous studies have revealed that celastrol can ameliorate mouse colitis and human Crohn's disease by inhibiting pro-inflammatory cytokine secretion [8–9].

In this study, we hypothesized that the anti-inflammatory activity of celastrol might be mediated by modulating the cellular necroptosis related signal pathway. Therefore, we established a mouse model of UC and explore the mechanism of celastrol in UC treatment.

2. Materials and methods

2.1. Mouse model of UC

C57BL/6J female mice (body weight: 19 ± 1 g) were provided by the Laboratory Animal Center of Soochow University. The animals were maintained on a 12-h light/dark cycle at 22 °C, given water *ad libitum*, fed standard laboratory chow, and allowed to acclimatize for a minimum of 1 week before the experiment. All experiment procedures were conducted with the approval of the Animal Research Committee at Soochow University.

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Thirty-six C57BL/6J female mice were randomly assigned to three groups: the saline group that was given oral administration of 5% DSS (MPbio, CA, USA) for 4 days and then normal saline for 7 days; the celastrol group that was treated with 5% DSS for 4 days followed by intraperitoneal injection with celastrol (1 mg/kg) (Zelang, China) [10–12] for 7 days; and the normal group that was treated with no DSS or celastrol.

2.2. Disease activity index (DAI)

The disease activity of UC mice was observed from days 0 to 11 and was evaluated based on the index shown in Table 1. The sum of the score for diarrhea, bleeding and body weight loss was used as a disease activity index.

2.3. Tissue dissection

On day 11, mice were euthanized. The abdomen was opened by a longitudinal incision and the GI-tract was exposed. Colon tissues were washed in normal saline and colon length was measured. Colon tissues from six mice of each group were homogenized for Western blotting analysis of proteins. Colons from the remaining six mice in each group were prepared as spiral "Swiss rolls" and fixed overnight in 4% formaldehyde for histological analysis.

2.4. Histology

Fixed "Swiss roll" colon tissues were processed according to standard procedures and mounted in paraffin blocks. Thin (3.0 μm) paraffin sections were cut continuously to have longitudinal sections of the entire length of the colon tissue on one slide. Slides were stained by H&E and analyzed in a blinded fashion, and scored according to the criteria listed in Table 2 [13–14].

2.5. Alcian blue staining

After dewaxing and hydration, paraffin sections were incubated in 3% glacial acetic acid for 5 min, followed by staining with alcian blue for 10 min.

2.6. Immunoblotting

Protein extracts were prepared using the mammalian protein extraction reagent (Thermo Scientific, PA, USA) supplemented with protease inhibitor tablets (Thermo Scientific, PA, USA). Protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, MA, USA). Membranes were probed with the following primary antibodies: TNF-α (Boster, China), RIP3 (ProSci, CA, USA), MLKL (kindly provided by Dr. Sudan He, Soochow University, China), caspase-8 (R&D, MN, USA), IL-1β (Boster, China), and IL-6 (Boster, China).

2.7. Immunohistochemistry

After dewaxing and hydration, paraffin sections were boiled in citrate for antigen retrieval. Endogenous peroxidase activity was blocked by 3% H_2O_2 for 15 min at room temperature. The slides were blocked by incubating in 5% bovine serum albumin (BSA) at 37 °C for 30 min. Sections were then incubated with primary antibodies against RIP3, MPO (RD, MN, USA), PCNA (Novusbio, CO, USA), CD68 (Novusbio, CO, USA), E-cadherin (Proteintech, China) overnight at 4 °C. The slides subsequently proceeded to the protocol of GTVision™ III Detection System/Mo&Rb (Genetech, China), followed by counterstaining with hematoxylin. Cell death was analyzed using cleaved caspase-3 (CellSignaling, MA, USA) and the *in situ* cell death detection kit for TUNEL assay (Beyotime, China). Positive immunohistochemistry signals were shown in brown. Four typical fields of high power (×400) were randomly selected from each slide for evaluation. For nucleus positive cells, percentage of positive cells was calculated in each image. For cytoplasm or cytomembrane positive cells, area of positive reactions was analyzed by Image J software.

2.8 Measurement of inflammatory cytokines by ELISA assay

The blood samples were collected from all mice in non-EDTA coated tubes. After 4000 rpm centrifugation for 15 min, serum were obtained and used to determine the levels of IL-1β, IL-6, TNF-α using ELISA kits (Lankebio, China) according to manufacturer's instructions.

2.9. Statistical analysis

Data were analyzed using GraphPad Prism Software and presented as mean ± standard error of mean (SEM). Differences among different group were analyzed by one-way ANOVA. Differences of body weight and DAI between saline group and celastrol group were analyzed by Student t-test. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Celastrol ameliorated clinical symptoms in DSS-induced colitis in mice

The anti-inflammatory effects of celastrol have been demonstrated in different animal models [11]. The chemical structure of celastrol is shown in Fig. 1A. To investigate whether celastrol is effective in reducing the disease activity of UC in mice, DAI was examined by monitoring body weight loss, diarrhea, and fecal blood during the experiment. Addition of 5% DSS in drinking water caused body weight loss, diarrhea, and rectal bleeding. After intraperitoneal injection of celastrol, the mice showed body weight rebound and the rate of increase was greater than that in the saline group, starting from day 7 (Fig. 1B). The DAI of the celastrol group was lower than that of the saline group, starting from day 6 (Fig. 1C). On day 11, colons were dissected and length was measured. The results showed that the colon length of the celastrol group was significantly longer than that in the saline group (Fig. 1D).

Table 1
Ulcerative colitis disease activity index.

Score	Diarrhea	Bleeding	Body weight score (% body weight loss)
0	Normal stool pellets	No rectal bleeding or blood in stool	<2%
1	Softer stool/stick to cage wall	Weak hemoccult-positive in stool	≥2%–<5%
2	Moderate diarrhea/unformed stool	Visual blood in stool/strongly hemoccult-positive spots in stool	≥5%–<10%
3	Diarrhea (watery stool)	Fresh rectal bleeding	≥10%–<15%
4			≥15%

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