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Indirubin ameliorates dextran sulfate sodium-induced ulcerative colitis in mice through the inhibition of inflammation and the induction of Foxp3-expressing regulatory T cells

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ABSTRACT

Indirubin, an active ingredient of a traditional Chinese medicine prescription named Danggui Longhui Wan, has been reported to exhibit abroad anti-cancer and anti-inflammation activities. However, the effect of indirubin on ulcerative colitis (UC) has not been addressed. Here, we investigated the therapeutic efficacy of indirubin on dextran sulfate sodium (DSS)-induced UC in mice and explored its underlying mechanisms. UC model was induced in BALB/c mice by administrating with 3% DSS in drinking water for 7 days. Subsequently, indirubin treatment (10 mg/kg) for 7 days obviously inhibited the loss of body weight, reversed the elevation of disease activity index (DAI), alleviated crypt distortion and mucosal injury, and reduced inflammatory cell infiltration in the colon mucosa, thereby ameliorating DSS-induced UC. Mechanically, the levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-2 as well as myeloperoxidase (MPO) activity in colon tissues were decreased significantly, while the levels of IL-4 and IL-10 were increased remarkably by indirubin treatment. Moreover, indirubin administration effectively suppressed $CD4^+$ T cell infiltration in the colon of DSS-induced UC mice and promoted the generation of Foxp3-expressing regulatory T cells. Additionally, further studies showed that indirubin obviously inhibited DSS-induced activation of nuclear factor (NF)-κB signaling. These results reveal that the significant anti-UC effect of indirubin may be attributable to its inhibition of inflammatory responses and promotion of $Foxp3^+$ T cells. Our studies provide the first evidence for the anti-UC effect of indirubin as well as the related molecular mechanisms and suggest a promising candidate drug for UC therapy.

1. Introduction

Ulcerative colitis (UC) is a chronic autoimmune inflammatory bowel disease characterized by weight loss, abdominal pain, bloody diarrhea, mucosal barrier dysfunction with inflammatory cell infiltration, and immune dysregulation (Ordas et al., 2012; Xavier and Podolsky, 2007). Over the past decade, the prevalence of UC has persistently increased in Asia, which is associated with the improvement of living levels and the deterioration of environment (Cosnes et al., 2011). If the UC is not treated in a timely manner, there will be a high risk for colorectal cancer (Cosnes et al., 2011). Although many therapeutic drugs such as 5-aminosalicylic acid (5-ASA) agents, corticosteroids, biologics, and immunosuppressive agents are commonly used for UC in clinical, these therapies show not only limited benefits but also severe side effects or high cost (Blonski et al., 2014). Therefore, it is necessary to discover novel therapeutic drugs with high efficacy and low toxicity for the treatment of UC.

Although the exact pathogenesis of UC remains unclear, uncontrolled immune responses and overwhelmed inflammation have been recognized. It is well accepted that overproduction of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-2, and interferon-γ as well as activation of the nuclear factor (NF)-κB signaling pathway play important roles in the development of UC (Atreya et al., 2008; Garside, 1999). Furthermore, the coordinated effect of various $CD4^+$ T lymphocyte subtypes has been considered to be fundamental to maintenance of intestine

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homeostasis (Shi et al., 2012). Kanai et al. pointed out that immune imbalance of $CD4^+$ T cells is likely to be an initial or an additional factor for the progression of UC (Kanai and Watanabe, 2005). Taken together, therapeutic approaches targeting excessive inflammation or/and abnormal immune responses will be intriguing for the treatment of UC.

Indirubin, firstly identified as the active compound of a well-known traditional Chinese medicine prescription named Danggui Longhui Wan, is widely used in the treatment of chronic myelogenous leukemia (Eisenbrand et al., 2004). Chemically, as a purple 3,2′-bisindole isomer of indigo, indirubin can also be extracted from *Indigoferal tinctoria* and *Polygonum tinctorium* (Xiao et al., 2002). Recently, several studies have shown that indirubin and its derivatives exhibit strong anti-tumor, anti-bacterial and anti-viral activities via targeting different protein kinases (Broecker-Preuss et al., 2015; Heredia et al., 2005; Ponnusamy et al., 2010). Additionally, another report by Zhang et al. suggested that indirubin has potential implication for the treatment of autoimmune diseases which is associated with the enhanced percentage of functional $CD4^+CD25^+Foxp3^+$ T cells (Zhang et al., 2007). Moreover, indirubin has also been found to inhibit inflammatory responses in several animal models through suppressing the release of inflammatory cytokines or the activation of NF-κB pathway (Kim and Park, 2012; Kunikata et al., 2000). All of these backgrounds attracted us to hypothesize that indirubin might have favorable therapeutic action in the treatment of UC. Therefore, to text this hypothesis, the present study was designed to evaluate the effects of indirubin on the dextran sulfate sodium (DSS)-induced UC in mice and explore its underlying mechanisms.

2. Materials and methods

2.1. Development of DSS-induced UC model and treatments

All animal care and experimental procedures were approved by the Animal Care Ethics and Use Committee of Shenyang Military Area Command and conducted in accordance with the guidelines of this Committee. Healthy male BALB/c mice (6–8 weeks old) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). After 1-week-adaption of the experimental conditions, the mice were randomly divided into five groups (n = 12 per group): the control group, indirubin (10 mg/kg/d) group, DSS group (the model group), DSS + indirubin (1 mg/kg/d) group, and DSS + indirubin (10 mg/kg/d) group. The model of UC was induced in mice by 7 days of 3% DSS in drinking water, in the meantime, each animal in the control group and the indirubin group received normal drinking water. From the 8th day, the mice in the indirubin experimental groups were orally administrated with 1 mg/kg or 10 mg/kg per day indirubin for 7 days. Meanwhile, each mouse in the control group and the UC model group were given equal volume of distilled water for 7 days. On day 8 after indirubin administration, the mice were sacrificed by cervical dislocation under anesthesia and colons were removed. Subsequently, the colon tissue was cut into several segments, some of them were fixed in 4% paraformaldehyde, and others were flash-frozen in liquid nitrogen and kept at −80 °C. For each technique (histological analysis, ELISA assay, immunohistochemical analysis, real-time PCR and western blot analysis), six animals were used per group and the animals were randomly selected.

2.2. Colitis evaluation

During the experimental period, body weight, stool condition, and hematochezia were observed and recorded. The severity of disease was assessed by the disease activity index [DAI = (weight loss score + stool consistency score + hematochezia score)/3], which was performed as previously described (Murano et al., 2000).

2.3. Histopathological examination

The paraformaldehyde-fixed colon tissues were dehydrated, embedded in paraffin, and cut into serial sections at 5 μm. After dewaxing and rehydration, the tissue sections were stained with hematoxylin and eosin (H&E) and observed under an optical microscope (DP73, OLUMPUS, Japan) for histological evaluation. Histological grading was performed by two experienced pathologists in a blinded manner as previously described (Xiong et al., 2013)

2.4. Enzyme-linked immunosorbent assay (ELISA) for inflammatory cytokines

The levels of TNF-α, IFN-γ, IL-2, IL-4, and IL-10 in colon tissues of mice were determined using respective commercial Mouse ELISA Kits (BOSTER, Wuhan, China). Briefly, the colon tissues from each group were homogenized with phosphate buffer saline (PBS) to extract total protein by centrifuging at 12,000g at 4 °C for 10 min. Then, the amount of protein in the supernatant was quantified using BCA protein assay kit (Wanleibio, Shenyang, China). The level of TNF-α, IFN-γ, IL-2, IL-4, and IL-10 in each sample was detected according to the manufacturers' protocols. The optical density at 450 nm was determined by using a plate reader (ELX-800, BIOTEK, USA).

2.5. Myeloperoxidase (MPO) activity assay

MPO activity is considered as an indicator of neutrophil infiltration. The MPO activity in colon tissues was determined using MPO Assay Kit (Jiancheng Biological Project Company, Nanjing, China). In brief, after weighting, colon tissue samples were homogenized on ice, and then the MPO activity in colon was assessed according to the instructions from manufacturer. The absorbance of each sample at 460 nm was measured by using spectrophotometer (UV752, Yoke, Shanghai, China), and the MPO activity was expressed in U/g wet weight.

2.6. Immunohistochemistry staining

The paraffin-embedded colon sections were deparaffinized, rehydrated, and microwaved for antigen retrieval. After washed with PBS, the tissue sections were incubated with 3% H_2O_2 for quenching the endogenous peroxidase activity, and then blocked with goat serum (Solarbio, Beijing, China). Subsequently, the sections were incubated with anti-CD4 primary antibodies (1:200 diluted, Rabbit Polyclonal CD-4, 11056-2-AP, Proteintech, Chicago, IL, USA; 1:50 diluted, Rabbit Polyclonal CD-4, sc-7219, Santa Cruz, Dallas, Texas, USA) at 4 °C overnight in a humidified chamber. These two antibodies were appropriate for immunohistochemistry and previously validated to be used with mice in our group. After washing with PBS, each slide was incubated with biotinylated goat anti-rabbit secondary antibody (1:200 diluted, A0277, Beyotime, Jiangsu, China) for 30 min at 37 °C and followed by activin-biotin-HRP (A0303, Beyotime). The sections were finally visualized using diaminobenzidine (DAB), counterstained with haematoxylin, and observed under an optical microscope. The negative controls were carried out by primary antibody omission and other steps were processed as described above.

The analysis of CD4 positive cells was carried out with Image-Pro Plus (IPP) software v 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA). For the immunohistochemical assessment, colon tissues from six animals per experimental group and three sections per colon