Salvianolic acid B decreases interleukin-1β-induced colitis recurrence in mice

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Abstract

Background: Degree of mucosal recovery is an important indicator for evaluating the therapeutic effects of drugs in treatment of inflammatory bowel disease (IBD). Increasing evidences has proved that tight junction (TJ) barrier dysfunction is one of the pathological mechanisms of IBD. The aim of this study was to observe whether enhancement of TJ can decrease colitis recurrence.

Methods: Eighty C57BL/6 mice were randomly divided into four groups including normal group, colitis group, sulfasalazine (SASP) treated group, and traditional Chinese drug salvianolic acid B (Sal B) treated group. Colitis was established in mice by free drinking water containing dextran sulfate sodium, after treatments by SASP and Sal B, recombinant human interleukin-1β (IL-1β) was injected intraperitoneally to induce colitis recurrence.

Results: Compared with sham control, cell apoptosis in colitis group was increased from 100.85 ± 3.46% to 162.89 ± 11.45% (P = 0.0038), and TJ dysfunction marker myosin light chain kinase (MLCK) was also significantly increased from 99.70 ± 9.29% to 296.23 ± 30.78% (P = 0.0025). The increased cell apoptosis was reversed by both SASP (125.99 ± 9.45% vs. 162.89 ± 11.45%, P = 0.0044), and Sal B (143.27 ± 6.09% vs. 162.89 ± 11.45%, P = 0.0059) and Sal B (104.27 ± 6.09% vs. 162.89 ± 11.45%, P = 0.0044). High MLCK expression in colitis group was reversely influenced by SASP (285.23 ± 41.04% vs. 296.23 ± 30.78%, P = 0.0028) but not influenced by SASP (285.23 ± 4.04% vs. 296.23 ± 30.78%, P > 0.05). The recurrence rate induced by recombinant human IL-1β in Sal B-treated group was significantly lower than that in SASP-treated group.

Conclusions: These results suggested a link between intestinal mucosal barrier dysfunction, especially TJ barrier dysfunction, and colitis recurrence. The TJ barrier dysfunction in remission stage of colitis increased the colitis recurrence. This study might provide potential treatment strategies for IBD recurrence.

Keywords: Colitis; Inflammatory bowel disease; Recurrence; Tight junctions

Introduction

Inflammatory bowel disease (IBD) is a chronic and recurrent gastrointestinal inflammatory disease which includes two main types of ulcerative colitis (UC) and Crohn disease (CD).[1] The incidence rate of IBD is between 0.1 to 11 and 0.5 to 24.5 per 100,000 people for CD and UC, respectively, in various areas around the world.[2]

The pathological mechanisms of IBD and the underlying mechanisms of IBD relapse remain under studied. Fecal calprotectin has been selected as one of the biomarkers to predict the recurrence of IBD.[3] The release of fecal calprotectin is induced by the interaction of activated monocytes with epithelial cells.[4] Compared with healthy people, the first-degree relatives of IBD (especially CD) have a significant increase in intestinal permeability, which may increase the susceptibility to the diseases.[5] Intestinal epithelial cells form a biochemical and physical barrier to prevent the invasion of luminal pathogens. The importance of bacterial flora in intestinal barrier has also become increasingly popular, and the decline in tolerance to microbiota is considered to be the main cause of barrier loss in IBD cases.[6] Besides, immune cell-related barrier loss, especially induced by exosomes, also influences the function of intestinal barrier.[7] The intestinal epithelial barrier is manipulated by epithelial cell secretory and transporting defenses (mucus, antimicrobial proteins, and immunoglobulin A), the apoptosis/proliferation of epithelial cells, and cell junctions like adherens junction and tight junction (TJ).[8]

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Intestinal epithelial TJ barriers are areas where the membranes of two adjacent cells join together to prevent molecules from getting through. It is formed by at least four transmembrane proteins including occludin, tricellulin, claudin, and junctional adhesion molecules. Epithelial cell loss (including apoptosis and necrocytosis) and TJ barrier dysfunction occur in IBD, but whether they contribute to initiation of inflammation or are the results of inflammation remains unknown. Increased expression level of myosin light chain kinase (MLCK) has been confirmed to destroy epithelial TJ function. Chronic alcohol abuse might activate tumor-necrosis factor α (TNF-α) receptors to phosphorylate MLCK resulting in intestinal barrier dysfunction.

IBD can be characterized by active disease (recurrence) with quiescent periods (remission). Recurrence of IBD cannot be ignored and it has been reported that 10-year recurrence rate in UC ranges from 70% to almost 100% in regional studies. Patients in their active, young, and middle age, with intestinal and extra-intestinal manifestations/complications in IBD like intestinal obstruction, fistula, colonic carcinoma, arthropathies, mucocutaneous, ophthalmological manifestations, as well as conditions affecting the hepatobiliary system may suffer from pain mentally and bodily. These problems may bring a heavy financial burden to their families and society. Commonly used therapeutic drugs such as non-steroidal anti-inflammatory drugs, although inhibiting excessive humoral and cellular immunity, do not reduce the recurrence of IBD.

Taken together, we proposed a hypothesis that IBD recurrence might be closely associated with intestinal barrier dysfunction. As one of the major water-soluble components of Radix Salvia miltiorrhiza (also known as traditional Chinese herb Danshen), salvianolic acid B (Sal B) has been testified its role in biological activities including anti-inflammatory, anti-oxidant, and anti-carcinogenic effects, and its protective effect on epithelial barrier function has already been reported to alleviate MLCK-related TJ barrier dysfunction in IBD. In this study, Sal B was used to induce enhancement of TJ. The selection of Sal B helped us to test the effects of TJ barrier dysfunction in colitis and colitis recurrence. We also chose sulfasalazine (SASP) as a classic treatment for IBD to compare with Sal B in IBD recurrence.

Methods

Ethical approval

This study was carried out in accordance with the recommendations of the National Institute of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985) and Dalian Medical University Animal Care and Ethics Committee.

Animals

C57BL/6J mice (weighing 18–22 g) were purchased from the Experimental Animal Center, Dalian Medical University (Certificate of Conformity: No. SYXX [Liao] 2013-0006). The animals were acclimatized to laboratory conditions (23°C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 weeks prior to experimentation and no animals died before the experiments. Based on previous publications and pre-experiments, the 100 mg/kg Sal B fulfills our requirements, the more dosages of Sal B were not selected in order to obey “3R” (reduction, replacement, and refinement) principles in animal use process.

Reagents

Sal B (purity speciation: ≥98%) was obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SASP was purchased from Tianjin Kingyork Group Co. Ltd. (Tianjin, China). MLCK antibodies and occludin antibodies were obtained from Abcam (Hong Kong) Ltd. (Hong Kong, China). Other agents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental design

Eighty mice were randomly divided into four groups, each consisting of 20 mice. Group I served as sham operation group. From day 1 to day 7, groups II, III, and IV received free drinking water containing 4% dextran sulfate sodium (DSS) to induce colitis. Group II served as the colitis control group. From day two, groups III and IV received treatment with SASP (80 mg/kg body weight, intragastric, dissolved in saline) and Sal B (100 mg/kg body weight, intragastric, dissolved in saline) respectively. SASP and Sal B were administered by gavage once a day for six consecutive days. Groups II, III, and IV were then treated with mice recombinant human interleukin-1β (IL-1β; 200 µg/kg) respectively for 48 h from day 7 to induce the recurrence of colitis according to the method reported previously with modifications. IL-1β produced by inflammatory cells has various biological activities. Infiltrating neutrophils recruited by adhesion molecules are up-regulated by IL-1β, which is closely related to the recurrence of colitis. Colitis symptoms were observed to test whether colitis recurrence was successfully induced on day 10. Group I (sham group) received the administration of equal volume of phosphate-buffered saline (PBS) continuously. The mice were allowed to eat and drink ad libitum from 1 h after the operation [Figure 1]. Distal colon samples were harvested for biochemical studies, which were performed to eliminate the severity of mucosal injury and inflammation.

Assessment of colitis symptoms

Inflammatory factors of colitis model including animal body weight, diarrhea incidence, and total food intake were recorded every day. After being sacrificed, the colon tissues of the animals were embedded in paraffin and fixed in 10% formalin for at least 24 h. The samples were then cut into 5 µm pieces and fixed on slides according to the routine procedure. Colon sections were hematoxylin and eosin (H&E)-stained (or used for other immunohistochemistry studies). Then, the samples were analyzed by light microscopy (Nikon Eclipse TE2000-U, Nikon Corp, Tokyo, Japan). Colon macroscopically visible damage was measured using a 0 to 10 scale by two observers.
Western blotting analysis

Colonic segments were isolated from mice and were then immediately stored in liquid nitrogen. Total protein was isolated from full thickness of intestinal wall using total protein extraction kit (Key GEN Bio TECH, Dalian, China). The blots on nitrocellulose filter membrane were probed with corresponding antibodies according to the manufacturer’s instructions, respectively, at 4°C with gentle shaking, overnight. Antibodies to Bcl-2 (ab59348), Bax (ab53154), MLCK (ab76092), and occludin (ab216327) were purchased from Abcam. The bands were detected and quantified using multispectral imaging system (UVP, Cambridge, UK). The western blotting images were quantified by Image Lab (version 4.0.1; Bio-Rad, CA, USA).

Apoptosis analysis by flow cytometry

Colonic segments were isolated from mice and were then extracted 1 × 10⁶ single cell suspension of intestinal epithelium, respectively. The cells were then fixed 70% ethanol at 4°C. Counted and balanced the number of cells in each group, and then stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) according to the manufacturer’s protocol (Annexin V-FITC apoptosis detection kit; BD Pharmingen, USA). Apoptotic cells (Annexin V-positive and PI-negative) were then quantified by flow cytometry using the BD analysis program (BD Pharmingen, NJ, USA).

Measurement of transmission electron microscopy

The integrity of intestinal epithelium was observed using transmission electron microscopy (TEM). Colonic segments were cut into 1 mm × 1 mm square pieces and then fixed in 3% glutaraldehyde-buffered fixative for 2 h at 4°C. After repeated rinsing with 1/15 mol/L PBS and 0.19 mol/L sucrose buffer, the tissue was then fixed in 1% osmium tetroxide for 1 h. Rinsed again with sucrose buffer for 15 min and gradually dehydrated with different concentrations of ethanol and acetone, the tissue was immersed in the solution consisting of 100% acetone and embedding agent (1:1) for 30 min, and then immersed in the pure embedding agent through the night. Areas of interest were selected from ultra-thin sections, which was stained with uranyl acetate for 10 min in the dark and then stained with lead citrate for 10 min. The sections were then observed under TEM.

Statistical analysis

The animal experiments, in vitro experiments, and data analysis were conducted according to a single-blind study design. Data were compared among three or more groups using one-way analysis of variance, and between two groups using Student t tests. Data were expressed as mean ± standard deviation. Data were normally distributed and each group showed similar variances. All experiments were repeated at least three times or in multiple animals and a P value <0.05 was considered statistically significant. SPSS software version 25.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Defecation and colitis symptoms

The defecation and colitis symptoms were evaluated. After the induction of colitis by free drinking water containing 4% DSS, mice in colitis group were found to have shapeless stools, increased stool frequency, outflow of red or dark red liquid from the anus, positive fecal occult blood test as well as loss of appetite. All these symptoms peaked on day 3 and were significantly relieved on day 7. The result indicated that the colitis models were successfully established. In colitis period, the DAI was significantly increased in colitis group, both SASP (10.10 ± 0.63) to (3.80 ± 0.78), P = 0.0013; and by Sal B from (8.20 ± 0.78) to (5.50 ± 0.72), P = 0.0036 [Table 1]. In the recurrence period induced by IL-1β, the increased DAI was reversed by Sal B from (8.60 ± 1.50) to (5.0 ± 1.25), P = 0.0017; and the increased macroscopically visible damage was also

Figure 1: Experimental flow chart of the current study. SASP: Sulfasalazine; ig: intragastric; Sal B: Salvianolic acid B; ip: intraperitoneally injection; IL-1β: Interleukin-1 beta.
reversed by Sal B from (7.10 ± 0.88) to (4.30 ± 1.34), P = 0.0035 [Table 1]. The increased DAI and macroscopically visible damage were not significantly influenced by SASP. These results suggested that Sal B may protect against IL-1β induced colitis recurrence.

**TJ barrier dysfunction in inactive stage of colitis**

Both TJ barrier dysfunction and epithelial barrier loss (induced by cell apoptosis) were evaluated in this study. Expressions of MLCK and TJ protein occludin were measured to test the integrity of TJ barrier function, and apoptosis-related proteins including Bax and Bcl-2 were measured to test the apoptosis dependent barrier loss. On day 3 and day 7, expression of Bax in colitis group was increased and Bcl-2 was decreased significantly compared with normal control group [Figure 2A]. The expression of MLCK was increased and occludin was decreased continuously compared with normal control group from day 3 to day 7 [Figure 2B]. All these results indicated that intestinal barrier dysfunction was getting worse with the development of colitis.

**Table 1: Colitis scale symptoms in different groups.**

| Groups          | Colitis period (day 7) | Recurrence period (day 10) | t  | P         |
|-----------------|------------------------|----------------------------|----|-----------|
| Disease activity index |                       |                            |    |           |
| Sham (group I)  | 0.80 ± 0.42            | 1.50 ± 0.52                | –  | –         |
| Colitis (group II) | 10.10 ± 1.20           | 8.60 ± 1.50                | 23,130 | <0.0010 |
| SASP (group III) | 4.70 ± 1.63            | 7.40 ± 1.35                | –8,438 | 0.0037   |
| Sal B (group IV) | 3.90 ± 1.01            | 5.0 ± 1.25                 | –12,500 | 0.0014   |
| Colonic macroscopically visible damage |           |                            |    |           |
| Sham (group I)  | 0                      | 0                          | –  | –         |
| Colitis (group II) | 8.20 ± 0.63           | 7.10 ± 0.88                | 46,165 | <0.0010 |
| SASP (group III) | 3.80 ± 0.78            | 6.70 ± 1.06                | –13,877 | 0.0013   |
| Sal B (group IV) | 5.50 ± 0.72            | 4.30 ± 1.34                | –8,926  | 0.0036   |

Data are expressed as mean ± standard deviation, n = 10 mice. *P < 0.01 compared with sham. †P < 0.01 compared with colitis. SASP: Sulfasalazine; Sal B: Salvianolic acid B; --: No data.

**Figure 2:** Expression of Bax, Bcl-2, MLCK, and occludin on day 3 and day 7 of colitis. (A) Expression of Bax and Bcl-2 on day 3 and day 7. (B) Expression of MLCK and occludin on day 3 and day 7. Data are expressed as mean ± standard deviation. *P < 0.01 compared with sham group (n = 3). Data in NC group are set to a relative value of 100%, and other data are calculated as a relative value of NC. †P < 0.01 compared with that in day 3 (n = 3). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NC: Normal control; MLCK: Myosin light chain kinase.
**TJ barrier dysfunction was reversed by Sal B but not SASP**

The effects of SASP and Sal B on intestinal epithelial barrier dysfunction were evaluated. Compared with normal control group, H&E staining results showed that the integrity of intestinal epithelium was destroyed including mucosal atrophy, partial microvilli loss, edema of the lamina propria and marked infiltration of inflammatory cells in colitis group. The colitis manifestations were reversed by application of Sal B and SASP [Figure 3].

The results of flow cytometry showed that apoptosis in colitis group peaked on day 3 and lasted till day 7. Compared with colitis group on day 7, apoptosis was significantly reduced in SASP-treated group (125.99 ± 8.45% vs. 162.89 ± 11.45%, P = 0.0059) and Sal B-treated group (104.27 ± 6.09% vs. 162.89 ± 11.45%, P = 0.0044) [Figure 4]. The expression of Bax was significantly increased and the expression of Bcl-2 was significantly decreased in colitis group. All these changes were reversed by Sal B and SASP [Figure 5A]. However, Sal B significantly decreased high MLCK expression in colitis (182.44 ± 89.42% vs. 296.23 ± 30.78%, P = 0.0028), while SASP did not affect MLCK expression (285.23 ± 41.04% vs. 296.23 ± 30.78%, P > 0.05). While the decreased expression of occludin was also reversed by Sal B (96.24 ± 15.71% vs. 39.33 ± 16.64%, P = 0.0030) but not SASP (40.55 ± 20.30% vs. 39.33 ± 16.64%, P > 0.05) [Figure 5B]. These results showed that TJ barrier dysfunction could be reversed by Sal B but not SASP.

**Sal B protected from IL-1β-induced colitis recurrence through maintaining TJ barrier**

After SASP and Sal B treatment, the colitis recurrence induced by IL-1β was studied. This was assessed by the number of mice with colitis symptoms (recurrence rate) and the severity of symptoms, respectively.

The alleviated symptoms and lower DAI in both SASP-treated and Sal B-treated groups were observed compared with colitis group [Table 1]. After IL-1β injection to induce colitis recurrences, compared with colitis group and SASP-treated group, mice in Sal B-treated group showed decreased recurrence rates, less severe colitis symptoms, as well as a reduced DAI [Table 1]. Macroskopically visible damage (from 562.29 ± 50.75% to 121.30 ± 10.66%, P < 0.001), colon weight to length (from 207.32 ± 27.09% to 107.58 ± 10.03%, P = 0.0014), MPO activity (from 803.60 ± 50.71% to 130.50 ± 10.06%, P < 0.001), and TNF-α (from 360.54 ± 42.01% to 120.49 ± 18.34%, P = 0.0011) were also significantly lower in Sal B-treated group [Figure 6]. As shown in TEM results [Figure 7], epithelial TJ was clear and integral, and the local circuitous and desmosome could be seen. The widen cell gap and disappeared local circuitous were observed in colitis group, which was not reversed by SASP in Sal B-treated group. All these results showed that TJ barrier function enhanced by Sal B leads to the prevention of colitis recurrence induced by IL-1β.
Discussion
In this study, enhancement of TJ in colitis by Sal B was studied by flow cytometry, western blot and TEM analysis in DSS-induced colitis models. Results showed that enhancement of TJ barrier by Sal B significantly decreased the colitis recurrence.

Intestinal epithelial barrier regulates interactions between luminal contents and mucosal immune cells, and the most common types of epithelial barrier dysfunction are believed as TJ barrier dysfunction and epithelial barrier loss induced by cell apoptosis. MLCK induces contraction of the peri-junctional actomyosin ring through MLCK-mediated MLC phosphorylation and subsequent cytoskeletal movement. The change of MLCK expression level always accompanies with internalization of transmembrane TJ proteins like occludin, claudin-2, and ZO-1, which could increase permeability of intestinal epithelial barrier, while MLCK inhibitor can rapidly reverse this barrier dysfunction and stabilize ZO-1 in TJ.

In our study, TJ barrier function was significantly enhanced by Sal B through Sal B induced down-regulation of MLCK and up-regulation of occludin. In the remission stage of colitis, TJ barrier dysfunction still existed. Sal B but not SASP could alleviate MLCK-related TJ barrier dysfunction.
Figure 5: Effects of SASP and Sal B on apoptosis and tight junction proteins. (A) Effects of SASP and Sal B on expression of Bax and Bcl-2. (B) Effects of SASP and Sal B on expression of MLCK and occludin. Data are expressed as mean ± standard deviation. Data in sham group are set to a relative value of 100%, other data is calculated as a relative value of sham. *P < 0.01 compared with sham group (n = 3); †P < 0.01 compared with DSS-colitis group, (n = 3). DSS: Dextran sulfate sodium; NC: Normal control; SASP: Sulfasalazine; Sal B: Salvianolic acid B; MLCK: Myosin light chain kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Figure 6: Analysis of the recurrence of colitis induced by recombinant human IL-1β (200 μg/kg) after SASP and Sal B treatment. (A) Macroscopically visible; (B) colon weight-to-length ratio; (C) MPO; and (D) proinflammatory cytokine TNF-α. Data are expressed as the mean ± standard deviation. Data in sham group are set to a relative value of 100%, and other data are calculated as a relative value of sham. *P < 0.01 compared with sham group (n = 10 mice). NC: Normal control; SASP: Sulfasalazine; Sal B: Salvianolic acid B; DSS: Dextran sulfate sodium; IL-1β: Interleukin-1 beta; MPO: Myeloperoxidase; TNF-α: Tumor necrosis factor-α.
dysfunction, which finally decreased colitis recurrence.\textsuperscript{16} Both excessive epithelial cell apoptosis and TJ barrier dysfunction could be observed in IBD.\textsuperscript{8} These results suggested that TJ barrier dysfunction might not be sufficient to lead to the occurrence of IBD. However, we believe that severe intestinal barrier dysfunction, in which both TJ barrier dysfunction and apoptosis are included, may directly trigger initiation of IBD.\textsuperscript{29}

IL-1\textbeta\textsuperscript{1} was used to induce colitis recurrence in mice models. In the treatment of recurrence colitis models, we observed that recurrent rate and disease severity of mice were significantly decreased in Sal B-treated group compared with SASP-treated group. Although apoptosis-related epithelial barrier dysfunction could be reversed by SASP and Sal B, intestinal TJ barrier dysfunction could be restored by Sal B but not SASP. The results showed that enhanced TJ barrier function can protect against colitis recurrence. In other words, TJ barrier dysfunction may be an indicator for colitis recurrence.

We provided an effective treatment strategy in the recurrence of IBD through Sal B induced enhancement of TJ, which indicates an excellent way in the both detection and prevention of IBD recurrence. Although TJ barrier dysfunction and epithelial barrier loss may occur at the same time, they are different types of epithelial barrier dysfunction. Thus, TJ barrier dysfunction is closely associated with the recurrence of IBD, which may serve as an ideal indicator for IBD recovery. However, intestinal epithelial barrier functions are formed by many factors including at least mucus, gut micro-organisms, single-layered epithelial cell formed physical barrier, as well as immune functions. Further studies are necessary to determine the relationship between these kinds of barrier functions and TJ barrier, which may also very interesting for IBD prevention.

There are now increasing evidence in recent years that intestinal epithelial barrier dysfunction is involved in IBD. TJ barrier dysfunction may not trigger the occurrence of...
colitis directly. However, it is closely related to recurrence of colitis. Stabilizing TJ as well as anti-MLCK treatments may hold significant value to prevent the recurrence of IBD.

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Conflicts of interest

None.

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