Effect of Astragalus polysaccharides on expression of TNF-α, IL-1β and NFATc4 in a rat model of experimental colitis

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\textbf{A R T I C L E  I N F O}

\textbf{Article history:}
Received 25 October 2013
Received in revised form 20 July 2014
Accepted 24 July 2014
Available online 15 August 2014

\textbf{Keywords:}
Astragalus polysaccharides
TNF-α
IL-1β
NFATc4
Experimental colitis

\textbf{A B S T R A C T}

\textbf{Aim:} Astragalus membranaceus is a Chinese medicinal herb and has been shown to improve hapten-induced experimental colitis. One of its major components is polysaccharides. We investigated the effect of Astragalus polysaccharides (APS) on expression of TNF-α, IL-1β and NFATc4 in a rat model of experimental colitis.

\textbf{Methods:} The experimental colitis model was induced by TNBS. Forty five rats were divided into five groups (n = 9): Normal control group, receiving ethanol vehicle with no TNBS during induction and IP saline injection during treatment; TNBS colitis model group (TNBS + IP saline), receiving only IP saline vehicle treatment; APS low dose group (TNBS + L-APS), receiving APS 200 mg/kg; APS high dose group (TNBS + H-APS), receiving APS 200 mg/kg; and positive control group (TNBS + Dexm), receiving dexamethasone 0.3 mg/kg. The clinical features, macroscopic and microscopic scores were assessed. The expressions of TNF-α, IL-1β and NFATc4 were measured by realtime PCR and ELISA assays.

\textbf{Results:} Compared to normal control rats, TNBS + IP saline had significant weight loss, increased macroscopic and microscopic scores, higher disease activity index (DAI) up-regulation of TNF-α, IL-1β and NFATc4 mRNA expression and up-regulation of TNF-α and IL-1β protein expression. Compared to TNBS + IP saline, treatment with APS or dexamethasone significantly reduced DAI, partially but significantly prevented TNBS colitis-induced weight loss and improved both macroscopic and microscopic scores; high dose APS or dexamethasone significantly down-regulated TNF-α and IL-1β expressions (both mRNA and protein) and up-regulated NFATc4 mRNA and protein expression. The effect of high dose APS and dexamethasone is comparable.

\textbf{Conclusions:} APS significantly improved experimental TNBS-induced colitis in rats through regulation of TNF-α, IL-1β and NFATc4 expression.

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1. Introduction

Inflammatory bowel disease (IBD), including Crohn’s disease and ulcerative colitis, is a chronic idiopathic inflammatory disorder that affects the gastrointestinal tract of children and adults. The precise etiology of IBD remains unclear. The current hypothesis states that complex interactions among various factors, including genetic factors, the host immune system and environmental factors, cause disruption of intestinal homeostasis, leading to dysregulated inflammatory responses of the gut [1–3]. Basic and translational research [4,5] has led to a better understanding of the role of inflammatory mediators including cytokines in the gut of patients with IBD. Cytokines carry signals between immune, epithelial and mesenchymal cells and play a pivotal role in the development of IBD. The inflammatory cytokines are the most logical targets for IBD treatment. Among various cytokines, tumor necrosis factor-α (TNF-α) is the cytokine that has been widely studied. Currently, several TNF-α blockers (infliximab, adalimumab and certolizumab) have been approved for IBD therapy in clinical practice [6]. The choice of treatment depends on severity, localization and the course of the disease [7]. Although these available agents have shown clinical benefits to some degree, they are not entirely effective and have multiple adverse effects.
Furthermore, IBD management requires long-term treatment that often leads to drug refractoriness or intolerance [8]. Therefore, it is necessary to develop novel therapeutic approaches.

Various natural products have been shown to safely suppress the pro-inflammatory pathway and control IBD. In vivo and/or in vitro studies suggest [9–11] that the anti-IBD effects exhibited by natural products are mainly caused by their ability to modulate cytokine production such as TNF-α, IL-1β, IL-6 and IL-17. Traditional Chinese medicine (TCM) has received great interest in recent years. Astragalus membranaceus is a Chinese herb and has been used in China for more than 2,000 years to strengthen human immunity. Ko et al. [12] demonstrated that root extract of Astragalus membranaceus administered orally and locally can protect rats against hapten-induced colitis through attenuation of TNF-α and IL-1β and up-regulation of IL-10. However, it is still unknown what active component is mainly responsible for the effect of Astragalus membranaceus on experimental colitis.

Astragalus membranaceus contains different active components, including polysaccharides, flavonoids, astragalosides I–VII (saponins), amino acids and trace elements [12–14]. Our previous studies have demonstrated that Astragalus polysaccharides (APS) has a potent effect on erythroid lineage differentiation and increases expression of NFATc4 mRNA by gene expression profile analysis in K562 cells [15]. NFATc4 is a key modulator of intestinal cell proliferation and differentiation. It is one of the crucial transcription factors that tightly control pro-inflammatory cytokine expression for adaptive immunity in T and B lymphocytes [16]. We hypothesize that APS is the main active component of Astragalus membranaceus that would improve experimental colitis. We therefore investigated the effect of APS on expression of TNF-α, IL-1β and NFATc4 in a rat model of experimental colitis.

2. Methods

2.1. Animals

45 pathogen-free male Wistar rats, weighting 180–220 g, were obtained from the animal facility of Nanfang Medical University (Guangzhou, China). All animals were allowed free access to water and standard chow diet. They were adapted to laboratory conditions for 7 days, with 12 h day/night cycles at 23 °C and 60% humidity. All animal protocols were reviewed and approved by Guangzhou Women and Children’s Medical Center Institutional Review Committee on Laboratory Animal Care and all animals were handled in accordance with institutional guidelines.

2.2. Animal model of colitis

Colitis was induced in the rats by rectal administration of Trinitrobenzene sulfonic acid (TNBS) (Sigma) into the colons in a dose of 150 mg/kg, dissolved in 50% solution of ethanol as described by others [17]. Briefly, the animals were anesthesitized with 10% Chlo- ral hydrate (300 mg/kg), and 3 ml/kg of TNBS – ethanol solution (50 mg/ml) was administered into the colon at the depth of 8 cm from the rectum with the use of a soft polyethylene catheter. The rats were positioned in the Trendelenburg position for one minute in order to avoid loss of TNBS solution via the rectum. Normal control animals received rectal administration of 50% ethanol solution at 3 ml/kg without TNBS during induction.

2.3. Experimental protocol and sample preparation

72 h after induction of colitis, animals were treated daily by intraperitoneal injection (IP) for 7 days. Animals in the normal control group (Normal control) received ethanol vehicle with no TNBS during induction and received IP saline injection during treatment. TNBS-induced colitis animals were randomly divided into 4 treatment groups: TNBS colitis control group, receiving only saline vehicle treatment (TNBS + IP saline); APS low dose treatment group, receiving APS 100 mg/kg (TNBS + L-APS); APS high dose treatment group, receiving APS 200 mg/kg (TNBS + H-APS); and dexamethasone treatment group, receiving dexamethasone 0.3 mg/kg (TNBS + Dexam).

Animals were then allowed to recover and observed daily throughout the duration of the study. Clinical symptoms, including the amount of food consumed, consistency and frequency of stools and the change of body weight were monitored until tissue harvest.

24 h after the last treatment, all animals were sacrificed after being deeply anesthetized with ether. The distal 8 cm of colon from rectum was removed, dissected along the longitudinal mesentery, rinsed with isotonic saline and assessed for extent of colonic mucosa injuries. Multiple tissue specimens about 0.3 cm × 0.5 cm were prepared for microscopic examination, real-time PCR and ELISA assays.

2.4. Disease activity index

Disease activity index (DAI) was based on weight loss, stool consistency and blood in stools. Briefly, score was assigned for each item to calculate DAI as follows: (i) Percentage of weight loss: 0, none; 1, 1–5%; 2, 6–10%; 3, 11–15%; 4, >15%. (ii) Stool consistency: 0, normal; 2, loose stool; 4, diarrhea. (iii) Blood in stools: 0, hemocult (–); 1, hemocult (+); 2, hemocult (+); 3, hemocult (++); 4, gross bleeding. All animals were monitored daily for the duration of the study to assess DAI changes in response to treatment.

2.5. Macroscopic and microscopic assessment

The removed colon was excised free of adherent adipose tissue, rinsed with ice-cold saline and dissected longitudinally. It was examined visually immediately and damage was scored on a scale of 0–5 by the pathologists who were blinded to the group, as previously described by others [18]. Briefly, scoring of macroscopic colon damage was as follows: 0, no colonic damage; 1, hyperaemia and no ulcer; 2, linear ulcer and no colonic wall thickening; 3, linear ulcer and colonic wall thickening in one area; 4, colonic ulcer at multiple areas; and 5, major ulcer and perforation.

The samples of colonic tissue were routinely fixed and stained for microscopic examination. Four samples for each group were selected randomly and their paraffin blocks were prepared. Four paraffin blocks were investigated. The colonic pathological changes were observed and evaluated by two trained independent researchers using a modified histopathological score formula [19]: (i) infiltration of acute inflammatory cells: 0 none, 1 mild, 2 severe; (ii) infiltration of chronic inflammatory cells: 0 none, 1 mild, 2 severe; (iii) fibrin deposition: 0 negative, 1 positive; (iv) submucosal oedema: 0 none, 1 focal, 2 diffuse; (v) necrosis of epithelial cells: 0 none, 1 focal, 2 diffuse; and (vi) mucosal ulcer: 0 negative, 1 positive.

2.6. Real-time PCR analysis

Total RNA was isolated from colonic tissue using the TRizol reagent. Reverse transcription of the isolated RNA was performed in a solution containing 4 μg of total RNA, 1 μg of Oligo (dT), 10 μL of 5× buffer, 2.5 μL of 10 mM dNTPs, and 400 U of MMLV reverse transcriptase. The final reaction volume was 50 μL. The reverse transcription was performed at 42 °C for 1 h. Real-time PCR was conducted according to the manual of SYBR Exscript™ RTPCR kit using a Stratagene MX3000P QPCR system. The primers
were designed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA). All primer sequences, thermal cycling conditions, and product sizes are shown in Table 1. Gene expression levels were recorded as threshold cycle (Ct) values. Each sample was amplified twice. A melting curve analysis was performed on each sample to ensure single amplification. The amount of target genes was standardized via comparison with the amount of β-actin mRNA.

The relative fold increase of gene expression was calculated by setting 2^{-ΔΔCt} value of normal control as 1.

2.7. ELISA assays

To quantify colonic tissue cytokines, 50 mg of colonic tissue was extracted using 500 μl of 5 M guanidine HCl and 50 mM Tris–HCl (pH 8.0) with a protease inhibitor. The extracts were centrifuged at 15,000 g for 30 min at 4 °C to remove insoluble materials. The supernatant fractions were analyzed by ELISA kit (ADL, San Diego, USA) according to instructions after total protein determination. To quantify TNF-α, IL-1β and NFATc4 protein in colon tissue, the 1:20 dilutions of the supernatants of colonic tissue homogenates were added into 96-well microplates. These microplates were coated with antibodies specific for rat. After the enzyme-substrate reaction, sample absorbances were measured at 450 nm with a microplate reader. Standard curves were prepared like the samples using antibodies specific for rat. After the enzyme-substrate reaction, sample absorbances were measured at 450 nm with a microplate reader. Standard curves were prepared like the samples using antibodies specific for rat.

2.8. Statistical analysis

All parameters were expressed as mean ± SE. Statistical analysis was performed using one-way ANOVA followed by the Duncan's multiple range test (SPSS version 10.0). A P-value of less than 0.05 was considered significantly different.

3. Results

3.1. Clinical features

After intracolonic instillation of TNBS, stool frequency was increased and stool characteristics were loose or watery with some mucus and/or blood. None of the rats died during the experimental period.

As shown in Table 2, compared to normal control animals, TNBS + IP saline rats had significant weight loss at the end of experiment from baseline (p < 0.001). Treatments with low dose APS, high dose APS or dexamethasone partially but significantly prevented TNBS colitis-induced weight loss (F = 226.3, p < 0.001). There was no significant differences in body weight changes in animals between TNBS + H-APS and TNBS + Dexm groups (p = 0.115).

Disease activity index was significantly higher in TNBS+IP saline animals than normal controls (p < 0.001). Treatments with low dose APS, high dose APS or dexamethasone significantly reduced disease activity index (F = 66.634, p < 0.001). There was no significant differences in disease activity index in animals between TNBS + H-APS groups and TNBS + Dexm groups (p = 0.489).

3.2. Macroscopic and microscopic assessment

As shown in Table 2, macroscopic and microscopic scores were significantly higher in TNBS + IP saline animals than normal controls (p < 0.001). Compared to TNBS + IP saline animals, treatment with low dose APS, high dose APS or dexamethasone significantly deceased both macroscopic and microscopic scores (F = 52.029, P = 0.000; F = 78.289, P = 0.000, respectively). The improvement of macroscopic and microscopic scores with H-APS were comparable to those with dexamethasone (P = 0.861 and P = 0.184, respectively).

In TNBS + IP saline animals, histological exam showed edema, mucosal necrosis, and loss of the epithelium. There were diffuse polymorphonuclear leukocyte infiltrations in lamina propria and muscularis mucosa (Fig. 1B). There were mild edema and moderate inflammatory cell infiltration in TNBS+L-APS rats (Fig. 1C). In high dose APS and dexamethasone groups, colonic structures were normal and there was marked reduction of inflammatory infiltrates, mild edema limited mainly to the lamina propria and minimal ulceration (Fig. 1D and E).

3.3. TNF-α, IL-1β and NFATc4 mRNA expression by real-time PCR

Real-time PCR detections were performed to evaluate the level of TNF-α, IL-1β and NFATc4 mRNA expression. Compared to
normal controls, TNF-α, IL-1β, and NFATc4 mRNA expressions were all significantly up-regulated in TNBS colitis rats (Fig. 2A–C). Treatments with high dose APS or dexamethasone significantly down-regulated TNF-α and IL-1β mRNA expressions but up-regulated NFATc4 mRNA expression in comparison to TNBS colitis group. APS at low dose significantly down-regulated IL-1β mRNA, up-regulated NFATc4 mRNA and only slightly down-regulated TNF-α mRNA (not reaching statistical significance). The effects of APS on TNF-α, IL-1β and NFATc4 mRNA expression appear dose-dependent.

3.4. TNF-α, IL-1β and NFATc4 protein expression by ELISA

ELISA assays were performed to evaluate the level of TNF-α, IL-1β and NFATc4 protein expression. Compared to normal controls, TNF-α and IL-1β protein expressions were significantly up-regulated in TNBS colitis rats (Fig. 2D–F). Treatments with high dose APS or dexamethasone significantly down-regulated TNF-α and IL-1β protein expressions in comparison to the TNBS colitis group. APS at low dose significantly down-regulated IL-1β protein but did not have a significant effect on TNF-α protein expression. NFATc4 protein expression did not differ between normal control and the TNBS colitis group. However, compared to TNBS colitis or normal control groups, high dose APS or dexamethasone, but not low dose APS, significantly up-regulated NFATc4 protein expression (2F).

4. Discussion

The search for active compounds in natural products used in traditional medicine has attracted great interest [9,20–22]. In Traditional Chinese Medicine, *Astragalus membranaceus* is a major component in a prescription to treat chronic phlegmatic disorders and general gastrointestinal disturbances including stomach ulcer, chronic diarrhea and intestinal inflammation. Recent studies suggested that *Astragalus membranaceus* have pharmacological effects on the activation of B cells and macrophages, regulation of both humoral and cellular immune responses, cardiovascular protection and prevention of inflammation and cancer [23–25]. *Astragalus membranaceus* has pharmacological effects on regulating epithelial cell proliferation [26], formation of intestinal epithelial cell connections and cell differentiation, alleviating intestinal pathological lesions. Astragalus polysaccharides (APS) is the main active ingredient in *Astragalus membranaceus*. The present study demonstrated that APS significantly improved TNBS-induced colitis with improvement of diarrhea and both macroscopic and microscopic scores, in a dose-dependent manner. The protective effect of high dose APS is comparable to that of dexamethasone.

Cytokines are key signaling molecules of the intestinal immune system, which play an important role in IBD. In vivo and/or in vitro studies indicate that the levels of proinflammatory cytokines, including interleukin IL-1β, IL-18, IL-6, TNF-α, and TL1A (tumor necrosis factor-like ligand) are increased in active IBD and correlate with the severity of inflammation [27,28]. TNF-α is one of the most important pro-inflammatory cytokines that directly influence intestinal epithelial tissue and IL-1β is also a key mediator of IBD progression [29]. Excessive TNF-α expression results in damage to the epithelial barrier, initiation of apoptosis in epithelial cells, and initiation of chemokine secretion by colonic epithelial cells. A persistent increase in TNF-α in colonic mucosa may contribute to the epithelial barrier defects associated with intestinal inflammation seen in IBD [30]. In our animal model of TNBS-induced experimental colitis, TNF-α and IL-1β expressions were significantly up-regulated, further confirming the critical role of these pro-inflammatory cytokines in the pathogenesis of intestinal inflammation.

Various natural products have been shown to suppress pro-inflammatory pathways and control IBD. Studies indicate that APS could suppress the production of TNF-α, IL-1β and IL-8 [31,32]. This study provided evidence that APS might protect rats against TNBS-induced colitis through modulation of TNF-α and IL-1β. The expression of TNF-α and IL-1β mRNA and protein were down-regulated after treatment with high dose (200 mg/kg) APS in TNBS-treated rats, which is consistent with results reported by others [32].

The intestinal epithelial barrier is the frontline between genetic, environmental, and immunological factors [33,34], and is maintained by normal intestinal flora, epithelial-cell proliferation, cell-matrix adhesion molecules, intact endoplasmic reticulum stress response, and prompt epithelial restitution after injury [35,36]. The inflammatory response often results in continued epithelial injury, which causes erosions, ulcerations, and a decrease in the production of α-defensin [37,38]. Studies have shown that nuclear
factor of activated T cell (NFATc1–c4) proteins are a family of transcription factors which involve in the regulation of cell differentiation and development in a number of cell types [15,39]. NFATs are crucial transcription factors that tightly control proinflammatory cytokine expression, including TNF-α and IL-1β, for adaptive immunity in T and B lymphocytes and NFATc3 and c4 is required for Toll-like receptor-initiated innate inflammatory immune response in bone marrow derived macrophages [16]. However, little is known about the role of NFATs in IBD.

In this study, we attempted to determine whether NFATc4 is involved in TNBS-induced colitis. NFATc4 mRNA and protein were detected in normal colonic mucosa, but only NFATc4 mRNA was up-regulated after induction of colitis, indicating NFATc4 may be involved in pathogenesis of colitis through regulation of downstream gene expression at the transcription level. Surprisingly, after APS treatment, while TNF-α and IL-1β expression was down-regulated, NFATc4 mRNA and protein expression was further up-regulated, not down-regulated towards that in normal controls as one would expect. This may suggest a protective mechanism of NFATc4 with APS treatment of TNBS-induced colitis. Wang et al [40] reported that NFATc4 is a key modulator of intestinal cell proliferation and differentiation. We speculate that up-regulated expression of NFATc4 by APS may represent a damage repairing process, leading to improvement of colitis. Further studies are needed to clarify the exact role of NFATs in colitis and how it interacts with TNF-α and IL-1β.

In conclusion, this study provides evidence that APS significantly improves TNBS-induced experimental colitis, through regulation of TNF-α, IL-1β and NFATc4 expression. It provides important new insight that may contribute to further development of APS as a novel therapeutic agent for inflammatory bowel disease.

Acknowledgement
The present study was supported by grants from Administration of Traditional Chinese Medicine of Guangdong Province Foundation (No. 2009030).

References