Tuning inflammatory responses in ulcerative colitis mice using tripterygium glycosides

Jihong Zhong, Yingchao Liu, Yan Shen

Department of Gastroenterology, The Second Affiliated Hospital of Zhejiang Chinese Medical University, No.318 Chaowang Road, Gongshu District, Hangzhou City 310005, Zhejiang Province, China

Received March 21, 2019; Accepted June 10, 2019; Epub August 15, 2019; Published August 30, 2019

Abstract: Ulcerative colitis (UC) is a relapsing inflammatory bowel disease with an incomplete pathogenesis. Existing studies have used immune suppressive medicine as an effective method of treatment for the disease. However, detailed mechanisms of the immune suppression are not well known. The current study investigated the roles of tripterygium glycosides, an immune suppressive compound, on modulating mice ulcerative colitis. BALB/c mice induced with UC and treated with different doses of tripterygium glycosides showed lowered pathological scores. Real-time PCR results showed decreased expression levels of NLRP3, ASC, and caspase-1 mRNAs in the colon tissues of mice treated with a high dose of tripterygium glycosides. In the colorectal mucosa, it was found that high dose tripterygium glycoside treatment led to downregulated ROS levels in the UC mice, as well as lowered NLRP3, ASC, caspase-1 mRNAs, and NADPH oxidase activity. Processing the mucosa tissue into homogenates for cell culturing, this study discovered lowered levels of inflammatory cytokines, such as IL-1α and TNF-α, but not IL-13, in samples from mice with high dose tripterygium glycoside treatment. Results of the current study indicate that tripterygium glycosides regulate UC pathological scores in a dose-associated manner. Specifically, tripterygium glycosides tune levels of caspase-1, NLRP3, and ACS mRNAs in the neutrophils from the mice. Additionally, tripterygium glycosides reduce secretion of IL-1α, TNF-α, and other proinflammatory cytokines in DSS-induced mice ulcerative colitis. Results also suggest that neutrophils are involved in immune suppression by decreasing the production of the abovementioned inflammatory cytokines.

Keywords: Tripterygium glycosides, dextran sulfate sodium, ulcerative colitis, reactive oxygen species, NADPH oxidases, NLRP3 inflammasome

Introduction

Human inflammatory bowel diseases (IBD), such as ulcerative colitis (UC), are characterized by chronic, relapsing, and remitting inflammatory responses associated with dysfunction in the mucosal immune system. UC patients face the possibility of developing colorectal cancer. Previous studies have indicated that parameters, such as prolonged UC duration for over 7 years, in-depth colonic involvement, or a family history of colorectal cancer, could contribute to occurrence of colorectal cancer [1, 2]. To lower the potential risks of developing CRC, clinical strategies, such as colonoscopic surveillance programs, have been adopted [3]. However, to significantly reduce the risks of CRC, fundamental studies are required, aiming to understand the pathological mechanisms of UC. Thus, the current study examined the mechanisms of an immune suppressive compound (tripterygium glycosides, TG) for treatment of UC.

UC is featured by increased levels of inflammatory cytokines, such as IL-1β, IL-8, and tumor necrosis factor-α. Disease severity is associated with the production of inflammatory cytokines [4-6]. In particular, IL-1β and tumor necrosis factor-α have been utilized as markers indicating inflammation in UC. These two cytokines can change tight junctions and intestinal permeability. Specifically, IL-1β plays a central role in the early stages of inflammation in UC [5, 7]. Upon the cascade of the disease, an increased amount of IL-1β will be discovered in the colonic mucosa and peritoneal immune cells (macrophages). Additionally, using caspase-1 knocked-
Tripterygium glycosides modulate inflammation

out mice, researchers have found that the increased secretion of IL-1β and IL-8 is driven by caspase-1 related pathways [6, 8]. Another study reported that the existence of TGF-β in colonic mucosa was associated with UC progression, promoting the expansion of this disease to an even more challenging state, such as tissue fibrosis or cancer [9].

TG is a chemical component extracted from Tripterygium wilfordii. TG has been approved by the Chinese Food and Drug Administration (Z32021007) for treatment of multiple diseases, including Crohn's disease, rheumatoid arthritis (RA), and UC [10-12]. Current studies have reported that TG could regulate inflammatory responses in different types of diseases [13, 14]. Multiple fundamental studies have demonstrated anti-inflammatory functions of TG. For example, in the rat synovial RSC-364 cell line treated with IL-1β, TG induced the secretion of IL-32 and matrix metalloproteases (MMP-1 and MMP-9), in a dose-dependent manner [12]. This study, thus, indicated that TG could be employed to suppress inflammatory immune responses in diseases such as RA [12]. Similarly, in ulcerative colitis, TG decreases the secretion of dextran sulfate sodium-induced IL-6 [15]. In type II collagen-induced arthritis rats, TG downregulated the secretion of IL-6, IL-8, and TNF-α significantly [10]. However, several studies have investigated the anti-inflammatory functions of TG, but few studies have looked into the detailed anti-inflammatory mechanisms of TG in different diseases. The current study examined the impact of TG on NOXs related inflammatory pathways, revealing the influence of TG on NLRP3, ASC, and caspase-1 mRNAs, as well as NADPH oxidase activity, in mice induced with UC.

Materials and methods

Materials and animals

A total of 50 female BALB/c mice (4-8 weeks old) were supplied by the Animal Center at Zhejiang Chinese Medical University. Each mouse was approximately 20 g in weight. DSS was obtained from Sigma-Aldrich (Fluka: 5000 MW, catalog no., 31404). It was diluted in water before use (5%, 5 g DSS diluted in 100 mL water). Tripterygium glycosides were purchased from Zhejiang DND Pharmaceutical Co., LTD. (Product no., Z33020422) and were diluted in water at three different dose levels, 9 mg, 27 mg, and 81 mg per kg of mice body weight per day, respectively. IL-1α, TNF-α, and IL-13 ELISA kits (MLA00, MTA00B, M1300CB) were purchased from R&D Systems Incorporation (USA). All animal studies followed regulations of the institute, as well as local and federal laws concerning animal protection.

Animals and samples

The mice were randomly divided into 5 groups, with 10 mice in each group. Mice in each group received the following treatment, respectively: They received a different dose of TG daily (i.e., 9 mg/kg, 27 mg/kg, 81 mg/kg). Mice with no disease induction or TG treatment were used as the control groups (i.e., CTRL group). Mice induced with the disease were used as the disease model group (i.e., Model group). For the model group (i.e., mice induced with the disease), the mice were fed with 5% DSS solution daily to induce the disease. In the control group, the mice were treated with 0.02 mL/g DI water, without being induced with the disease. In the treatment group, the mice were treated with different doses of TG, respectively. Daily establishment of the mice UC model was tested on day 8 using HE staining. Briefly, mice in the three different groups were treated with 9 mg/kg, 27 mg/kg, and 81 mg/kg TG for 21 days, respectively. After treatment, the mice were sacrificed, with colon samples collected for further testing. Standards for selection of colon tissues were as follows: 8 cm away from the anus with 0.5 cm forward and backward.

HE staining

Colon samples were fixed in 10% paraformaldehyde, then embedded in wax. HE samples were cut at around 3-4 mm in thickness. This was followed by HE staining and observation under a microscope.

Neutrophil isolation and testing

Neutrophils were isolated using a standard isolation kit (EasySep™ Mouse Neutrophil Enrichment Kit, STEM Technologies, USA), following manufacturer instructions. Briefly, the kits were used to isolate neutrophils from colon tissues by negative selection [16, 17]. During selection, the unwanted cells were targeted with biotinylated antibodies against non-neu-
Tripterygium glycosides modulate inflammation

Table 1. Real-Time PCR Primers and Conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank Accession</th>
<th>Primer Sequences (5’ to 3’)</th>
<th>Size (bp)</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse GAPDH</td>
<td>GU214026.1</td>
<td>GAGGTCGGTGTGAACGGATTG</td>
<td>127</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATGTAGACCATGTAGTTGAGGCTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Asc</td>
<td>NM_023258.4</td>
<td>GGCACAGAAGTGACGGAGTG</td>
<td>103</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATCTTGCTTGGGCTGTTGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse caspase 1</td>
<td>NM_009807.2</td>
<td>CCAGGCAAGCCAAATCTTATCAC</td>
<td>125</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTCTCCCTTATCGATCTTTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Nlrp3</td>
<td>NM_145827.4</td>
<td>GGGAGACCGTGGAGAAGG</td>
<td>129</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCCAAAAGGATCGGACAAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

trophils. Those labeled cells were then recognized with tetrameric antibodies, targeting against biotin and dextran. Targeted cells were then attached with magnetic particles and separated with a magnet. The desired cells were then collected in a new tube for testing. All studies followed manufacturer instructions.

ROS testing

Levels of ROS in the colon samples, as well as neutrophil samples, were tested using ROS test kits (Product no., E004, Nanjing Jiancheng Bioengineering Institute). Testing was carried out according to manufacturer instructions.

NOXs activity testing

NOXs activity was measured using a colorimetric assay kit, following manufacturer instructions (Abcam, Cambridge, MA). In the standard isolation kit, NADPH extraction buffer, NADP standard, enzyme cycling mix, and stop solution were included. Briefly, neutrophils from colonic mucosal were first treated with trypsin (Bibco Incorporation) at 4°C at for 5 minutes. The cells were then centrifuged at 2,500 × g for 5 minutes. For NAPDH measurement, cellular samples were heated to 60°C for 30 minutes to decompose NADP+. They cooled on ice for another 30 minutes. The reaction mix from the kit was then added to the samples for 5 minutes at room temperature, converting NADP+ to NADPH. An NADPH developer was then added to the samples for 3 hours. NOXs activity was then measured with a microplate reader.

PCR analysis of NLRP3, ASC, and caspase-1 mRNA levels in neutrophils

Total RNA was extracted from the neutrophils using an RNA isolation kit (Invitrogen™ TRIzol™). The extraction was performed according to manufacturer instructions. An ultraviolet spectrophotometer was employed to assess RNA concentrations. Each measurement was performed 5 times. Briefly, 500 ng RNA were reverse transcribed to cDNA using the reverse transcription kits. The cDNAs were then amplified using ABI 7500 PCR amplification equipment. The following procedures were adopted: Pre-denaturation (95°C for 30 seconds), circulation amplification, denaturation (95°C for 5 seconds), annealing at 60°C for 30 seconds, for a total of 40 cycles. The 2^−ΔΔCt method was employed to assess relative expression levels of target genes. Primers used for each target gene in PCR are listed in Table 1.

Assessment of caspase-1 expression via immunohistochemical staining

EnVision™ kits were used to assess expression levels of caspase-1 in the colon tissues. Briefly, the tissues were cut into sections around 4 μm and dried at 80°C for 15 minutes. The samples were then treated with 3% H2O2 for 10 minutes. Primary antibodies were then added to the samples for 60 minutes, followed by the addition of goat-anti-rabbit IgG for 40 minutes. DAB solution was used to treat the samples for 3 minutes under a microscope. This was followed by washing with DI water. Cells that had brown or yellow particles were considered to be caspase-1 positive cells.

Measurement of IL-1α, TNF-α, and IL-13 in the cell culture supernatant

Colon tissues were cut into pieces, approximately 2-3 mm³, and added with 1 mL PBS. The samples were then grounded using mechanical forces, obtaining isolated cells on ice. Grounded samples were then centrifuged at 12,000 × g.
Tripterygium glycosides modulate inflammation

for 15 minutes, with the supernatant collected and stored at -80°C. ELISA tests were performed according to manufacturer instructions.

Statistical analysis

SPSS17.0 was used to analyze data. Data are expressed as mean ± SD. ANOVA was used for one-factor analysis of variance. \( P < 0.05 \) indicates statistical significance.

Results

TG treatment influenced UA pathological scores in mice in a dose-dependent manner

After treatment of the mice with DSS for 8 days, the establishment of the UA model was assessed using histochemical staining (Figure 1A-E). Using histochemically stained images, this study assessed pathological scores, analyzing the impact of TG on the UA pathology degree. Results indicated that mice treated with DSS had higher pathological scores, compared to mice with no treatment, suggesting the establishment of the UA mice model (Figure 1F). TG treatment caused lowered pathological scores, compared to the model group (UA mice with no treatment, \( P < 0.05 \)). Doses of UA also had an obvious impact on UA treatment. All three doses of TG treatment (high, medium, and low dose of TG) produced a significant impact on lowering UA pathological scores, compared with the model group (\( P < 0.05 \)) (Figure 1F). Compared to normal mice (mice with no treatment), mice treated with high doses of UG had similar levels of pathological scores, compared to the mice with no disease (\( P < 0.05 \)), indicating that UG treatment can repair UA disease to almost normal levels. However, the current study showed that a medium or low dose of UG had significantly lower pathological scores, compared to mice with no disease (Figure 1F). Comparing the mice treated with different doses of TG, there were significant differences between high doses and medium doses, as well as significant differences between high doses and low doses, respectively (\( P < 0.05 \)). However, there were no significant differences between medium doses and low doses, indicating that high doses are more appropriate for effective treatment (Figure 1F).

TG treatment resulted in a reduction of ROS and NOXs levels in diseased mice

After establishing the model, the current study examined levels of ROS in the neutrophils from mice in different groups. Briefly, neutrophils from mice treated with different doses of TG had lower ROS levels than the model mice, sug-
Tripterygium glycosides modulate inflammation

Gestiging that treatment can reduce ROS production in the neutrophils. Western blot analysis showed a dose-dependent reduction in ROS and NOXs activity in the mice (Figure 2A, 2B). This study also quantified levels of ROS and NOXs protein in Western blot gels. Similar to
Tripterygium glycosides modulate inflammation

The results in Figure 2A, 2B, quantification illustrated statistical differences in expression of ROS and NOXs between the treated groups and model group (mice induced with disease) (Figure 2C, 2D). ROS levels in neutrophils from mice treated with different levels of TG were higher than those from normal mice (Figure 2E). Present data indicates that TG treatment could reduce levels of ROS in the diseased mice. Similarly, different doses of TG treatment led to lower levels of NOXs, compared to the model mice (Figure 2F). For both ROS and NOXs levels, a higher dose of TG treatments resulted in lower levels, indicating that high doses of TG are more effective in regulating ROS and NOXs levels in neutrophils.

Using colon tissues from mice in different groups, this study investigated levels of caspase-1, NLRP3, and ASC mRNAs in neutrophils (Figure 3A-C). There were no statistical differences in levels of caspase-1 mRNAs between the high dose group and normal group. Neutrophils from the medium and low dose groups led to lower levels of caspase-1, NLRP3, and ASC mRNAs, compared to the model group. Levels of these mRNAs were still higher than those in the normal group (Figure 3A-C). In addition to testing levels of caspase-1, NLRP3, and ASC mRNAs, this study also tested the activity of these three proteins with Western blotting. Results suggest that TG treatment could upregulate the levels of these mRNAs (Figure 3A-C). Using Western blotting, this study also analyzed levels of caspase-1, NLRP3, and ACS levels in cells with different treatments (Figure 3D). Western blot analysis is quantified in Figure 3D. In all three proteins, treatment cau-

Figure 3. Assessing expression of (A) Caspase-1, (B) NLRP3, and (C) ACS mRNAs via TG treatments. TG treatment downregulated levels of NLRP3, ASC, and caspase-1 mRNAs in the neutrophils from different groups. (D) Western blot analysis of caspase-1, NLRP3, and ACS activity in neutrophils from different groups. Quantification of the (E) caspase-1, (F) NLRP3, and (G) ACS activity in neutrophils from different groups in the Western blot gel. The following mice groups were included: Mice with induced with UC disease and mice treated with low, medium, and high doses of TG, respectively. Mice with no treatment were used as control. *p < 0.05, **p < 0.01.
Tripterygium glycosides modulate inflammation

Compared to the normal group (mice with no disease), different treatments (low, medium, and high groups) still had higher levels of expression in the three proteins, respectively (Figure 3E-G). Collecting the colon tissues from mice in different groups, histochemical staining was used to assess levels of caspase-1 expression (Figure 4A). Staining illustrated increased expression levels of caspase-1 in the diseased mice (Figure 4A). Using a different dose of TG, expression levels of caspase-1 were reduced, respectively (Figure 4B-D). Compared to the diseased mice, either with or without treatment, caspase-1 expression was relatively low in mice with no disease (normal mice) (Figure 4E). Statistically, there were significant differences between the model mice and normal mice (p < 0.01 between model mice and normal mice) (Figure 4F). Treatment also caused statistical differences between treated mice (low, medium, or high doses of TG) and model mice (p < 0.01 between model mice and mice

Figure 4. Assessing the impact of TG treatment on caspase-1 expression in colon tissues from different groups. Histochemical staining of colon tissues from mice with (A) Induced with UC disease and treated with (B) Low; (C) Medium; (D) High doses of TG, respectively; (E) Mice with no treatment were used as control; (F) Quantification of a caspase-1 scores of samples from different groups.

Figure 5. Secretion of (A) IL-1β, (B) TNF-α, and (C) IL-13 in cellular culture supernatant from different groups. To prepare the cell culture samples, colon tissues were cut into pieces at sizes of 2-3 mm³ and added with 1 mL PBS. The samples were then grounded using mechanical forces to obtain isolated cells on ice. The cells were then washed with PBS and collected for culturing. Secretion of IL-1β, TNF-α, and IL-13 was analyzed. Treatment caused significant differences in the secretion of IL-1β and TNF-α but not IL-13. *p < 0.05, **p < 0.01.
Tripterygium glycosides modulate inflammation

treated with low or medium doses of TG; p < 0.05 between model mice and mice treated with high doses of TG (Figure 4E).

Next, the current study compared levels of IL-1β, TNF-α, and IL-13 in the cell culture supernatant. Results indicated that TG treatment (low, medium, or high dose, respectively) led to lowered secretion of IL-1β, compared to the model mice (p < 0.05, Figure 5A). No significant differences were observed between the normal and high or medium dose groups, respectively (p > 0.05, Figure 5A). There were significant differences between the low dose group and normal group (p < 0.05, Figure 5A). Similarly, treatment also caused dramatic differences in levels of TNF-α between the model group and treated group (low, medium, and high dose group, respectively) (Figure 5B). Different from the other two cytokines, treatment did not lead to significant differences in levels of IL-13, indicating that treatment did not affect the secretion of this cytokine (Figure 5C).

Discussion

Current clinical studies are unclear concerning the pathological mechanisms of UC. UC is considered to be a mixed result from multiple factors, including genetics, immunity, environment, and bacteria in the body. Immune disorders are considered to be a major factor. Medicines for treatment of UC include 3 types, aminosalicylates, corticosteroids, and immune-suppressants. Even though a certain level of effectiveness has been achieved using these medicines, the side effects are a huge concern, especially when long-term usage of the medicines is involved. Thus, it is necessary to search for effective medicines that can be used for treatment of UC for long-term usage, investigating the fundamental mechanisms of these medicines.

TG was originally extracted from certain plants, showing strong anti-inflammation effects. Existing studies have reported that TG could regulate the secretion of multiple immune effects including, IL-1, IL-12, and TNF-α [18]. For example, in one study, it was reported that TG could inhibit expression of IL-32 and matrix metalloproteinases (MMP-1 and MMP-9) in rat synovial RSC-364 cell line treated with interleukin-1β [12]. In humans, it has been reported that TG could reduce the severity of rheumatoid arthritis [19]. Despite multiple studies on the immune suppressive functions of TG, the detailed mechanisms remain unclear.

Many studies have attempted to reveal the inflammatory mechanisms in colitis. In one study, it was reported that colitis induced in mice with DSS is regulated through NLRP3 inflammasome pathways [20]. Specifically, the authors reported that DSS could promote the secretion of IL-1β from macrophages, in a caspase-1 dependent manner. Additionally, on one hand, macrophages incubated with DSS in vitro secreted high levels of IL-1β, in a caspase-1-dependent manner. On the other hand, existing studies have revealed that secretion of IL-1β was abrogated in the macrophages that were absent from NLRP3, ACS, or caspase-1, indicating that activation of caspase-1 by DSS may be associated with the NLRP3 inflammasome [20]. Moreover, IL-1β secretion was dependent on phagocytosis, lysosomal maturation, cathepsin B and L, and reactive oxygen species (ROS), with ORS remaining as the main parameter [20]. In patients with colitis disease, neutrophils and macrophages can promote the secretion of ROS upon the impact of NOXs, with neutrophils remaining the major immune cells for inflammatory damage [21]. In inflammatory tissues in UC, neutrophils are major cells that produce ROS and inflammatory cytokines [22, 23]. Thus, they were selected for the current work.

Conclusion

In summary, upon oral administration of DSS, a UC mice model, resembling human UC, was established. Using this model, the current study examined the production of ROS, NOXs activity, and NLRP3 related signals, including NLRP3, caspase-1, and ACS. Using in vitro and in vivo models, results showed that neutrophils play vital roles in NLRP3 related inflammation in UC.

Acknowledgements

This work is supported by The National Natural Science Fundation (#81403335).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yan Shen, Department of Gastroenterology, The Second Affiliated
Tripterygium glycosides modulate inflammation

Hospital of Zhejiang Chinese Medical University, No.318 Chaowang Road, Gongshu District, Hangzhou City 310005, Zhejiang Province, China. Tel: (86) 0571-85288252; E-mail: shenyan161103@163.com

References


