Therapeutic Potential of Oxymatrine in DSS-Induced Inflammatory Bowel Disease Models

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Abstract

Oxymatrine (OMT), an alkaloid derived from the root of the Sophora flavescens, has been reported to possess a significant effect on relieving UC owing to its anti-inflammatory property. But the other therapeutic mechanism of OMT remains unclear. Recent studies have found, PI3K/AKT signaling pathway is involved in the pathogenesis of UC by pro-inflammatory effects and activating T cells. Moreover, PI3K/AKT pathway is one of the most important pathways for regulating cell apoptosis. Thus, we aim to explore whether OMT protects against UC by targeting PI3K/AKT pathway. We established the UC mice models, using LY294002 (a specific inhibitor of PI3K/AKT) as a positive control, to observe the effect of low, medium and high dose of OMT on UC and its influence on PI3K/AKT signaling pathway. Our data indicated that OMT can significantly ameliorate UC through anti-inflammatory, pro-apoptotic, down-regulating the differentiation of Th1 and Th17 cells via PI3K/AKT pathway. This study reveals that PI3K/AKT signaling pathway is a potential mechanism of OMT-induced UC remission and suggests that OMT is a promising therapeutic agent for the treatment of UC.

1. Introduction

Ulcerative colitis (UC), a kind of inflammatory bowel disease (IBD), is a chronic recurrent disease that results in inflammation and ulcers in the lining of the colon and rectum, and accompanied by a certain rate of colon cancer [1]. The typical symptoms of active disease are abdominal pain and diarrhea mixed with mucus and blood. The etiology of UC is still uncertain and may involve genetics, immune system dysfunction, changes of the intestinal microbiota and environmental factors [2], [3]. Current therapies including aminosalicylates, corticosteroids, immunosuppressants and infliximab have more or less some side effects, and they cannot maintain long-term ease of UC patients. Thus, there is an urgent need for new therapeutic drugs to conquer these defects. At present, the extracted components from Chinese medicinal herbs are the research focus and directions.

Sophora flavescens, a traditional Chinese herb, has a long clinical treatment history for various human diseases such as virus infection, cutaneous pruritus, inflammatory disease, arrhythmia and liver fibrosis. Clinically, we found that Sophora flavescens can significantly alleviate the acute phase of UC. Thus, OMT, as a main active ingredient extracted from Sophora flavescens, has become our research target automatically. OMT has been shown to possess anti-virus [4], anti-inflammation [5], pro-apoptosis [6], anti-fibrosis [7], immune regulation [8], anti-proliferation [9], anti-tumor [10], anti-oxidation [11], anti-allergy [12] and cardiovascular protective properties [13]. In recent years, studies have shown that OMT can alleviate UC and attention is attracted to its definite anti-inflammatory effects [5]. However, the further mechanism and other effects of OMT in the treatment of UC need to be clarified.

It is known that nuclear factor-kappa B (NF-κB) is a classical transcription factor that regulates the transcription of genes controlling cell apoptosis, proliferation, the cellular-stress response, the immune and inflammatory responses [14]. NF-κB has been involved in the pathogenesis of a variety of inflammation-related diseases, including UC [15], [16]. As an upstream activator of NF-κB, the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is implicated in multiple cellular processes, such as survival, proliferation, differentiation, apoptosis and autophagy [17], [18]. Active PI3K mediates phosphorylation of AKT at Thr308 and Ser473 respectively and activated AKT can inhibit apoptosis by directly phosphorylating apoptosis-related proteins or by regulating the activity of transcription factors [19]. Although the study of PI3K/AKT signaling pathway is mainly focused on the field of anti-tumor [20], [21], its role in UC and other inflammatory and autoimmune diseases has obtained more and more attention [22], [23]. It has recently been reported that PI3K/AKT signaling pathway plays a crucial role in relieving DSS-induced colitis via immune regulation and anti-inflammatory effects [24], [25], [26].

Considering the pivotal role of PI3K/AKT signaling pathway and OMT in UC, we demonstrate that whether OMT relieves dextran sulfate sodium (DSS)-induced colitis through anti-inflammatory, pro-apoptotic and immune regulation three aspects and its further mechanism is related to downregulation of PI3K/AKT signaling pathway.

2. Materials and methods

2.1. Animals

Specific pathogen-free (SPF) male BALB/c mice (weight 18–22 g) were purchased from the experimental animal center of Huazhong University of Science and Technology (HUST, Wuhan, China). Animal experimental procedures were performed strictly in accordance with the Animal Research Institute Committee guidelines of HUST. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of HUST.

2.2. Establishment of UC models and treatment protocol

UC was induced in mice by 3.0% DSS (36–50 kDa; MP Biomedicals) for 7 days [27]. From the first day of the model induction, the mice of treatment groups were administered through an intraperitoneal injection of 25, 50 or 100 mg/kg OMT (Aladdin, A111285) and 50 mg/kg LY294002 (Abmole Bioscience, Hong Kong, China) respectively daily for 7 days [28]. LY294002 was dissolved in DMSO and then diluted by PBS. To avoid toxic side effects of DMSO on animals, we ensured the concentration of DMSO was 5% at most. In the control and DSS groups, equal volume of PBS were intraperitoneal injected.

2.3. Evaluation of UC

Body weight loss, stool consistency and fecal blood loss were observed daily. The disease activity index (DAI) was evaluated as previously described [29]. On day 8, mice were sacrificed, and colons were

collected for histological scores. Part of the colons were fixed and stained with hematoxylin and eosin (H&E) for histopathological analysis [29].

2.4. Western blot

In order to quantify the expression of specific protein, western blot was performed as previously described [29]. Briefly, colon samples were homogenized in RIPA lysis buffer containing a protease inhibitor cocktail (Goodbio Technology, Wuhan, China). Then, the protein concentrations were detected by BCA protein assay kit (Goodbio Technology) according to the manufacturer's procedures. Protein extracts were separated on SDS-PAGE gels and further electro-transferred to PVDF membranes. After being blocked with 5% nonfat skim milk, the membranes were incubated overnight at 4 °C with appropriate primary antibodies against AKT (1:3000; Cell Signaling Technology), phospho-AKT (Thr308), phospho-AKT (Ser473) (1:1500; Cell Signaling Technology), Bcl-2, Bad, cleaved-caspase3, cleaved-caspase9 (1:1000; Cell Signaling Technology), NF-κBp65 (1:3000; Abcam), T-bet (1:1000; Abcam), ROR-γt (1:1000; eBioscience) and β-actin (1:1000; Goodbio Technology), respectively. Membranes were then washed with TBST and incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, the bands on the membranes were visualized and analyzed. The expression of β-actin was used as control.

2.5. Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded colonic tissue sections complying with a standard procedure [29]. Slices were then incubated with specific primary antibodies for anti-Bcl-2, anti-Bad, anti-caspase3, anti-caspase9 (1:400; Cell Signaling Technology) and anti-NF-κBp65 (1:400; Abcam) overnight at 4 °C, followed by a biotinylated secondary antibody. Sections were developed with diaminobenzidine (DAB) and counterstained with hematoxylin. Images of IHC slides were visualized and analyzed using an optical microscope.

2.6. Flow cytometry

For measuring intracellular amount of IFN-γ and IL-17A positive cells, monocytes were obtained from spleen and mesenteric lymph nodes (MLNs) [30]. Firstly, cells were incubated and stimulated with 200 ng/ml phorbol myristate acetate (PMA) (Abcam, Cambridge, UK), 1 μg/ml ionomycin (Abcam), 10 μg/ml brefeldin A (BD Biosciences, San Diego, USA) at 37 °C for 6 h. Then, cultured cells were stained surface marker with FITC-anti-CD4 antibody (BD Biosciences) for 15 min, followed by fixation and permeabilization, and then stained intracellularly with PE-anti-IFN-γ (BD Biosciences) and PE-anti-IL-17A (BD Biosciences) respectively for 30 min. Finally, acquisition and analysis were performed by flow cytometry.

2.7. Real-time quantitative PCR

Total RNA was extracted from colonic tissue using TRIZOL reagent (TaKaRa, Shiga, Japan). PrimeScript™ RT Master Mix (TaKaRa) was used to produce complementary DNAs (cDNAs) by reverse transcription. Subsequently, Real-time quantitative PCR analyses were performed using the SYBR Premix Ex Taq™ (TaKaRa). Expression of target gene was normalized to the level of β-actin and expressed relatively to the control group based on the 2−△△Ct method. All primer sequences were described in Table 1.

Table 1. Primer sequences for RT-PCR.

Gene	Primer sequences (5′-3′)	Length (bp)
β-Actin	Sense	
	Anti-sense	GTGACGTTGACATCCGTAAAGA
	GTAACAGTCCGCCTAGAAGCAC	287
TNF-α	Sense	
	Anti-sense	TGTCTCAGCCTCTTCTCATTCC
	CAGACACTCACCTCATCCCTTT	104
IL-6	Sense	
	Anti-sense	CCCCAATTTCCAATGCTCTCC
	CGCACTAGGTTTGCCGAGTA	141
IL-1β	Sense	
	Anti-sense	GGGCCTCAAAGGAAAGAATC
	TACCAGTTGGGGAACTCTGC	183
IFN-γ	Sense	
	Anti-sense	CTCAAGTGGCATAGATGTGGAAG
	TGACCTCAAACTTGGCAATACTC	251
IL-17A	Sense	
	Anti-sense	ATGAGTGCCGACAAACAACG
	TTCTGCCATCTCCTCAGCCT	179
T-bet	Sense	
	Anti-sense	TCAATACCCGCCCAAGATG
	TCGCCTAGTCCTGAGTCGC	162

ROR-γt Sense

Anti-sense TCAGGAGTGCTTACTGTCGGTC

AGTTCTTCGGGGCTGGAAT 136

2.8. ELISA analysis

Colon homogenate supernatants in each group were collected, and the concentrations of TNF-α, IL-6, IL-1β, IFN-γ and IL-17A in colonic tissues were determined by ELISA kits (NeoBioscience, Shenzhen, China) as per the manufacturer's protocols [29].

2.9. Statistical analysis

SPSS 20.0 software was used for statistical analysis. All results are presented as mean ± SD. The statistical significances were assessed using a one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. OMT relieved DSS-induced colitis

The molecular formula of OMT is C15H24N2O2 and its structure is presented in Fig. 1A. To explore the role of OMT in UC, we constructed a mouse model of DSS-induced colitis. After DSS induced, mice suffered a significant body weight loss, decreasing activity, severe diarrhea and bloody stools mixed with mucus. As shown in Fig. 1B, the mice in the DSS group had obvious weight loss while mice treated with OMT at the doses of 25, 50 and 100 mg/kg and LY294002 (50 mg/kg) were protected from the body weight loss. DSS caused severe colitis that was associated with a shortened colon, while such change was improved by OMT and LY294002 (Fig. 1C and D). Meanwhile, the DAI score and histological score, reflecting the severity of UC, also decreased obviously in the OMT and LY294002 treated groups (Fig. 1E and F). Histopathological analysis showed no damage in the control group, while mucosal damage, ulceration, gland destruction, loss of goblet cells, inflammatory cells infiltration were observed in the DSS group. The damage was significantly reduced by administration of OMT, which was similar to the treatment of LY294002 (Fig. 1G). Thus, OMT reflected its wonderful therapeutic efficacy against UC.

Fig. 1

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Fig. 1. OMT protects against DSS-induced colitis of mice. (A) Chemical structure of OMT. (B) Changes of body weight were recorded daily. (C) Representative photographs of colons were provided. (D) The colonic length was measured. (E) DAI of mice was evaluated. (F) Histological scores of colonic lesions were assessed. (G) Colon samples were stained with H&E for histological analysis (amplification, × 100). Values represent means ± SD (n = 8). ###$P < 0.001$, vs control group. ∗$P < 0.05$, ∗∗$P < 0.01$, ∗∗∗$P < 0.001$, vs DSS group.

3.2. OMT regulated the expression of apoptosis-related protein downstream of the PI3K/AKT pathway

Located downstream of the PI3K/AKT pathway, anti-apoptotic protein Bcl-2, pro-apoptotic proteins Bad, caspase3 and caspase9 are regulated by PI3K/AKT [17]. Studies have indicated that activated AKT not only phosphorylates Bad, but also blocks phosphorylation of caspase9 and caspase3, which leads to the inactivation and suppression of pro-apoptotic function. Moreover, PI3K/AKT exerts anti-apoptosis effect via elevating the anti-apoptotic protein Bcl-2. NF-κB, governing the expression of genes with anti-apoptotic activity, can also be activated by AKT though phosphorylating the IκB kinase (IKK). Western blot analysis in combination with IHC in our study showed the proteins of Bcl-2 and Bad were significantly elevated in the DSS group. On the contrary, the levels of cleaved-caspase3 and cleaved-caspase9 were down-regulated by DSS treatment. Whereas, OMT and LY294002 triggered apoptosis by activated cleaved-caspase3 and cleaved-caspase9 and lowed the expression of Bcl-2 and Bad in colonic tissues (Figs. 2A, B and 3).

Fig. 2

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Fig. 2. OMT regulates the expression of apoptotic proteins and inflammatory factors downstream of PI3K/AKT pathway. (A) The colonic protein levels of NF-κBp65, Bcl-2, Bad, cleaved-Caspase3 and cleaved-Caspase9 were detected by western blot. (B) Relative ratio levels were determined by densitometric analysis normalized to β-actin. Values represent means ± SD (n = 3). ###P < 0.001, vs control group. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001, vs DSS group.

Fig. 3

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Fig. 3. Effects of OMT on the levels of apoptotic proteins and inflammatory factors in colon. Expressions of NF-κBp65, Bcl-2, Bad, Caspase3 and Caspase9 in colon sections were assessed by IHC (magnification, × 200).

3.3. OMT suppressed the secretion of inflammatory factors in impaired colon

NF-κB, represents a central factor in the activation of a variety of inflammatory mediators [15]. As depicted in Fig. 2A, B and Fig. 3, DSS administration increased the protein level of NF-κBp65 in colonic tissues whereas it was decreased obviously after treatment with OMT and LY294002. To further investigate the effects of OMT on pro-inflammatory cytokines production, we detected the expression of TNF-α, IL-1β and IL-6 in inflamed colon tissues of each group by RT-PCR (Fig. 4A) and Elisa (Fig. 4B). Data showed that, higher level of IL-6 was found in the DSS group compared with the control group, and OMT at all doses as well as LY294002 could reduce its level markedly. The similar trends were shown in the protein level of TNF-α and IL-1β. However, the LY294002 group failed to show a significant

improvement in the mRNA level of TNF-α and IL-1β. Our results indicated that treatment of UC with OMT remarkably prevented the inflammation of colon.

Fig. 4

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Fig. 4. Effects of OMT on the changes of pro-inflammatory cytokines in colon. (A) The mRNA expression levels of TNF-a, IL-6 and IL-1 β in colon were quantified. (B) ELISA analysis of TNF-a, IL-6 and IL-1 β in colon. Values represent means $\pm$ SD ($n \geq 6$). ##P < 0.01, ###P < 0.001, vs control group. $*$P < 0.05, $**$P < 0.01, $***$P < 0.001, vs DSS group.

3.4. Th1 and Th17 cells differentiation and function were restrained by OMT

T helper (Th) lymphocytes, Th1 and Th17 cells are considered to be key roles in the pathogenesis of UC [31]. To test whether OMT could repress Th1 and Th17 cells differentiation, the frequency of CD4+ IFN-γ+ T cells (Th1 cells) and CD4+ IL-17A+ T cells (Th17 cells) isolated from MLNs and spleen of mice were measured by flow cytometry. Results showed that the proportions of Th1 and Th17 cells were up-regulated in the DSS group in comparison with the control group. However, OMT and LY294002 reduced the percentage of Th1-polarized and Th17-polarized CD4+ T cells (Fig. 5A and B). Since T-bet and ROR-γt are signature transcription factors for Th1 and Th17 differentiation, we further investigate the influence of OMT on the activation of T-bet and ROR-γt. Western blot and quantitative RT-PCR analysis revealed that OMT and LY294002 treatment resulted in dramatic reduction of T-bet and ROR-γt (Fig. 6A and B). Consistently, as symbolic cytokines secreted by Th1 and Th17 cells, the levels of IFN-γ and IL-17A mRNA and protein expression in colons were also observably down-regulated by OMT and LY294002 (Fig. 6A and C). These results supported the notion that OMT decreased the proportion of Th1 and Th17 cells in UC models.

Fig. 5

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Fig. 5. OMT suppresses Th1 and Th17 cells differentiation. (A) The differentiation of Th1 and Th17 cells in MLNs and spleen of mice was analyzed by flow cytometry. (B) The average percentage of Th1 and Th17 cells in MLNs and spleen. Values represent means $\pm$ SD (n = 3). ###P < 0.001, vs control group. $*$P < 0.05, $**$P < 0.01, $***$P < 0.001, vs DSS group.

Fig. 6

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Fig. 6. OMT affects transcription factors and function of Th1 and Th17 cells. (A) The mRNA expressions of T-bet, IFN- γ, ROR- γt and IL-17A were determined by quantitative RT-PCR. Values represent means $\pm$

SD (n ≥ 6). (B) Western blot analysis of ROR- γ t and T-bet expressions in colon. Values represent means ± SD (n = 3). (C) The levels of IFN-γ and IL-17A in colonic tissues were measured by ELISA. Values represent means ± SD (n ≥ 6). ##P < 0.01, ###P < 0.001, vs control group. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001, vs DSS group.

3.5. OMT improved UC mainly through PI3K/AKT inhibition

PI3K/AKT participates in the regulation of apoptosis, inflammation and immunity [32]. To further verify whether OMT relieves UC via the PI3K/AKT pathway, we use western blot to detect the effect of OMT on AKT phosphorylation, and LY294002 was considered as a positive control. Data indicated there was no significant difference in the amount of total AKT in each group. However, elevated levels of phosphor-AKT (Thr308) and phosphor-AKT (Ser473) in the model group suggested DSS was responsible for the activation of PI3K/AKT and OMT treatment could reverse this trend as expected (Fig. 7A and B). Hence, OMT and LY294002 could strikingly inhibit the phosphorylation and activation of AKT, which was consistent with the results of OMT on the regulation of inflammation, apoptosis and immune response. Taken together, our results clarified OMT played a potential therapeutic role in UC through suppressing PI3K/AKT activation.

Fig. 7

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Fig. 7. OMT ameliorates DSS-induced colitis relying on PI3K/AKT signaling pathway. (A) Protein levels of total AKT, phosphor-AKT (Thr308) and phosphor-AKT (Ser473) in colon were assessed by western blotting. (B) Representative protein levels of p-AKT (Thr308)/AKT, p-AKT (Ser473)/AKT. Values represent means ± SD (n = 3). ###P < 0.001, vs control group. ∗∗∗P < 0.001, vs DSS group.

4. Discussion

Inflammation, immune dysfunction, apoptosis are known to be involved in the pathogenesis of UC. Classical pro-inflammatory cytokines TNF-α, IL-1β and IL-6, regulated by NF-κB, are reported to play central roles in the induction and persistence of chronic inflammation in UC [33]. Th1 and Th17 cells, are thought to mediate IBD pathogenesis [34]. And apoptosis is also dysregulated during UC progression including pro-apoptotic process of intestinal epithelial cells and anti-apoptotic effect of inflammatory and immune cells [35], [36]. It is more convincing to explore OMT reliable curative effect on UC from the three aspects above.

As the most commonly activated pathway in cancer, PI3K/AKT is thought to be one of the most potential targets for human cancer therapy [37]. Activated PI3K/AKT pathway contributes to cancer cell proliferation, survival, angiogenesis and inhibition of apoptosis, which are important factors of tumorigenesis. Apart from regulation of tumors, Current evidence suggests PI3K/AKT can also modulate inflammation, apoptosis and immune cells proliferation, differentiation through regulating the activity

of downstream effector molecules, such as NF-κB, Bad, caspase9, m-TOR and so on [17], [23], [25]. Given that PI3K/AKT extensive regulatory function, its role in UC and other inflammatory and autoimmune diseases has been reported. Based on these results, we have a hypothesis that PI3K/AKT may participate in the pathogenesis of UC and OMT protects against UC partly through PI3K/AKT.

LY294002, an inhibitor of PI3K/AKT, mainly suppresses the ATP binding site of PI3K competitively [28]. It is reported LY294002 has pro-apoptotic and anti-proliferative activities through blocking the PI3K/AKT pathway. To evaluate whether OMT can affect PI3K/AKT pathway, we intentionally utilize LY294002 as a positive control to make the results comparable. In our experiments, LY294002 significantly inhibited the phosphorylation of AKT, induced apoptosis through the regulation of apoptosis-related proteins downstream of PI3K/AKT. In addition, LY2940002 could also modulate inflammation and T cells differentiation, similar to the effect of OMT in UC. Thus, LY294002 is not only promising in the treatment of tumors [28], but also meaningful in the inflammatory and immune disorders.

Our study indicates that OMT does have a significant alleviating effect on DSS-induced colitis. Firstly, symptoms were improved obviously, including weight loss, mucosanguineous feces, diarrhea. Next, histopathological analysis showed that OMT, especially the medium and high dose groups, markedly reduced inflammatory injury, tissue edema, gland damage. Furthermore, OMT also had significant regulatory effects on UC-related inflammation, apoptosis and immunity. The up-regulation of Bcl-2, Bad and the down-regulation of caspase3, caspase9 in colonic tissues induced by DSS suggest that the Bcl-2 family and the mitochondrial pathway of apoptosis are both involved in the pathogenesis of UC. Our results show anti-apoptotic effect exerted by inflammatory and immune cells may be superior in the pathology of UC compared to the pro-apoptotic effect of intestinal epithelial cells. And OMT can reverse this advantage by inactivating PI3K/AKT signal pathway.

Multiple indicators in our experiment showed the efficacy of 50 mg/kg OMT group was better than the 100 mg/kg OMT group, though there was no statistic difference between the two groups. On one hand there may be individual differences among the mice and free drinking of DSS water may lead to severity bias of symptoms in mice between the two groups, on the other OMT may have an optimal dose in the treatment of UC and the efficacy will be declined either above or below this dose. But this result doesn't affect the conclusion that OMT can really alleviate UC significantly. We also found compared with LY294002, OMT relieved the symptoms of UC more remarkable. This may be either correlated with the distinct multi-target effect of OMT or attributed to the certain side effects of LY294002 on animals.

Therefore, OMT shows potential therapeutic efficacy against DSS-induced colitis: alleviation symptoms, suppression inflammation, induction apoptosis as well as regulation T cells. All of these effects are closely related to its unique mechanism of suppressing PI3K/AKT signaling. In conclusion, our data demonstrates PI3K/AKT signaling pathway, involved in the pathogenesis of UC, may be an attractive therapeutic target, and OMT might be a promising candidate for the treatment of UC. The mechanisms

of OMT intervened in specific molecules downstream of PI3K/AKT and OMT on the regulation of UC related cell apoptosis in vitro are our further research directions.

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