Int J Clin Exp Pathol 2019;12(2):498-506
www.ijcep.com /ISSN:1936-2625/IJCEP0084139

Original Article

Role of mast cells and eosinophils in different stages of trinitrobenzenosulphonic acid-induced rat colitis

Ping Zhao[1], Haitao Guan[2], Lei Dong[1], Jinyan Luo[1], Jun Gong[1]

Departments of [1]Gastroenterology, [2]Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, P. R. China

Received August 16, 2018; Accepted November 21, 2018; Epub February 1, 2019; Published February 15, 2019

Abstract: The present study aimed to elucidate the effect of mast cells (MCs) and eosinophils (Eos) in trinitrobenzenosulphonic acid (TNBS)-induced colitis in SD rats. A rat model of ulcerative colitis (UC) was established by intracolonic injection of 100 mg/kg TNBS (in 0.3 ml 50% ethanol). At 6, 11, 16, 21 days after TNBS injection, the rats were sacrificed to determine the colon injury scores, the counts, distribution, and ultrastructure of mast cells (MCs) and eosinophils (Eos), the concentration of whole blood, and colon histamine. The results showed that after TNBS injection, for 6 days, colon injury score was significantly increased in the distal colon of the rats ($P < 0.01$ vs. control), accompanied by markedly increased whole blood histamine level and Eos count ($P < 0.01$), but decreased colon histamine concentration ($P < 0.01$). At the following 11, 16, 21 days' detection, MCs count and colon histamine level were gradually increased while Eos count and blood histamine were decreased during 21 days' detection period. Furthermore, the correlation analysis revealed that the Eos counts were positively correlated with the colon injury score and blood histamine content ($P < 0.05$, respectively). The MCs count was negatively associated with the blood histamine content ($P < 0.05$), but positively associated with the colon tissue histamine content ($P < 0.01$). In conclusion, though no correlation was found between MCs and Eos counts in the TNBS-induced colitis in this study, their relationship with whole blood and colon histamine appear to play different roles in both the acute and repair stages of colitis.

Keywords: Mast cells, eosinophils, histamine concentration, ulcerative colitis, trinitrobenzene sulfonic acid

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is referred as a chronic inflammatory disorder of the gastrointestinal tract [1]. External environment, interstitial microbial flora, genetic factors, and immune system are proven to be involved in the initiation of UC reactions, but the pathogenesis of this disorder still remains undefined [2, 3]. UC typically affects individuals at the ages of 15 to 35 with periods of quiescence and flares. Recently, researchers have observed that cumulative risk of colon cancer in patients diagnosed with initial UC was rising from 8% to 18% in the following years [4]. Increasing evidence has suggested the crucial role of the immune system in the initiation and progression of UC by modulating a series of inflammatory mediators such as nitric oxide, cytokines, and oxygen free radicals in response to allergen exposure [5, 6].

A distinct morphologic feature of UC is the presence of various inflammatory cells including mast cells (MCs) and eosinophils (Eos) in the gut mucosa [7]. Previous studies have showed that Eos play an essential role in numerous diseases including the generation of UC [8]. Eos could synthesize and secret a broad range of biologically active mediators such as antimicrobial or anti-inflammatory associated factors to maintain the homeostasis of the intestinal epithelial environment [9]. Eos as vital innate immune cells, are located at the mucosal surfaces of the gastrointestinal tract to participate in the host immunity against external pathogens and contribute to the tissue injury or repair processes, which may result in organ dysfunction [10]. Azad et al. have suggested that the

increased number of Eos in lamina propria is associated with the frequent relapse rate of UC in 26 Indian patients [11].

Furthermore, MCs as another type of inflammatory cell, also play an important role in the inflammatory process [12]. MCs is distinguished by their cellular cytokine and enzyme content and three types of MCs have been identified: the MC_{T-C} typecontains typtase, chymase, carboxypeptidase, and a cathepsin G-like proteinase; MC_T type shows only typtase, and MC_C type contains chymase and carboxypeptidase, with no tryptase, and they all release histamine [13]. Researchers have found that MC_T type is predominant in the lung and bowel mucosa and plays a crucial role in mediating immune responses in IBD progression [14]. Recent studies have suggested an increased number of MCs in the submucosa, lamina propria, and colorectal mucosa of patients with UC. Furthermore, the complexity and degranulation of mucosal MC is altered in IBD patients, and accompanied by increased levels of IL-6, TNF-α, histamine and tryptase, indicating the involvement of MC in the inflammatory UC progression [15]. Although studies have demonstrated both the potential beneficial and destructive roles of MCs/Eos in the UC patients, exact roles for them to participate in the initiation and repair processes of UC need discovery [16].

Intrarectal injection of 2, 4, 6-trinitrobenzenesufonic acid (TNBS) in ethanol is widely accepted to induce a colitis in rats; TNBS could transmit into the bowel wall and result in colon lesions including ulcerations, necrosis, and the bowel wall thickening for lasting several weeks, which ideally mimics many of the characteristics of macroscopy and histology in human UC [17]. Thus, TNBS-induced colitis in rats was used in this study to investigate the associations of MCs and Eos in the initiation and recovery progression of UC at various time periods.

Materials and methods

Animals

Male Sprague-Dawley rats (200-250 g) were purchased from the Laboratory Animal Center of Xi'an Jiaotong University. They were housed in a restricted access room with controlled temperature and light/dark (12 h/12 h) cycle. Standard food and tap water were provided *ad libitum* before experimental procedures. The study received the approval of the Animal Care Committee of the Xi'an Jiaotong University.

Induction of colitis

Rats were randomly divided into 2 groups: TNBS group (n = 20) and Control group (n = 5). Colitis was induced in 24-h fasted rats and then under ether inhalation anesthesia, TNBS (100 mg/kg), dissolved in 50% ethanol, was intrarectally injected in a volume of 0.3 ml, via a silicone catheter inserted 8 cm proximal to the anus. After removing the silicone catheter smoothly, we raised the rat tail and pressed on the anus by hand for a few seconds until they recovered from anesthesia, and then returned them to their cages with free access to water and food. Control group received normal water to drink.

Histological evaluation of colitis

Animals from control and TNBS treated group (n = 5 for each time) were anaesthetized with 20% urethane (7 ml/kg) by intraperitoneal injection at different time points (6, 11, 16, and 21 days). The abdomen was opened and the appearance of the colon was examined. Then, the distal colon was opened longitudinally and we removed 1.5 cm distal colon (7 cm from the anus), gently cleaned it of fecal content, and fixed it with 10% buffered formalin, and it was embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). The colonic damage score a was assessed according to previous report [18]. This system takes into consideration the absence or presence of hyperemia, the area of necrosis and ulcers, and the presence or absence of adhesions between the colon and other organs. Scoring of damage was performed by two observers unaware of the experimental protocol. After scoring, the net weight of the distal colon (7 cm from the anus) was recorded.

Eosinophil counts

Eos counts were performed on serial, H&E stained, 4 μm thick, transverse sections of the left colon and were expressed as the average Eos count per full transverse section of the colon. The results were confirmed by counting the numbers of Eos per mm^2 of lamina propria using video image analysis.

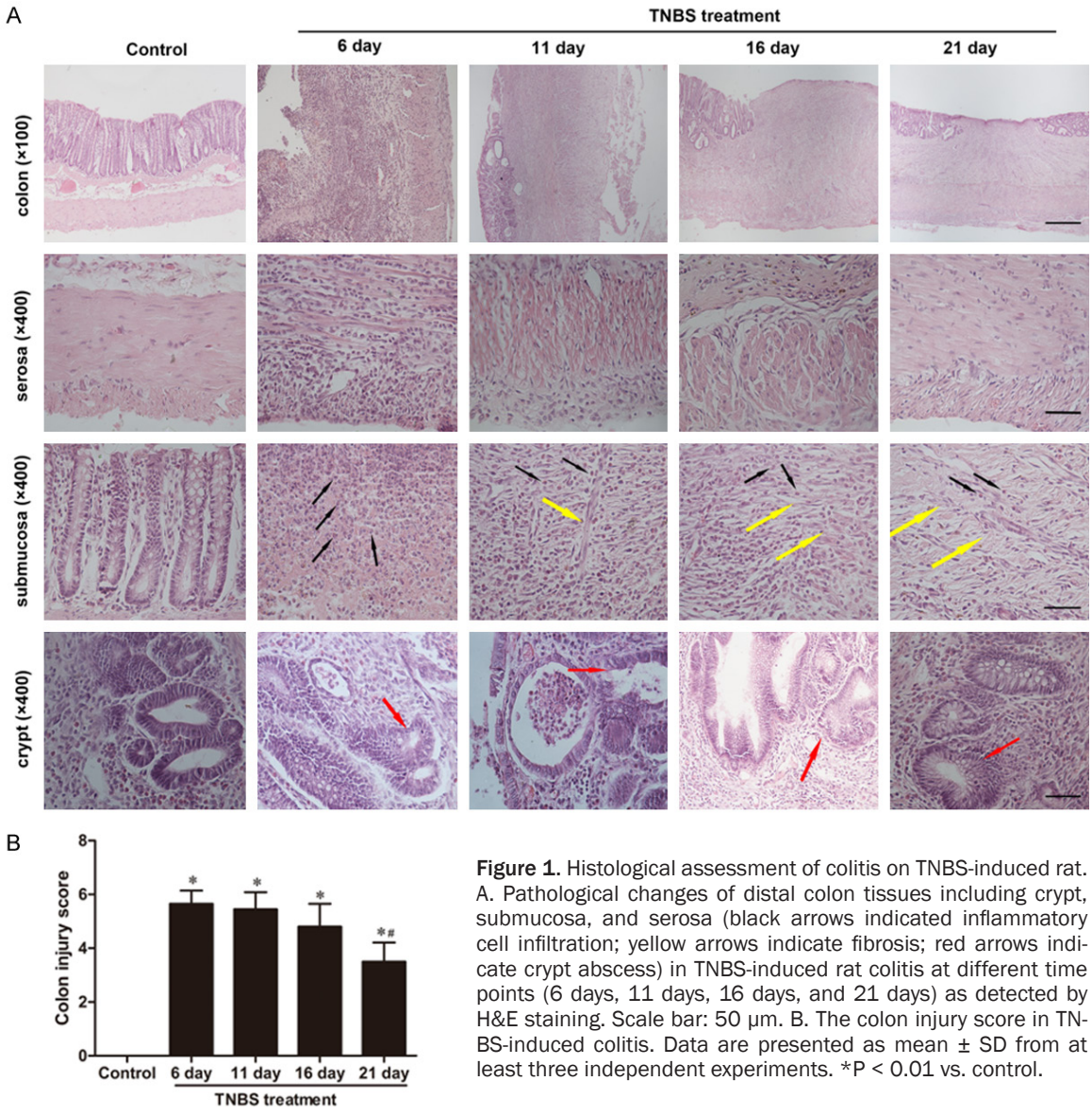

Figure 1. Histological assessment of colitis on TNBS-induced rat. A. Pathological changes of distal colon tissues including crypt, submucosa, and serosa (black arrows indicated inflammatory cell infiltration; yellow arrows indicate fibrosis; red arrows indicate crypt abscess) in TNBS-induced rat colitis at different time points (6 days, 11 days, 16 days, and 21 days) as detected by H&E staining. Scale bar: 50 μm. B. The colon injury score in TNBS-induced colitis. Data are presented as mean ± SD from at least three independent experiments. *P < 0.01 vs. control.

MC staining

To assess the number of MC, colon tissues were separated and fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with toluidine blue. The number of MC granules was counted per mm of the vertical section serosa (number/mm) and the degranulated MCs were identified.

Tissue and whole blood histamine concentration

The total histamine concentration in the colon and whole blood were determined by fluorescence measurement. Briefly, to obtain complete hemolysis, 2.8 ml volume of deionized water was added to 0.5 ml volume of heparin anticoagulant treated whole blood, and then we gradually added 0.7 ml volume of 25% trichloroacetic acid, and centrifuged at 4000 rpm/min for 10 min. Moreover, colon tissue samples (approximately 100 mg) were weighed, 4 ml volume of 2.5% trichloroacetic acid was added, and the tissue homogenate was centrifuged at 4000 rpm/min for 10 min. The histamine concentration of the supernatant was determined by an automated continuous-flow system [19].

Immunohistochemistry staining

Paraffin-embedded tissue sections were sectioned, deparaffinized, then treated with 3% H_2O_2 at room temperature for 10 min to block

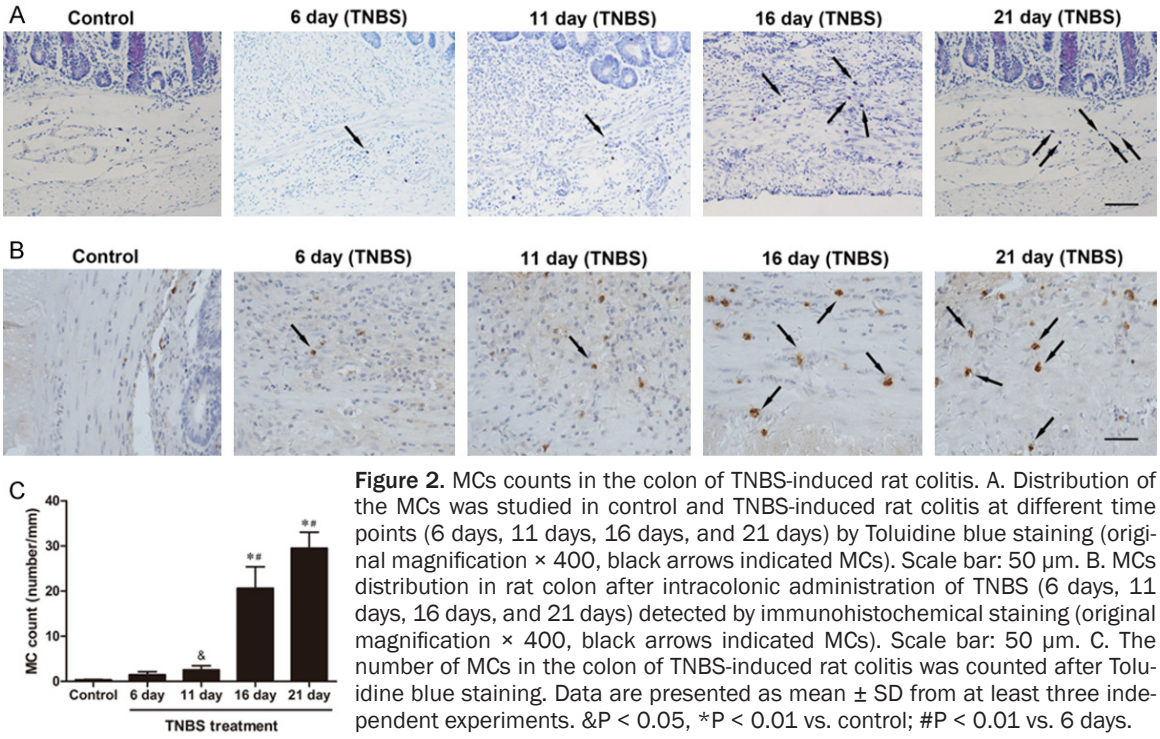

Figure 2. MCs counts in the colon of TNBS-induced rat colitis. A. Distribution of the MCs was studied in control and TNBS-induced rat colitis at different time points (6 days, 11 days, 16 days, and 21 days) by Toluidine blue staining (original magnification × 400, black arrows indicated MCs). Scale bar: 50 μm. B. MCs distribution in rat colon after intracolonic administration of TNBS (6 days, 11 days, 16 days, and 21 days) detected by immunohistochemical staining (original magnification × 400, black arrows indicated MCs). Scale bar: 50 μm. C. The number of MCs in the colon of TNBS-induced rat colitis was counted after Toluidine blue staining. Data are presented as mean ± SD from at least three independent experiments. &P < 0.05, *P < 0.01 vs. control; #P < 0.01 vs. 6 days.

endogenous peroxidase, and then in a microwave oven for 30 min to restore antigen. Sections were incubated with goat serum for 10 min before adding mouse anti-tryptase Ab-2 antibody (1:200) at 4°C for 48 h, then incubated with Avidin-binding secondary antibody and Streptavidin-biotin-peroxidase complex for another 1 h. Visualization was performed by incubation of the sections in a solution of 3,3'-diaminobenzidine (DakoCytomation, Denmark). After washing, the sections were counter-stained with hematoxylin and coverslipped. Photomicrographs were acquired with an inverted microscope (Leica, Germany).

Transmission electron microscopy

Distal colon mucosa tissues (7 cm from the anus) were cut into 1 mm^2 bulk, and immediately fixed into 2.5% glutaraldehyde fixation fluid at 4°C for 2 h, then embedded in Dow epoxy resin DER332 (Unione Chimica Europea, Milan Italy) as previously described. Ultrathin sections were prepared with an Ultratome III, double-stained with lead citrate and uranyl acetate, and observed by transmission electron microscopy (TEM, H-600; Hitachi, Japan).

Statistical analysis

Data are presented as mean ± SD. Comparison of more than two groups was made with the one-way analysis of variance ANOVA followed by Dunnett's *t* test (SPSS 13.0). Correlation coefficients were calculated using Kendall's tau-b method. The Mann-Whitney U test was used where appropriate. A *P* value of < 0.05 was considered significant.

Results

Histologic evaluation of colonic damage

Colonic injury determined 6 days after intrarectal administration of 100 mg/kg TNBS was represented by obvious ulcer formation, dilatation of the colon, stiffness and thickening of colon wall, hyperemia, and edema of surrounding colon mucosa. The main lesions were observed within 8 cm from anus; full-thickness of the colon wall was involved in inflammation, accompanied by epithelial exfoliation, crypt destruction and abscess, inflammatory cell infiltration, and even fibrosis, which was consistent with pathological changes of acute inflammation (**Figure 1A**). The histologic damage observed 11 and 16 days after TNBS administration was similar in colons from TNBS-treated rats at 6 days (**Figure 1A**). In the colons of rats receiving TNBS for 21 days, local hyperemia, edema, hyperemia, and some macroscopically visible inflammation of the colon wall were observed, accompanied by epithelial regenera-

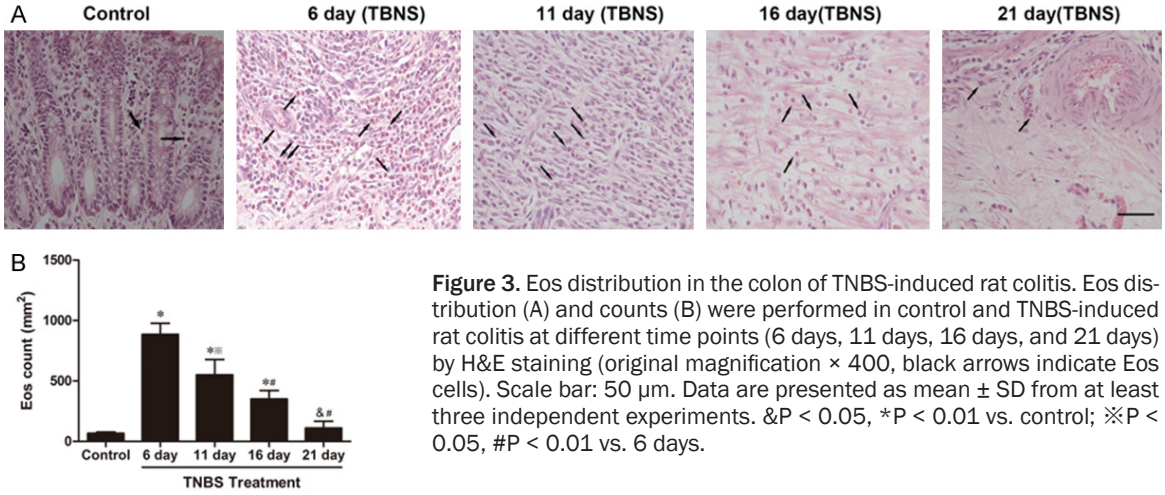

Figure 3. Eos distribution in the colon of TNBS-induced rat colitis. Eos distribution (A) and counts (B) were performed in control and TNBS-induced rat colitis at different time points (6 days, 11 days, 16 days, and 21 days) by H&E staining (original magnification × 400, black arrows indicate Eos cells). Scale bar: 50 μm. Data are presented as mean ± SD from at least three independent experiments. &P < 0.05, *P < 0.01 vs. control; ※P < 0.05, #P < 0.01 vs. 6 days.

tion and gland hyperplasia, granulation tissue formation, and neutrophilic infiltration, which were characterized as the chronic repair stage of TNBS-induced rat colitis. The injury score was significantly reduced when compared to that in the 6th day ($P < 0.01$), though still higher than the control group ($P < 0.01$, **Figure 1B**).

Effects of TNBS on mast cell number and distribution in the colon

In colon tissues from control rats, mast cells (MCs) were localized in the mucosa and submucosa around small blood vessels with slight degranulation, and occasionally seen in serosa, but with no distribution in the muscularis (**Figure 2A** and **2B**). 6 days after induction of colitis there was an increase in the number of MCs near the anus of TNBS-treated colons, and aggregation around the dilated small blood vessels and colon muscularis with degranulation; but no significance of MC number was counted when compared with that in control group. At day 11, 16, and 21, total MCs number gradually increased near the anus in the muscularis of colons accompanied by obvious degranulation. The immunohistochemical staining of MCs after TNBS treatment further illustrated that the increasing population of MCs was mostly distributed in the colonic muscularis, especially beside the nerve fiber and nerve plexus, positive for tryptase staining, accompanied by obvious degranulation (**Figure 2B** and **2C**).

The population changes of eosinophils in TNBS-induced colitis

Under normal conditions, eosinophils (Eos) counts were often distributed in the mucosa of the rat colon, whereas after treatment with TNBS for 6 days, the number of Eos was obviously increased (**Figure 3A**, black arrow, $P < 0.01$) and mainly distributed in the submucosa and sporadically in the muscularis propria of the distal colon. After induction of colitis for 11 and 16 days, the Eos counts in the distal colon were gradually decreased when compared to those on the 6th day ($P < 0.05$ and $P < 0.01$, respectively), but still higher than that in control group ($P < 0.01$, respectively). When treated with TNBS for 21 days, the population of Eos was significantly reduced compared to that in 6th day ($P < 0.01$).

Ultrastructural changes of MC and Eos during TNBS-induced colitis

MC, a type of round mononuclear cell, was filled with many high electron density granules. The morphology of these granules contains condensed materials to make them appear as a crystal shape or finely granular. During the process of colitis induction, the number of MC was increased and accompanied by activated degranulation. Taking the typical ultrastructure of MC and Eos at 16 days after TNBS administration for example, the TEM results showed that the cytoplasmic empty chambers of MC were filled with vesicles after degranulation, and many rounded granules were secreted from the intracellular to the external environment (**Figures 4A** and **2B**). Moreover, under normal conditions, Eos were usually small and contained a bi/poly-lobed nucleus with condensed peripheral nuclear chromatin. During the process of colitis induction, the TEM assay showed no significant ultrastructural changes

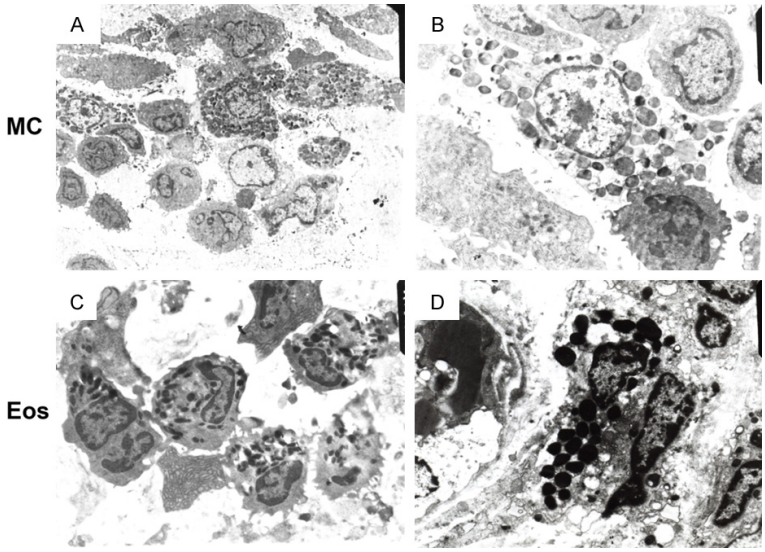

Figure 4. Transmission electron micrograph of sections of MCs and Eos. Ultrastructure detection of MCs (original magnification × 2000) (A) and its degranulation (original magnification × 5000) (B) in the colon of TNBS-induced rat colitis at day 16. Eos (original magnification × 4000) (C) and its condensed peripheral nuclear images (original magnification × 8000) (D) in the colon of TNBS-induced rat colitis at day 16.

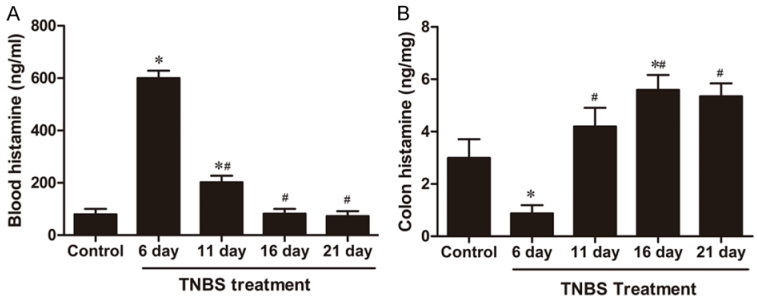

Figure 5. Assessment of histamine concentrations in the whole blood and colon from different stages of TNBS-induced colitis. Concentrations of blood histamine (A) and colon histamine (B) were measured by fluorescence measurement from whole blood and colon tissues, respectively. Data are presented as mean ± SD from at least three independent experiments. *P < 0.01 vs. control; #P < 0.01 vs. 6 days.

among the morphology of Eos (**Figure 4C** and **4D**).

Effect of TNBS on histamine concentrations from blood and colon tissues

Compared to the control group, the blood histamine content increased and reached to peak level around the 6th day of colitis induction ($P < 0.01$, **Figure 5A**); whereas the distal colon histamine concentration was significantly reduced ($P < 0.01$, **Figure 5B**). After treatment with TNBS for 11 days, the blood histamine concentration was gradually decreased compared to that on the 6th day, but still higher than that in the control group ($P < 0.01$); while the content of distal colon histamine was increased compared to the normal level. At day 16, the concentration of blood histamine was nearly decreased to normal levels, while the concentration of distal colon histamine increased to the peak level ($P < 0.01$ vs. control group). 21 days after induction of colitis, the concentrations of blood histamine and distal colon histamine both recovered to normal levels according to the results.

Correlation analysis among colon injury score, MC, Eos, and histamine content of blood and colon tissues in TNBS-induced colitis

Correlation analysis revealed that the Eos counts were positively correlated with the colon injury score and blood histamine content ($P < 0.01$, respectively), but had no correlation with MC count and colon histamine content. While the MC count was negatively associated with the blood histamine content ($P < 0.05$), but positively associated with the colon tissue histamine content ($P < 0.01$), it had no correlation with colon injury score. Furthermore, the blood histamine content was inversely related to the colon tissue histamine content ($P < 0.01$) during the TNBS-induced colitis repair progression (**Table 1**).

Discussion

Previous studies indicated that MCs and Eos can regulate the mucosal barrier of gastrointestinal tract [13]. Araki et al. suggested that when given a 3% dextran sulphate sodium (DSS) aqueous solution orally for 10 days, control (+/+) rats showed severe edema and hyper-

Table 1. Spearman rank order correlation between selected measures in the recovery process of TNBS-induced rat colitis

	Eos	MC	Blood histamine	Colonic histamine
vs. colon injury score	r = 0.670; *P* < 0.01	r = -0.011; *P* = 0.942	r = 0.525; *P* < 0.01	r = -0.125; *P* = 0.421
vs. Eos		r = -0.107; *P* = 0.455	r = 0.629; *P* < 0.01	r = -0.177; *P* = 0.216
vs. MC	r = -0.107; *P* = 0.455		r = -0.281; *P* < 0.05	r = 0.411; *P* < 0.01
vs. blood histamine	r = 0.629; *P* < 0.01	r = -0.281; *P* < 0.05		r = -0.431; *P* < 0.01
vs. colonic histamine	r = -0.177; *P* = 0.216	r = 0.411; *P* < 0.01	r = -0.431; *P* < 0.01	

P < 0.05 is significant.

emia with sporadic erosions in mucosal damage when compared to Ws/Ws rats which indicated that MCs play an important role in the development of DSS colitis [20]. Stasikowska et al. also showed that the accumulation of toluidine blue-stained and tryptase immunopositive MCs was significantly increased in the active stage of UC compared with non-active UC [21]. Consistently, we found that MCs and Eos were essential infiltrating cells in UC in the present study. The number of toluidine blue stained MCs was gradually increased in TNBS induced rat colitis at day 6, 11, 16, 21, and accompanied by obviousdegranulation. Furthermore, the distribution of MCs was transformed from the submucosal lamina propria to the full-thickness of the wall, mostly distributed at the muscular layer when compared to control group. The immunohistological staining assay showed that the type of increasing number of MCs were tryptase immunopositive in TNBS induced colitis models. Therefore, these findings indicated that MCs were directly involved in the mucosal inflammation damage during the colitis development induced by TNBS.

Tryptase, a tetrameric serine proteinase, is observed in all MCs and constitutes approximately 20% of total cell protein. It has been reported that UC could directly induced tryptase expression in MCs, and the MC tryptase inhibitor APC2059 was effective and safe for UC treatment, which also emphasized the crucial role of tryptase secretion during UC pathogenesis [22, 23]. Histamine has frequently been used as a biochemical marker for MC calculation in multiple tissues, because MCs represent the major peripheral tissue repository of this amine [12]. MC number is strongly correlated with tissue histamine levels in either normal tissues or those undergoing fibrosis or inflammation. Histamine as the proinflammatory mediator was located in the nuclei of MC and Eos granules and released from the cellular surface when activated [24]. In our current study, the colonic histamine concentration was obviously lower than that in control group in the TNBS-induced rat colitis in the acute stage; whereas the whole blood histamine concentration was significantly higher than that in the control group, which was also positively correlated with colon injury score. Thus, we supposed that when colitis was stimulated by TNBS, the histamine was released from activated MC during the acute stage, and directly participated in the damage of colonic mucosa. In addition, the colonic histamine concentration was very reduced at day 6, which might be because the released histamine in the colonic mucosa rapidly decomposed and resulted in lower concentration in the acute inflammation condition. A previous study has reported that the histamine level of colonic mucosa was significantly increased in allergic enteropathy and UC patients [25]. In addition, during the chronic repair stage of TNBS-induced colitis, the count of MCs in colonic mucosa and colonic histamine concentration were both distinctly increased, which implied that MC count was associated with the chronic inflammation process of TNBS induced rat colitis.

In the present study, we found that during the repair process of TNBS-induced rat colitis, MCs count was positively correlated with colonic histamine concentration, but negatively correlated with blood histamine concentration. The colon injury score was decreased while the MC count was increased after the 6th day of the experiment, which showed opposed alternation. These results demonstrated that MCs participated in mucosal injury by releasing transmitters like histamine during the initial acute stage of TNBS induced rat colitis; while at the subsequent late repair process, MCs could be still

involved in the repair process of this inflammation through some mechanism. Thus, Galli et al. proposed a "mast cell-leukocyte cytokine cascade", which illustrated that a series of biological responses are initiated by MC activation, resulting in the MC-regulatory release of various cytokines which can subsequently facilitate the recruitment of neutrophils, Eos, and other effector cells [26]. Indeed, a protective role for MCs has been recently supposed by some researchers, in view of the observation that MC accumulation is frequently involved in the repair process of some fibrotic and inflammatory diseases such as scleroderma or liver cirrhosis [27, 28]. Therefore, increasing evidence suggests that MCs participate in not only the initial fibrotic stage but also in the fibrosis-mediated reparative process [29].

The normal colonic Eos were mainly distributed among the mucous layer. Recent clinical and animal experiments have demonstrated that Eos infiltration was obviously increased in UC inflammatory tissues, especially in the submucosa, indicating that Eos participated in the UC inflammatory reaction through releasing various cytotoxic proteins and inflammatory mediator [30, 31]. In our study, we found that Eos were significantly increased at the acute stage of TNBS-induced colitis, and mostly distributed in the mucosa and submucosa, but the Eos infiltration was alleviated in the inflammatory remodeling process. We also revealed that the Eos count was positively correlated with blood histamine concentration and colitis injury score, indicating the direct involvement of Eos in TNBS induced colitis. Smyth et al. demonstrated that an increased number of Eos was observed in the mucosa and muscularis mucosae of patients [32]. Stasikowska et al. also discovered that the number of Eos was significantly increased in active UC compared to non-active UC; moreover, the number of Eos was significantly correlated with number of MCs in active stage of UC [21]. However, no significant correlation was found between Eos count and MCs count in the present study, which might be due to the small animal sample data we collected not being enough to determine significance as Stasikowska et al. reported. Thus, further studies on the correlations of MCs and Eos on the different stages of UC are needed.

In summary, though no correlation was found between MCs and Eos counts in the TNBS-induced colitis in this study, our findings confirmed that increased MCs with high colon histamine concentration were more likely to be involved in the chronic repair stage of TNBS-induced rat colitis, while decreased Eos with low whole blood histamine more likely participated in the initial acute stage of TNBS-induced rat colitis.

Acknowledgements

This study is supported by Scientific and Technological Project of Shaanxi Province (2016-SF-090). The authors would like to thank Neo-Markers Company (USA) for providing us free mouse anti-tryptase Ab-2 antibody.

Disclosure of conflict of interest

None.

Address correspondence to: Haitao Guan, Department of Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, 157 West NO. 5 Road, Xi'an 710004, Shaanxi Province, P. R. China. Tel: +86-29-87679526; Fax: +86-29-87678599; E-mail: Guanhaitao86@163.com

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