Protective effects of tranilast on oxazolone-induced rat colitis through a mast cell-dependent pathway

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ABSTRACT

Background. Mast cells in the gut play an important role in the innate and adaptive immune responses that are relevant to human inflammatory bowel disease. However, the contribution of mast cells to the development of inflammatory bowel disease is not well understood. This study aimed to determine the role of mast cells in oxazolone-induced colitis and to explore whether the mast cell membrane stabiliser tranilast could ameliorate colonic inflammation.

Methods. Wild-type rats and mast cell-deficient rats were sensitised and challenged with oxazolone, then treated with tranilast after challenge. Controls were treated with saline.

Results. Mast cell-deficient rats presented a weak response to oxazolone, while wild-type rats showed severe ulcerative colitis after stimulation with oxazolone. The mast cell-deficient rats model had a significantly lower disease activity index score than wild-type rats model (1.8±1.64 vs. 8.3±0.58 respectively; P<0.01). Tranilast could reduce the secretion of cytokines, immunoglobulins and myeloperoxidase activity in tranilast treatment groups compared with the model group. The number of mast cells in the wild-type model was higher than in the other groups. There was no significant change in mast cell-deficient rats.

Conclusion. Mast cells play an important role in oxazolone-induced colitis. The mast cell membrane stabiliser tranilast can ameliorate oxazolone-induced colitis via a mast cell-dependent pathway.

Keywords: Mast cell; Ulcerative colitis; Tranilast; IL-33
INTRODUCTION

Inflammatory bowel disease (IBD), which consists of ulcerative colitis (UC) and Crohn’s disease (CD), is characterised by inflammatory cell infiltration in the bowel and crypt destruction [1]. Animal models are essential in understanding the pathogenesis of IBD. Three major chemical-induced colitis models have been widely used: 2,4,6-trinitro benzene sulfonic acids (TNBS), oxazolone (OXA) and dextran sodium sulphate (DSS) colitis. TNBS- and DSS-induced colitis models involve T helper cell type 1 colitis; however, the OXA-induced model is similar to human UC, which is mainly T helper cell type 2 colitis [1,2].

The role of inflammatory and immune cells in IBD has aroused widespread attention. Mast cells (MCs), a type of inflammatory cell, play a key role in the inflammatory process. The role of MCs in regulating immune responses in IBD has been researched for decades. However, the contribution of MCs to the development of IBD is still not well understood [3-6]. Recent evidence of an increased number of MCs in colorectal mucosa, lamina propria and submucosa inpatients with UC and CD shows the relationship between human IBD and MCs [7]. In addition, mucosal MC complexity and degranulation is altered in patients with IBD. The levels of TNFα, IL-6, substance P and the expression of histamine, prostaglandins, leukotrienes and tryptase are increased in IBD patients [8-10]. These studies suggest that MCs might be involved in chronic intestinal inflammation. To confirm this, Coldwell et al. have shown inflammatory interstitial accumulation of MCs in the vicinity of afferent fibres, including calcitonin gene-related peptide (CGRP) in DSS-induced rats, and colon visceral afferent fibres in response to 5-HT during acute inflammation (7 days) [11]. Menozzi et al. found that the number and activity of MCs are enhanced in DSS-
induced rat colitis. However, the number of MCs in TNBS-induced colitis was reduced in the acute stage, but increased after 60 days, suggesting that MCs might play an important role in the process of tissue repair in the later stage [12].

The IBD therapeutic effects of MC stabilisers are worthy of further study. Some drugs currently used to treat IBD, such as 5-ASA, hormones and methotrexate, can affect the activity of MCs in vitro. On the other hand, some preliminary data suggest that the MC stabiliser ketotifen has a positive effect on IBD patients [12]. The evidence Eliakim et al. provided shows that ketotifen can significantly reduce the severity of colitis [14]. A recent multi-centre phase II open clinical trial observed the efficacy of the tryptase inhibitor APC 2059 (a specific inhibitor of MC mediators) in UC patients. In a total of 56 cases of mild to moderate UC patients, the disease activity score improved or reached normal after treatment in more than half of the subjects [14]. Although further evidence is required, clinical and experimental data have confirmed that MC-related treatment, whether using MC stabilisers or inhibitors of specific mediators of MCs, is likely to become the adjuvant treatment for IBD. This is worthy of further study.

The MC membrane stabiliser tranilast, which inhibits MC degranulation and is approved for clinical use in Japan, is widely used in clinics for the treatment of allergic disorders such as bronchial asthma and allergic rhinitis [15]. Tranilast can also prevent fibroblast proliferation in vitro and suppresses collagen production in vitro and in vivo [16-18]. Tranilast-related research has mainly concentrated on the role of tranilast in respiratory diseases [19-21]. So far, there has only been one study on the role of tranilast in acute colitis, in which tranilast ameliorated DSS-induced colitis in mice through increasing the expression of heme oxygenase-1 [22].

Therefore, in the present study, we studied the role of MCs in OXA-induced colitis
in wild-type rats (WsRC +/+, +/+ ) and MC-deficient rats (WsRC Ws/Ws, Ws/Ws) and reported that the MC membrane stabiliser tranilast can reduce OXA-induced colitis, which is similar to human UC.

MATERIALS AND METHODS

Animals

Mast cell-deficient rats (WsRC Ws/Ws, Ws/Ws) and their wild-type controls (WsRC +/+, +/+ ), 10–12 weeks of age, weighing 150–180 g, were purchased from Japan TGC Inc. (Kanagawa, Japan). All animals were housed in a barrier system (temperature: 20–26°C; relative humidity: 40–70%) with a 12 h light/12 h dark cycle and fed with standard rat feed and tap water. At the end of the experiment, rats were sacrificed by cervical dislocation under isoflurane anaesthesia. The Institutional Committee of Animal Research approved all the experiments.

Induction of colitis by rectal administration of OXA

To sensitise the rats, a 2 cm×2 cm field was shaved on their back, following anaesthesia with isoflurane, and 0.3 mL of 5% (w/v) solution of OXA (Sigma) in acetone/olive oil (4:1) was applied. Seven days after sensitisation, rats were challenged rectally under ethyl ether general anaesthesia with either 0.45 mL 5% OXA in 50% ethanol to induce colitis or 50% ethanol alone (negative control) [23-27].

Groups and treatments

The groups and treatments of rats are described in Fig. 1. Wild-type rats (WsRC
+/-, +/+) were divided into five groups (n=7) as follows: negative control group, tranilast control group, model group and two tranilast treatment groups (30 mg/kg/day or 60 mg/kg/day). MC-deficient rats (WsRC Ws/Ws, Ws/Ws) were divided into three groups (n=7) as follows: negative control group, model group and tranilast treatment group (60 mg/kg/day). Seven wild-type rats treated with 60 mg/kg tranilast only were established as the tranilast control group to exclude the effects of tranilast on rats. Saline and tranilast were administered rectally from day 8 and given continuously for 7 days.

**Disease activity index evaluation**

The disease activity index (DAI) was used to evaluate intestinal inflammation according to the nature protocol scoring system published by Kihara et al. [2,28]. The scoring system includes three parts: weight loss (0, ≤1%; 1, 1–5%; 2, 5–10%; 3, 10–15%; 4, ≥15%), stool consistency (0, normal; 2, loose stools; 4, diarrhoea) and occult/gross rectal bleeding (0, no blood; 2, positive hemoccult; 4, gross bleeding). All scores are combined together, and then averaged for the final DAI score.

**Histological assessment of colitis**

For histopathological assessment, colon tissues were separated and fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. The severity of inflammation of the stained sections was examined by infiltration of lymphocytes and macrophages, distortion of crypts, crypt abscesses, frank ulceration and a reduction in goblet cell number.

**Mast cells staining**

To assess the number of MCs, colon tissues were separated and fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with toluidine blue. The number of MC granules were counted and the degranulated MCs were...
identified.

**MPO activity, cytokine and immunoglobulin analysis**

Myeloperoxidase (MPO) activity reflects granulocyte infiltration in colonic tissues after induction of colitis. Colon tissues (100mg) were excised and put into 1 ml PBS for homogenates, then centrifuged at 3000 rpm/min for ten minutes and the supernatant was prepared for ELISA testing. Cytokines (IL-6, IL-13, IL-33) and immunoglobulin (IgA, IgG, IgE) levels in colon tissue homogenates and serum were tested using ELISA kits. All of the kits were purchased from Abcam Co. Ltd. (UK). The detailed method of detecting mRNA expression of cytokines is described in supplementary file (Appendix A, Supplementary methods).

**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, Peking University). A *P* value of less than 0.05 was considered significant.

**Results**

**Colitis model established in wild rats**

Body weight was recorded from the day of sensitisation until three days after rectal OXA challenge. As is shown in Fig. 2A, after challenge with OXA, the body weight of wild-type rats (WsRC +/+ , +/+ ) decreased significantly compared with the negative control group. For wild-type rats, it is clear that the model group presented a higher DAI score than the negative control group (Fig. 2B graph a). For wild-type rats, the organ coefficient of the model group was significantly higher than that of the negative control group. Moreover, the colon length of the model group was shorter than that of the control and tranilast treatment groups (Fig. 3A).
Together with the histological data shown in Fig. 4, rectally administered OXA resulted in severe changes of colon architecture in wild-type rats. Epithelial layers and lamina propria were disrupted, accompanied by mucosal oedema, infiltration of inflammatory cells (including lymphocytes and neutrophilic granulocytes) into mucosa, and goblet cell disruption and disappearance (Fig. 4C). These changes suggested that the colitis model was successfully established.

**Effect of tranilast on the severity of colitis induced by OXA in wild-type rats**

Tranilast treatment groups (30 mg/kg or 60 mg/kg) presented a lower DAI score compared with the model group (Fig. 2B graph a). Additionally, there was no difference in DAI score between the two tranilast treatment groups (30 mg/kg or 60 mg/kg; Fig. 2B graph a). Meanwhile, it was also found that tranilast itself could not influence the DAI score of rats (Fig. 2B graph a). Also, tranilast itself could not influence the colon organ coefficient and colon length (Fig. 3A-B). The disruption of the colon architecture and inflammatory cell infiltration was ameliorated after treatment with tranilast (30 mg/kg or 60 mg/kg) (Fig. 4D, E). These results suggest that tranilast might ameliorate the severity of colitis induced by OXA in wild-type rats.

Myeloperoxidase (MPO) symbolises the activation of neutrophil and is an important indicator of colitis. MPO level of colon tissue homogenates was tested using an ELISA kit. As is shown in Fig. 2C, the MPO level of the model group was significantly higher than that of the negative control group and tranilast treatment groups (30 mg/kg or 60 mg/kg), this means that tranilast could ameliorate the severity of OXA-induced colitis. The results were consistent with the DAI score. It was also found that tranilast itself could not influence the MPO level of rats without OXA treatment (Fig. 2C graph a). From the results above, we can conclude that tranilast can ameliorate the severity of colitis induced by OXA.
Effect of OXA on MC-deficient rats

At the same time, MC-deficient rats were used to further research whether MCs participated in the progression of OXA-induced colitis. The body weight of MC-deficient rats (WsRC Ws/Ws, Ws/Ws) was decreased significantly compared with the negative control group (Fig. 2A graph b). The model group presented a higher DAI score than the negative control group, but there was no difference between the model group and the tranilast treatment group (Fig. 2B graph b). Interestingly, the organ coefficient was significantly higher than the negative control group, but there was no change in colon length (Fig. 3C, D). Meanwhile, in MC-deficient rats, the MPO level showed no difference in either group (Fig. 2B graph b).

Notably, there was no significant change in colon histology in MC-deficient rats after challenge with OXA or treatment with tranilast (Fig. 4F-H). These data suggest that MC-deficient rats might represent a weak response to OXA. As is expected, MCs can promote the progression of OXA-induced colitis, and the knockout of MCs can prevent this progression. Otherwise, from the result of histological examination, we predict that the MC membrane stabiliser tranilast might alleviate OXA-induced UC.

Treatment with tranilast and the number of colon MCs

Toluidine blue was used for staining MCs to detect whether MCs infiltrate the colitis model and whether mast cell numbers changed after treatment with tranilast. As shown in Fig. S1, for wild-type rats, compared with controls, MCs infiltrate the colon epithelia in the model group, and MCs under-degranulation was observed. Also, after treatment with tranilast, the number of MCs reduced significantly compared with the model group (Fig. S1 C, D, E). There were no MCs present in MC-deficient rats (Fig. S1 F, G, H).
Effect of tranilast on the secretion of cytokines and immunoglobulins caused by OXA

To further elucidate the mechanism underlying the inhibitory effects of tranilast on OXA-induced colitis, we investigated whether tranilast could influence the secretion of cytokines and immunoglobulin in the colon using an ELISA assay.

As shown in Fig. 5, in wild-type rats, after challenge with OXA, the level of cytokines (IL-6, IL-13 and IL-33) in the colon increased significantly compared with the negative control group. However, a significant down-regulation of cytokines was observed after treatment with tranilast compared with the model group (Figs. 5A graph a, 5B graph a, 5C graph a). It means that OXA could induce the secretion of Th2 cytokines in colon tissues, while tranilast could reduce this secretion. However, in MC-deficient rats, cytokine levels showed no difference among the negative control group, model group and tranilast treatment group (60 mg/kg/day; Figs. 5A graph b, 5B graph b, 5C graph b). In addition, as shown in supplementary Fig. S2 and Fig. S3, the IL-1, TNF-α mRNA expression and histamine secretion were also inhibited after treatment with tranilast in the wild-type model group.

Consistent with the result of cytokines above, as is shown in Fig. 6, was the result of immunoglobulin expression in colon homogenates. After challenge with OXA, the level of immunoglobulins (IgA, IgE and IgG) in the colon increased significantly compared with the negative control group. However, a significant down-regulation of these three immunoglobulins was observed after treatment with tranilast compared with the model group (Fig. 6A graph a, 6B graph a, 6C graph a). In addition, in MC-deficient rats, there was no difference in each group (Fig. 6A graph b, 6B graph b, 6C graph b). The levels of HO-1, TGF-beta mRNA expression were also inhibited after treated with tranilast in the wild-type model group, and there were no significant
changes in MC-deficient rats (Fig. S4).

From the results above, it is clear that OXA could increase the secretion of cytokines (IL-6, IL-13 and IL-33) and immunoglobulins (IgA, IgE and IgG) in wild-type rats, but there was no change in MC-deficient rats. MC membrane stabiliser tranilast could decrease the OXA-induced high secretion of cytokines and immunoglobulins in wild-type rats, but had no effect on MC-deficient rats.

**Comparison between two model groups**

The DAI score of the wild-type model group was significantly higher than that of MC-deficient rats (Fig. 2B graph c). The organ coefficient of MC-deficient rats had no significance compared with wild-type rats (Fig. 3E). The colon length of the wild-type model group was shorter than that of the MC-deficient model group (Fig. 3F). Additionally, the disruption of colon structure was more severe in wild-type rats than in MC-deficient rats (Fig. 4C, 4G). Moreover, after staining colon with toluidine blue, MCs infiltrate in the wild-type model and no MCs infiltrate in MC-deficient rats (Fig. S1C, S1F). The MPO activity of the model group in MC-deficient rats was lower compared with wild-type rats (Fig. 2C graph c).

Consistent with the result above, the three cytokine (IL-6, IL-13, IL-33) levels in homogenates of the wild-type model group were significantly higher than that in MC-deficient rats (Fig. 5A graph c, 5B graph c, 5C graph c). There was no difference among the groups in all three cytokines in serum (Fig. S5). The immunoglobulin levels in homogenates of the wild-type model group were significantly higher than those of MC-deficient rats (Fig. 6A graph c, 6B graph c, 6C graph c). There was no difference among the groups in any of the immunoglobulins in serum.

**DISCUSSION**
Mast cells, a type of inflammatory cell, play an important role in inflammation. The role of MCs in chemically-induced colitis is still not clear. Also, the search for drugs to treat colitis has increased enormously in recent years. Tranilast, a type of MC membrane stabiliser, has been widely used for bronchial asthma and allergic rhinitis [16]. Darakhshan and Ghanbari confirmed that tranilast can enhance the activity of tamoxifen and the TGF-β pathway to prevent breast cancer [29]. As discussed above, there have been few studies about the therapeutic effects of tranilast on colitis. In the present study, we confirmed the role of MC in OXA-induced UC by using MC-deficient rats, and that tranilast can ameliorate the severity of colitis-induced by OXA. We investigated the effects of tranilast on cytokine (IL-6, IL-13 and IL-33) and immunoglobulin (IgA, IgE and IgG) expression by ELISA assay, we also assessed the change in MCs in different groups and explored the underlying mechanisms. OXA was first used in MC-deficient rats inducing colitis to explore the role of MCs in UC, and to explore the curative effect of tranilast in this model. In particular, by comparison with the wild-type rats, the curing effect of tranilast is direct and provided evidence of the beneficial effects of tranilast in the treatment of colitis.

Challenge with OXA caused up-regulation of IL-6, IL-13 and IL-33 in colon homogenates and promoted MC infiltration in the colon epithelia. IL-6 has a positive correlation with IBD disease activities and plays a key role in apoptosis in the lamina propria at the inflamed site [30]. Furthermore, it plays a key role in DSS-induced experimental colitis [31-33]. It is also reported that IL-6 participates in TNBS-induced colitis and the nerve centre regulates the development of IBD via IL-6 [34]. However, the detailed mechanism of how IL-6 participates in the colitis was not clear. MCs are effector cells that can regulate allergic inflammation and participate in the process of immune response, and activated MCs can secrete pro-inflammatory cytokines such
as IL-6 and IL-13. In this study, the hapten OXA can cause colon MC degranulation and trigger immediate hypersensitivity. Therefore, the level of IL-6 was higher in the wild-type model than in the MC-deficient rats. Tranilast can inhibit MC degranulation; thus, the wild-type rats (WsRC +/+, +/+) that were treated with both OXA and tranilast showed no significant change in IL-6 level compared with the negative control group.

In this study, in wild-type rats, the level of IL-13 and IL-33 in the model group was significantly higher than that of the control and tranilast treatment groups. In addition, IL-13 and IL-33 levels of the model group in MC-deficient rats (WsRC Ws/Ws, Ws/Ws) were significantly lower than those of wild-type rats. IL-13 is an important effector for allergic inflammation, which is produced by several kinds of cell types including MCs [35,36]. IL-33 expression is specifically enhanced in the inflamed mucosa of ulcerative colitis [37] and MCs produce IL-13 only when challenged by certain cytokines such as IL-33 and IL-3 [35,37,38]. Furthermore, IL-33 could induce the production of cytokines IL-6 by MCs [39]. Thus, the changes in IL-6, IL-13 and IL-33 levels were due to MCs.

Mast cells have been regarded as key effector cells in IgE-associated allergic disorders and hypersensitivity [40]. After challenge with OXA, the IgE level of the wild-type model group was significantly higher than that of MC-deficient rats. IL-33 was associated with pathological changes of gut mucosa and also induces IgE-independent production of IL-13 in MCs [41,42]. B cells can produce IgE and further mediate colitis in mice [43]. This suggests that tranilast might inhibit the secretion of IgE in MC-mediated OXA-induced colitis. Therefore, we may connect this result with IL-33 inducing the IgE-independent production of IL-13, as discussed above.

Elevated levels of colon homogenate immunoglobulin (IgA, IgG) in the wild-type
model group showed that there was a humoral immune response after challenge with OXA and that they were secreted by B cells. However, treatment with tranilast may down-regulate the IgA and IgE levels, as with MC-deficient rats. Whether tranilast has the effect of inhibiting B cell immune response needs to be studied further.

The discrepancy of cytokine and immunoglobulin secretion is most likely due to their difference in MCs. Cytokine levels were higher in wild-type models than in the MC-deficient model because of OXA-induced MC degranulation; thus, the symptoms were much more severe in wild-type models. OXA could cause the high secretion of IgE and then trigger the IgE-mediated MC degranulation. Tranilast may inhibit MC degranulation and ameliorate the severity of colitis; this may be due to its effects on inhibiting the secretion of IgE. Tranilast plays a role upstream of MCs and the key factors are IgE and IL-33.

By using wild type (WsRC +/+, +/+), and MC-deficient rats (WsRC Ws/Ws, Ws/Ws), we demonstrated the key role of MCs in OXA-induced UC. We can confidently conclude that a MC membrane stabiliser may alleviate OXA-induced colitis, and that this effect may be mediated by Th2 cytokine. Thus, we provide a theoretical basis for the clinical treatment of colitis. Further research is needed to explore the specific mechanism of the role played by tranilast in OXA-induced colitis.

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FIGURE LEGENDS

**Fig. 1** Induction of colitis (groups and treatments). Animals were sensitised on day 1 and challenged on day 8, then treated with either saline or tranilast for 7 days. Animals were divided into 8 groups, wild-type (negative group, model group, tranilast control group, and two tranilast treatment groups (30 mg/kg or 60 mg/kg)) and mast cell-deficient rats (negative control group, model group and 60 mg/kg/day tranilast treatment group)

AOO, acetone/olive oil; OXA, oxazolone.

**Fig. 2** The disease activity index score and myeloperoxidase activity of rats. (A) The body weight of wild-type and mast cell-deficient rats. (B) Disease activity index score of rats and the comparison between two model groups. (C) Myeloperoxidase level of wild-type, mast cell-deficient rats and a comparison of the two models. (n=7, *P* ≤ 0.05, **P* ≤ 0.01).

MPO, myeloperoxidase; OXA, oxazolone.

**Fig. 3** Effects of tranilast on colon organ coefficient and colon length of rats. (A) Organ coefficient of wild-type rats. (B) Colon length of wild-type rats. (C) Organ coefficient of mast cell-deficient rats. (D) Colon length of mast cell-deficient rats. (E) Comparison of colon organ coefficient between the two types of rats. (F) Comparison of colon length between the two types of rats. *P* ≤ 0.05 vs model group, **P* ≤ 0.01 vs model group.

**Fig. 4** Histological assessment of colitis and the effects of tranilast on colon
histology (H&E). (A) Wild-type control group. (B) Wild-type tranilast control group (C) Wild-type model group. (D) Wild-type tranilast treatment group (30 mg/kg/day). (E) Wild-type tranilast treatment group (60 mg/kg/day). (F) Mast cell-deficient control group. (G) Mast cell-deficient model group. (H) Mast cell-deficient tranilast treatment group (60 mg/kg/day). Data are representative of three independent experiments (original magnification, 200×).

**Fig. 5** The level of cytokines in colon tissue homogenates of wild-type rats, mast cell-deficient rats and a comparison between the two model groups. (A) The level of interleukin-6 in colon tissue homogenates. (B) The level of interleukin-13 in colon tissue homogenates. (C) The level of interleukin-33 in colon tissue homogenates. Data are representative of three independent experiments. (n=7, *$P < 0.05$, **$P < 0.01$).

IL6, interleukin-6; IL13, interleukin-13; IL33, interleukin-33.

**Fig. 6** The level of immunoglobulins in colon tissue homogenates of wild-type, mast cell-deficient rats and a comparison between the two model groups. (A) The level of immunoglobulin A in colon tissue homogenates. (B) The level of immunoglobulin E in colon tissue homogenates. (C) The level of immunoglobulin G in colon tissue homogenates. Data are representative of three independent experiments. n=7, *$P < 0.05$, **$P < 0.01$.

IgA, immunoglobulin A; IgE, immunoglobulin E; IgG, immunoglobulin G.
Figure [1]

Wild Rats

A
Negative Control group

Day0
Dehair

AOO

Day1

Ethanol

Saline

Day8

Day14

B
Tranilast Control group

Day0
Dehair

AOO

Day1

Tranilast

Tranilast

Day8

Day14

C
Model group

Day0
Dehair

OXA

OXA

Saline

Day8

Day14

D
Tranilast Treatment group

Day0
Dehair

OXA

OXA+Tranilast

Tranilast

Day8

Day14

Mast Cells Deficient Rats

E
Negative Control group

Day0
Dehair

AOO

Day1

Ethanol

Saline

Day8

Day14

F
Model group

Day0
Dehair

OXA

OXA+Ethanol

Saline

Day8

Day14

G
Tranilast Treatment group

Day0
Dehair

OXA

OXA+Tranilast

Tranilast

Day8

Day14
Figure [2]
Figure [3]