Association between gastrointestinal motility and macrophage/mast cell distribution in mice during the healing stage after DSS-induced colitis

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Abstract. Irritable bowel syndrome (IBS) frequently occurs after infectious colitis or inflammatory bowel disease in patients with complete remission. This suggests that post-inflammation-associated factors may serve a role in the pathophysiology of IBS; however, the mechanism responsible remains unclear. In the present study, the involvement of macrophages and mast cells in alteration of gastrointestinal (GI) motility was investigated in mice in the remission stage after acute colitis. C57BL/6 mice were administered 2% dextran sulfate sodium in drinking water for 5 days and their intestinal tissues were investigated at intervals for up to 24 weeks. Expression of the mannose receptor (MR) and tryptase was examined by immunohistochemistry, and the GI transit time (GITT) was measured by administration of carmine red solution. A minimal degree of inflammatory cell infiltration persisted in the colon and also the small intestine of mice in remission after colitis and the GITT was significantly shorter. The number of muscularis MR-positive macrophages was significantly increased in the small intestine of mice in remission after colitis and negatively correlated with GITT. Furthermore, results indicated that the number of muscularis tryptase-positive mast cells was significantly increased throughout the intestine of mice during the healing process after colitis and was positively correlated with GITT. The present findings suggested an increased number of macrophages and/or mast cells in the intestinal muscular layer may be associated with the pathophysiology of GI dysmotility after colitis.

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Abbreviations: GI, gastrointestinal; IBS, irritable bowel syndrome; DSS, dextran sulfate sodium; MR, mannose receptor; GITT, gastrointestinal transit time; FGID, functional gastrointestinal disorder; IBD, inflammatory bowel disease; ENS, enteric nerve system

Key words: colitis, mast cell, macrophage, gastrointestinal motility, irritable bowel syndrome, dextran sulfate sodium, functional gastrointestinal disorder

Introduction

Irritable bowel syndrome (IBS) is a type of functional gastrointestinal disorder (FGID) characterized by symptoms such as abdominal pain or discomfort and stool irregularities, unassociated with metabolic or organic abnormalities (1,2). A number of factors are involved in the pathophysiology of IBS, such as visceral sensitivity, gastrointestinal (GI) motility, brain-gut interaction and psychosocial stress (1,2). Interestingly, IBS occurs frequently in patients recovering from infectious colitis, (3,4) and we have reported that IBS-like symptoms are often observed in patients with inflammatory bowel disease (IBD) even after the bowel inflammation has been eliminated (5). In such patients, despite macroscopic healing of the intestinal mucosa, IBS-like symptoms persist. Although it has not been fully clarified how IBS symptoms are manifested after infectious colitis or during remission after IBD, it is tempting to speculate that a minimal degree of inflammation and associated gut immune activation may play a pathophysiologic role (3,4,6,7). Among immune cells, mast cell is highlighted in the pathophysiology of IBS since the number of mast cells is increased in the colonic tissues in patients with IBS and moreover correlated with the severity of their clinical symptoms (8,9). In addition, recent evidences have revealed that macrophages play pivotal roles in GI motility via acting on myenteric neural cells, (10-13) and indeed, the infiltration of macrophages in the colonic tissues may be enhanced in IBS patients (9,14). Accordingly, in order to clarify the pathophysiologic roles of immune cells, the investigation of mast cells and macrophages in experimental IBS model appears to be important.

Dextran sulfate sodium (DSS)-induced colitis is an animal model used widely to investigate the pathophysiology of various types of human colitis (15). It has been shown that mice treated with DSS for 5-7 days develop severe acute colitis, and then undergo healing of the damaged colonic tissue. However, the pathophysiology and mucosal immune alteration after remission has not been fully studied in this

animal model. Then, to examine whether the mice in remission after DSS-induced colitis are useful as a model for IBS after acute colitis, we have observed those mice in a time dependent manner. Subsequently, this animal model showed significant alterations of GI motility and immune cell infiltration in the GI tract, being possibly resemble to the subjects with post-inflammatory IBS. In the present study, we therefore investigated the involvement of immune cells, focusing mast cell and macrophage that are highlighted in IBS studies, and analyzed its relation to the alteration of GI motility in mice in remission after acute colitis.

Materials and methods

Animal model. C57BL/6 mice (8-week-old females) were used in this study. All the mice were maintained in cages on a 12 h light/dark cycle under specific pathogen-free conditions and allowed free access to food and water. The mice were administered 2% DSS (molecular weight 36,000-50,000; ICN Biomedicals Inc, Aorano, OH, USA) in drinking water for five days and sacrificed at various time points thereafter. Their GI tissues were removed, cut open along the longitudinal axis, rinsed with saline, and fixed in neutral aqueous phosphate-buffered 10% formalin for histological examinations. This animal experiment was carried out with the approval of the Animal Use and Care Committee at Hyogo College of Medicine.

Histological evaluation. The fixed tissues were embedded in paraffin, cut perpendicularly to the surface at a thickness of 4 μm, and stained with hematoxylin and eosin. The degree of inflammatory cell infiltration in the small intestine and colon was scored on a scale of 0 to 3 as follows: 0, normal; 1, inflammatory cell infiltration into the mucosal layer; 2, up to the submucosal layer; 3, beyond the submucosal layer. The scores were evaluated for all of the slides from the small intestine and colon of each mouse, and the results were averaged.

Immunohistochemistry. Immunohistochemical staining for the mannose receptor (MR; a marker of M2-polarized macrophages) and tryptase (a marker of mast cells) was performed using an Envision kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) as described previously (16). The primary antibodies applied were: anti-MR (dilution 1:5,000; cat. no. ab64693; Abcam, Cambridge, UK) and anti-tryptase antibody (dilution 1:4,000; cat. no. ab2378; Abcam). The sections were deparaffinized, rehydrated and placed in PBS for MR staining or placed in 1X Dako REAL Target Retrieval Solution (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) followed by microwave treatment for tryptase staining. Then, to quench endogenous peroxidase activity, the sections were pretreated with 0.3% H_2O_2 in methanol for 25 min at room temperature. The sections were washed in PBS and incubated with the primary antibodies at 4˚C overnight. Thereafter, the slides were incubated with horseradish peroxidase-conjugated secondary antibodies (ready-to-use; cat. nos. K4001 or K4003; Dako; Agilent Technologies, Inc.) at room temperature for 30 min, visualized using 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 3 min, and counterstained with Mayer's hematoxylin. Under a light microscope (Olympus CX41; Olympus Corporation, Tokyo, Japan), MR- and tryptase-positive inflammatory cells were counted in a 200-μm stretch of the entire length of well-oriented tissue sections in at least 4 randomly selected fields from the small intestine to colon of each mouse, and the average was calculated.

GI transit time (GITT). GITT was measured as described previously (17). In brief, the mice orally received 0.3 ml of 0.5% methylcellulose solution including 6% carmine red (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were then allowed access to food and water *ad libitum* until the first red fecal pellet appeared. GITT was determined as the time period between oral gavage and the appearance of the first red fecal pellet.

Statistical analysis. All values were expressed as the mean ± SEM. Significance of differences between two animal groups was analyzed by Mann-Whitney *U*-test. Correlations among GITT, MR expression and tryptase expression were assessed by linear regression analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inflammatory cell infiltration in the intestinal tract of mice after DSS-induced colitis. As shown in Fig. 1, inflammatory cell infiltration in the intestinal tract of mice after DSS-induced colitis was evaluated. Strong infiltration of inflammatory cells was observed in not only the mucosal but also the muscular layer in the colonic tissues of mice in the acute phase of DSS-induced colitis (Fig. 1A). The severity of colonic inflammatory cell infiltration peaked at 2-4 weeks after DSS induction (Fig. 1E). Thereafter, it gradually declined but remained at a very weak level in the resolving phase (Fig. 1E). Thus, although most parts of the colonic mucosa appeared macoscopically normal, a minimal degree of inflammatory cell infiltration was still evident in some parts of colonic tissues in the resolving phase (Fig. 1B).

In the small intestine, weak infiltration of inflammatory cells was microscopically evident in the acute phase of DSS-induced colitis (Fig. 1C), although the macroscopic appearance was normal. Moreover, it was noteworthy that a minimal degree of inflammatory cell infiltration was sustained in some parts of the small intestine in the resolution phase (Fig. 1D and F).

GITT in mice after DSS-induced colitis. In normal mice, GITT was prolonged with increasing age (from 8 to 32 weeks) (Fig. 2). At two weeks after the start of the experiment, GITT was shorter in mice with DSS-induced colitis than in normal controls, although the difference was not significant. On the other hand, at four weeks later, GITT was significantly longer in mice with DSS-induced colitis than in normal controls. In contrast, however, GITT again became significantly shorter in mice in the resolution phase (at 24 weeks) of DSS colitis.

Expression of MR and tryptase in mice after DSS-induced colitis. We next examined the localization and population of MR-positive macrophages and tryptase-positive mast cells in the small intestine and colon of mice after DSS-induced colitis. In normal mice, MR-positive macrophages were scattered in

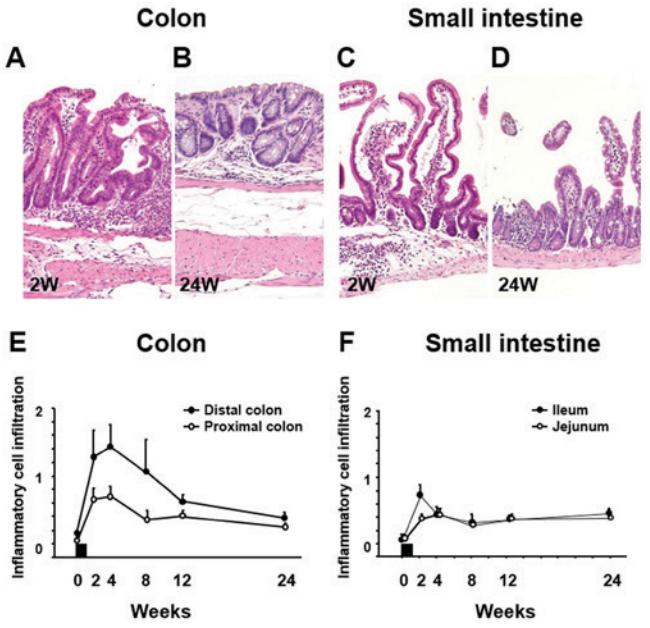

Figure 1. (A-F) Changes in inflammatory cell infiltration in the intestinal tract of mice after DSS-induced colitis. Colonic and small-intestinal tissues at 2 (A and C, respectively) and 24 weeks (B and D, respectively) after DSS induction (original magnification x200). Serial scores of inflammatory cell infiltration in the colon (E) and small intestine (F). Black bars indicate DSS treatment. DSS, dextran sulfate sodium.

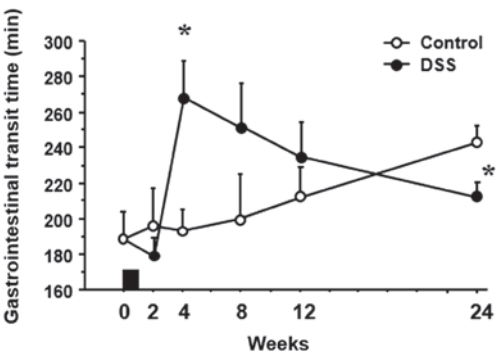

Figure 2. Changes in gastrointestinal transit time in mice without or with DSS-induced colitis. All the results are expressed as the mean ± SE. *P<0.05 vs. control at the same time point. Black bar indicates DSS treatment. DSS, dextran sulfate sodium.

both the mucosal and muscular layers of the colon (Fig. 3). In the distal colon, the number of MR-positive macrophages in the mucosal layer increased with age, whereas it remained very small in the muscular layer (Fig. 4). In mice with DSS-colitis, the number of MR-positive macrophages was significantly increased in the muscular layer throughout the small intestine and colon at 2 weeks after DSS induction (Fig. 4). Furthermore, it was significantly increased in the small-intestinal muscular layer at 24 weeks after DSS induction, and similar findings were observed in the muscular layer of the colon (Figs. 3 and 4).

In normal mice, tryptase expression was detected in the immune cells in the lamina propria but was hardly evident in the muscular layer throughout the small intestine and colon (Fig. 5). In those mice, the number of tryptase-positive cells in the mucosal layer increased with age (Fig. 6). In mice with DSS-colitis, the number of tryptase-positive cells was

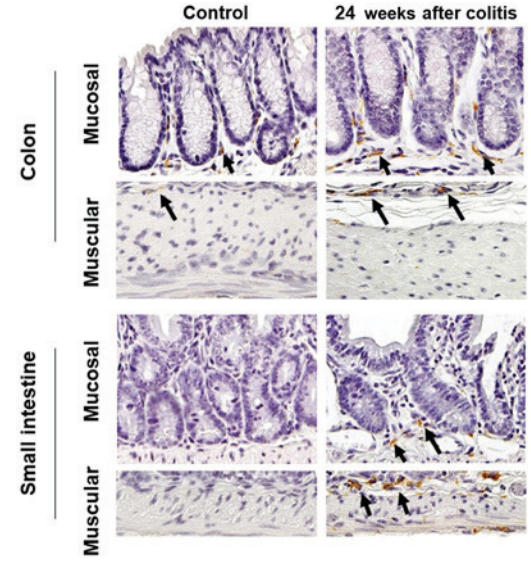

Figure 3. Expression of MR in the intestinal tract of untreated mice or mice in remission after DSS-induced colitis. Representative immunostaining of MR in the small intestine and colon of untreated control mice and mice in remission after DSS-induced colitis at 24 weeks after the start of the experiment (original magnification x400). Arrows indicate MR-positive cells. MR, mannose receptor; DSS, dextran sulfate sodium.

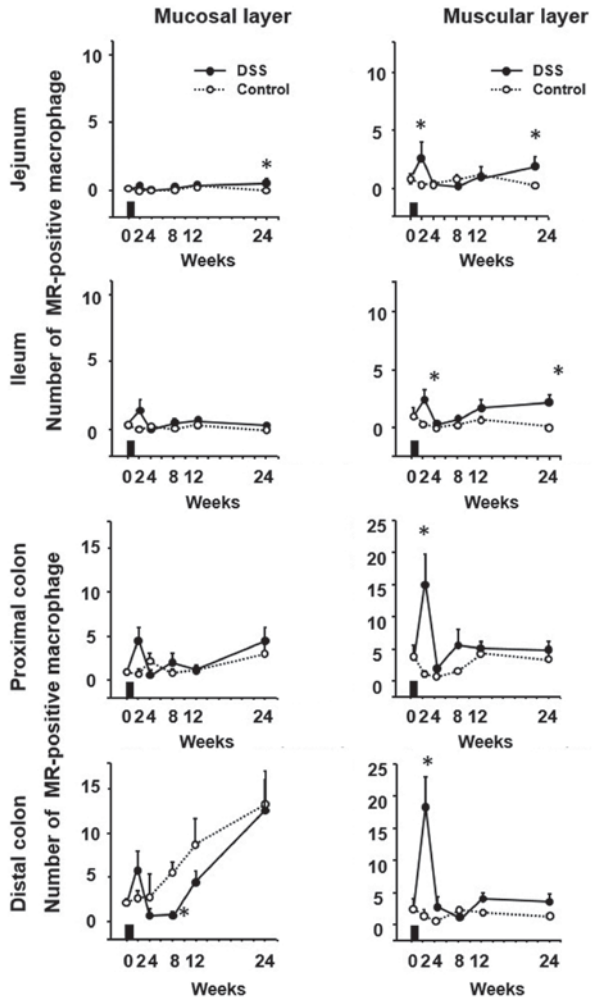

Figure 4. Changes in the number of MR-positive cells in the intestinal tract of mice without or with DSS-induced colitis. Black bars indicate DSS treatment. Results are expressed as the mean ± SE. *P<0.05 vs. Control at same time point. MR, mannose receptor; DSS, dextran sulfate sodium.

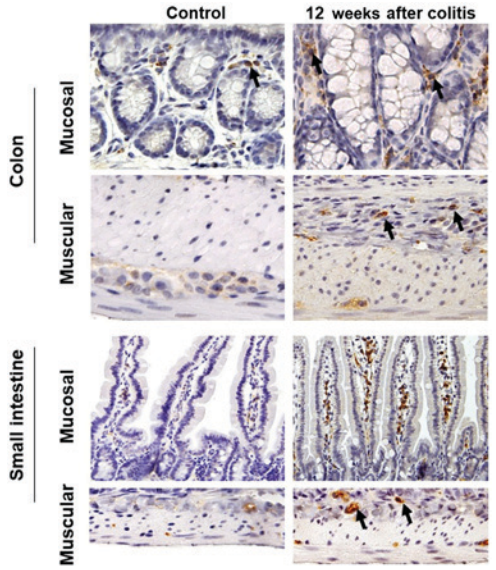

Figure 5. Expression of tryptase in the intestinal tract of untreated mice or mice in remission after DSS-induced colitis. Representative immunostaining of tryptase in the small intestine and colon of untreated control mice and mice in remission after DSS-induced colitis at 24 weeks after the start of the experiment. Colon tissues, original magnification of x400; small intestinal mucosa, original magnification of x200; and small intestinal muscular layer, original magnification of x400. Arrows indicate tryptase-positive cells. DSS, dextran sulfate sodium.

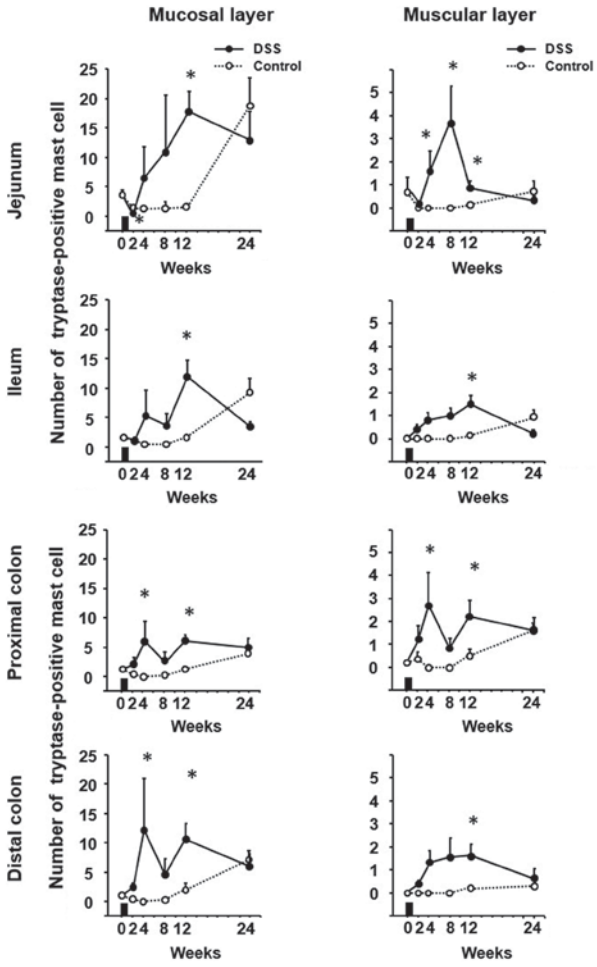

Figure 6. Number of tryptase-positive cells in the intestinal tract of mice without or with DSS-induced colitis. Black bars indicate DSS treatment. All the results are expressed as the mean ± SE. *P<0.05 vs. Control at same time point. DSS, dextran sulfate sodium.

significantly greater in not only the mucosal but also the muscular layer in the small intestine or colon between 4 and 12 weeks after DSS induction (Figs. 5 and 6).

GITT and its association with MR or tryptase expression. The correlation between GITT and MR or tryptase expression was evaluated in the experimental mice (DSS-treated and untreated) by linear regression analysis. GITT was negatively correlated with the number of MR-positive cells in the muscular layer of the jejunum (Fig. 7). In terms of tryptase expression, GITT was positively correlated with the number of tryptase-positive cells in the muscular layer of the jejunum and colon (Fig. 8).

Discussion

FGIDs frequently occur in patients after infectious colitis (3,4) although endoscopic examinations reveal no apparent abnormality in the enteric lumen, suggesting that some form of cryptic molecular alteration plays a pathophysiologic role. In this context, it is tempting to speculate that post-inflammation-associated factors are central to the mechanism of FGID development after inflammation (3,4,6,7). Although DSS-induced colitis is a well-established animal model, the associated GI motility has not been examined intensively. In the present study, we investigated GITT in mice with DSS-induced colitis at various time intervals. As shown in Fig. 1, GITT was shortened in the acute phase of DSS-induced colitis, reflecting the fact that diarrhea occurs during this period in this model (15). On the other hand, GITT was conversely prolonged during the healing process from 4 to 12 weeks after DSS induction. Histopathologic examination using microscopy demonstrated mild infiltration of inflammatory cells, implying that alteration of the immune system may affect GI motility. Furthermore, we found that a minimal degree of inflammatory cell infiltration remained in the intestine in the resolution phase after 24 weeks and that GITT became significantly shortened again at this time point. These findings suggest that alteration of the immune system certainly affects GI motility, although further studies of the infiltrating immune cells and the mediators they produce would be warranted.

In the present study, we investigated mice after induction of DSS-colitis focusing on macrophages and mast cells as these have received attention as key players in FGIDs after inflammation (8,10). MR-positive macrophages and tryptase-positive mast cells were observed in not only the mucosal but also the muscular layer of the intestinal tract. Interestingly, at 2 and 24 weeks after DSS-colitis induction, the number of macrophages was increased in the muscular layer of the intestinal tract, and GITT was simultaneously shortened. Moreover, from 4 to 12 weeks after DSS-colitis induction, the number of mast cells was increased, and GITT was prolonged. These finding strongly suggest that macrophages are involved in the acceleration of GI motility whereas mast cells are associated with the suppression of GI motility. Indeed, we showed that GITT was negatively correlated with the number of muscule-associated macrophages and positively correlated with that of mast cells. Although it is difficult to explain how GI motility is affected by these infiltrating immune cells, some alteration in their

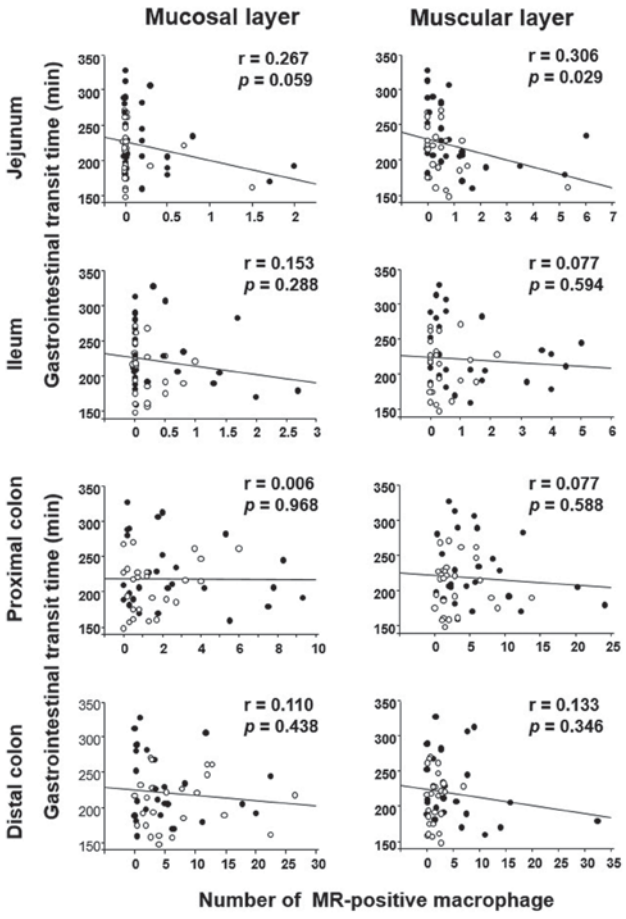

Figure 7. Correlation between GITT and MR expression in the intestinal tract of experimental mice. Results from DSS-treated mice are presented as black circles, whereas results from untreated control are presented as white circles. P-values were obtained by linear regression analysis comparing GITT with the number of MR-positive macrophages in the mucosal/muscular layer of the intestine. GITT, gastrointestinal transit time; MR, mannose receptor; DSS, dextran sulfate sodium.

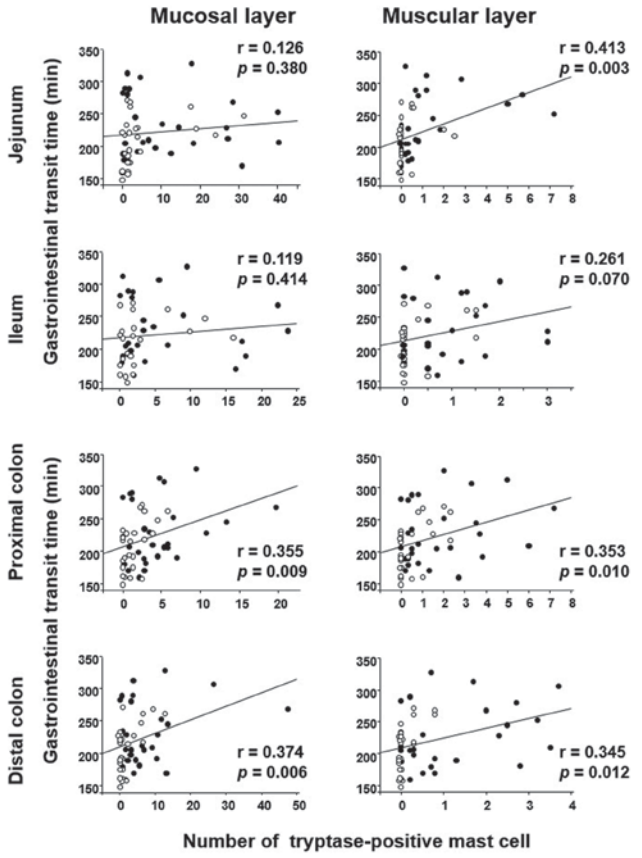

Figure 8. Correlation between GITT and tryptase expression in the intestinal tract of experimental mice. Results from DSS-treated mice are presented as black circles, whereas results from untreated control are presented as white circles. P-values were obtained by linear regression analysis comparing GITT with the number of tryptase-positive mast cells in the mucosal/muscular layer of the intestine. GITT, gastrointestinal transit time; DSS, dextran sulfate sodium.

profile may be associated with a change in GI motility during the healing process after acute colitis.

With regard to the involvement of macrophages and mast cells in post-inflammation GI dysmotility, interaction between the enteric nerve system (ENS) and smooth muscle (18,19) is greatly affected by immune cell-producing mediators such as cytokines, chemokines, neuropeptides or proteases (11,20,21). Indeed, mast cells are able to release histamine, serotonin, tryptase and prostaglandins, and those mediators are possible to act on their specific receptors of myenteric neural cells, leading to altered motor function (8). Similar to mast cells, macrophages are likely to affect ENS and smooth muscles with various mediators (10-13). Interestingly, macrophages have been recently classified into the M1 and M2 type that mainly produce Th1 and Th2 cytokines, respectively (22,23). In detail, M1 macrophages release proinflammatory cytokines such as TNFα, IL-1β and IL-6 and their stimulation acts on ENS and smooth muscle, resulting in the suppression of GI motility (10,12). On the other hand, M2 macrophages may suppress the expression of proinflammatory cytokines in M1 macrophage by release anti-inflammatory cytokines including IL-10, (10,24) possibly resulting in the acceleration of GI motility. In this context, we showed in the present study that M2 macrophage were increased in the muscular layer of the intestinal tract after remission of colitis, supporting the possibility that these may be involved in the acceleration of GI motility observed during the period of colitis remission. On the other hand, not only M2 macrophages but also mast cells are known to infiltrate into the muscle layer in the intestine and may accelerate GI motility through stimulation with Th2 cytokines, histamine or serotonin (8). Therefore, we expected that the increase of muscle-associated mast cells would result in acceleration of GI motility. Conversely, however, the data we obtained indicated the opposite situation, i.e., that muscle-associated mast cells might be involved in delayed GI motility. In humans, mast cells in the colonic mucosa are increased in patients with not only diarrhea but also constipation predominant IBS, (25,26) suggesting that these mast cells may not be a factor in the modification of intestinal movements. Although this study did not obtain any evidence for a mechanical role of muscle-associated mast cells in intestinal motility, the present animal model may be useful for investigating the role of mast cells in post-inflammatory FGIDs.

In summary, we have shown that GI dysmotility occurs in mice that are in remission after colitis, and that histopathologically, there is a significant increase of macrophages and mast cells in the muscular layer of the intestinal tract. Moreover, we have demonstrated that muscle-associated macrophages may be involved in the acceleration of GI motility, whereas

muscle-associated mast cells may be associated with its delay. Together, our findings suggest that the increased number of macrophages and/or mast cells in the intestinal muscular layer plays a pathophysiologic role in post-colitis GI dysmotility.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HF conceived a designed research and interpreted results of experiments. TT, TO, JW and HM also contributed to the design of the study and the interpretation of experimental results. MK and HF performed experiments, analyzed data, prepared figures and drafted manuscript. MK, HF, TT, TO, JW and HM approved final version of manuscript. HF, TT, TO, JW and HM edited and revised manuscript.

Ethics approval and consent to participate

This study involved no human data or tissues. The animal experiments were carried out with the approval of the Animal Use and Care Committee at Hyogo College of Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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