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ORIGINAL PAPER

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Irsogladine maleate ameliorates inflammation and fibrosis in mice with chronic colitis induced by dextran sulfate sodium

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Abstract Intestinal fibrosis is a common and severe complication of inflammatory bowel disease (IBD), especially Crohn's disease (CD). To investigate the therapeutic approach to intestinal fibrosis, we have developed a mouse model of intestinal fibrosis by administering dextran sulfate sodium (DSS) and examining the effects of irsogladine maleate (IM) [2,4-diamino-6-(2,5-dichlorophenyl)-*s*-triazine maleate], which has been widely used as an antiulcer drug for gastric mucosa in Japan, on DDS-induced chronic colitis. In this experimental colitis lesion, several pathognomonic changes were found: increased deposition of collagen, increased number of profibrogenic mesenchymal cells such as fibroblasts (vimentin$^+$, α-SMA$^-$) and myofibroblasts (vimentin$^+$, α-SMA$^+$) in both mucosa and submucosa of the colon with infiltrating inflammatory cells, and increased mRNA expressions of collagen type I, transforming growth factor (TGF)-β, matrix metalloproteinase (MMP)-2, and tissue inhibitor of matrix metalloproteinase (TIMP)-1. When IM was administered intrarectally to this colitis, all these pathological changes were significantly decreased or suppressed, suggesting a potential adjunctive therapy for intestinal fibrosis. IM could consequently reduce fibrosis in DSS colitis by direct or indirect effect on profibrogenic factors or fibroblasts. Therefore, the precise effect of IM on intestinal fibrosis should be investigated further.

Key words Irsogladine maleate · Inflammatory bowel disease · Crohn's disease · Fibrosis · Mesenchymal cells · TGF-β

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Introduction

Inflammatory bowel disease (IBD), which comprises ulcerative colitis (UC) and Crohn's disease (CD), is characterized by chronically relapsing inflammation of the bowel of unknown origin.[1] The induction of biologics such as infliximab, a chimeric immunoglobulin IgG1 monoclonal antibody to tumor necrosis factor, dramatically improved treatment of patients with IBD. These agents are beneficial and are established as a standard therapy for IBD; however, they have limited response rate and serious side effects.[2] In addition, about one-third of patients with IBD show an intractable clinical course. A frequent complication of CD is fibrosis, leading finally to intestinal obstruction. Fibrosis in CD is the result of transmural chronic inflammation with repeated episodes of immune-mediated damage and repair.[3,4] About 75% of all patients with CD have to undergo surgery at least once during the course of their disease,[5] and these obstructions recur in more than 45% of the patients.[5] In other diseases including radiation colitis, chronic ischemic colitis, or cystic fibrosis, recurrent mucosal damage with subsequent chronic inflammation may also lead to colonic wall thickening, intestinal fibrosis, and stricture formation.[3,4,6] Conventional treatments of intestinal fibrosis and strictures such as surgery and

endoscopic balloon dilatation are insufficient and unsatisfactory because postoperative reoccurrence of stenosis is highly prospective and requires a repeated operation. Therefore, new therapeutic approaches are required for the treatment of intestinal fibrosis.

Irsogladine maleate (IM) [2,4-diamino-6-(2,5-dichlorophenyl)-*s*-triazine maleate], an antiulcer drug widely used in Japan, is known to protect the gastric mucosa by enhancing the mucosal integrity of the stomach.[7,8] Studies with various models of gastric mucosal injury[9–12] revealed the mechanisms of its efficacy as activation of intercellular communication,[13] prevention of reduction of gastric mucosal blood flow,[9,14] an antiinflammatory effect,[15] and prevention of the loss of mucosal hydorophobicity[16] by increasing the intracellular levels of 3′,5′-cyclic adenosine monophosphate (cAMP) through inhibition of phosphodiesterase (PDE), especially type 4.[16] Additionally, IM treatment has been shown to ameliorate small intestinal lesions of rats induced by indomethacin,[17] and it also protects neutrophil migration and E-cadherin expression in gingival epithelium stimulated by periodontopathogenic bacteria.[18] However, it remains unknown if IM affords a prophylactic effect on intestinal fibrosis as observed in colonic lesions such as inflammatory bowel disease.

Because mechanistic studies of fibrosis are difficult to perform in humans, animal models have been developed to analyze the pathophysiological mechanism of intestinal fibrosis, as well as to make a platform for the development of new therapeutic approaches.[19–21] Therefore, developing ideal animal models of intestinal fibrosis is a prerequisite for the research. Melgar et al.[22] have reported that a single-cycle administration of dextran sodium sulfate (DSS) induced acute colitis leading to chronic colitis in C57BL/6 (B6) mice but not in BALB/c mice.[22] We have recently reported that in the chronic colitis model induced by a single-cycle administration of DSS, profibrogenic mesenchymal cells including fibroblasts and myofibroblasts play a key role in the intestinal fibrosis under profibrogenic milieu such as overexpression of transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α in the colon of mice.[23] Thus, the chronic DSS-induced colitis model could become a powerful and convenient tool for the development of new therapeutic approaches as well as to analyze the pathophysiology of intestinal fibrosis, especially with special relevance to these profibrogenic mesenchymal cells.[24,25]

In this study, we analyzed the therapeutic effect of rectal administration of IM on chronic fibrosing DSS-induced colitis and elucidated the mechanism as well as the involvement of profibrogenic mesenchymal cells for this effect.

Materials and methods

Animals and induction of colitis

Female C57BL/6(B6) mice (7–9 weeks old) were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and maintained in the Animal Center of Niigata University School of Medicine under specific pathogen-free conditions. To induce chronic fibrotic colitis, B6 mice were given 4% DSS (molecular weight, 36–50 kDa; Wako, Osaka, Japan) orally in distilled water ad libitum for 5 days (from day 0 to 5). On day 5, they were switched to regular drinking water. Normal control mice received regular drinking water throughout the experiment. All animal experiments were performed according to the *Guide for Animal Experimentation of Niigata University School of Medicine*.

Drug administration

IM suspension (supplied by Nippon Shinyaku, Kyoto, Japan) was prepared with 1.5% carboxymethyl cellulose (Dai-Ichi Chemical Industries, Tokyo, Japan) in water. These enema suspensions were administered rectally to 3 cm in a volume of 1 ml/kg body weight at days 5, 7, 9, 11, 13, 15, and 17. We used four groups of mice for the experiment: (i) DSS colitis mice treated with intrarectal administration of IM; (ii) DSS colitis mice treated with intrarectal administration of vehicle; (iii) normal mice treated with intrarectal administration of IM; and (iv) normal mice with intrarectal administration of vehicle.

Evaluation of colitis

Body weight loss (scores: 0, none; 1, 0–5%; 2, 6–10%; 3, 11–15%; 4, 16–20%; 5, 21–25%; 6, 26–30%), stool consistency (scores: 0, normal stools; 1, soft stools; 2, liquid stools), Hemoccult (Beckman Coulter, CA, USA) positivity, and presence of gross blood (scores: 0, negative fecal occult blood; 1, positive fecal occult blood; 2, visible rectal bleeding) were assessed daily for each mouse. The disease activity index (DAI) was calculated as a sum of the scores of the three parameters according to the scoring criteria as described above.[23,26]

Evaluation of histology

The entire colon (ten mice per group) was sampled and its length was immediately recorded. The entire colon was fixed in 4% formalin, embedded in paraffin, and 4-μm-thick transverse sections were stained with hematoxylin and eosin to address the degree of inflammation. For evaluating the degree of fibrosis, the sections were also stained with Masson's trichrome. The stained sections were then examined by light microscopy. We analyzed the distal colon tissue section located approximately 10 mm from the anal verge to calculate the mean number of infiltrating cells of five different points in the lamina propria of the colon in a high-power field (400×) under a microscope. The length of the whole colon wall, thickness of muscularis mucosa, and thickness of muscularis propria of each mouse were also calculated as a mean value of five different points.

Evaluation of extracellular matrix content in the colon

To measure the fibrosis area of the lamina propria and submucosal layer of the colon, we performed quantitative

digital morphometric analysis of extracellular matrix (ECM) for colonic sections with Masson's trichrome staining according to a protocol adapted from that described in detail by others.[25] In brief, five to ten randomly chosen high-power fields (HPF; 200× magnification) for each cross section were photographed with a digital camera equipped to an Olympus microscope system (Olympus, Tokyo, Japan). The color wavelengths of the copied image were transformed into digital readings using Image-Pro Plus Version 7.0 software (Media Cybernetics, Bethesda, MD, USA), allowing for quantification of the various color wavelength with pixels as the unit of measure. By using the original image for comparison, the color spectra were analyzed and those corresponding to ECM (blue wavelength) were quantified. The percentage of the ECM tissues in lamina propria and submucosal layers were calculated by dividing the total pixel area of the ECM by the total pixel area corresponding to the total colonic tissue in the field of view. At a minimum, the colons of three to five mice were analyzed in each group.

Staining for fibrogenic mesenchymal cells with multicolor immunofluorescence

To evaluate the expression of vimentin and α-smooth muscle actin (α-SMA) in the colon, formalin-fixed and paraffin-embedded colon sections were deparaffinized with xylene and ethanol, and then incubated with goat polyclonal anti-vimentin antibody (sc-7557; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit monoclonal anti-αSMA antibody (E184; Epitomics, Burlingame, CA, USA), respectively. To evaluate the number of proliferating crypt epithelial cells of the colon, the sections were then incubated with Alexa Fluor 647-labeled mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (PC10; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Alexa Fluor 555-labeled donkey anti-goat IgG and Alexa Fluor 488-labeled chicken anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) were next reacted with DAPI nuclear counterstain (mounting medium with DAPI; Vector Laboratories, Burlingame, CA, USA). The sections were observed by immunofluorescent microscopy (Axiovision; Carl Zeiss, Jena).

Quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from colon specimens with Trizol (Invitrogen, Carlsbad, CA, USA) according to the standard protocol, then reverse transcribed. Thereafter, cDNA was amplified using the ABI 7700 sequence-detector system (Applied Biosystems, Foster City, CA, USA) with a set of primers and probes corresponding to TNF-α, interferon (IFN)-γ, interleukin (IL)-10, TGF-β, collagen type I, matrix metalloproteinase (MMP)-2, tissue inhibitor of matrix metalloproteinase (TIMP)-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously.[23]

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis among groups was determined by unpaired Student's *t* test or nonparametric Mann–Whitney test. Differences were considered significant at $P < 0.05$.

Results

Rectal administration of IM attenuated mice from chronic DSS colitis

Effects of IM on colon injury

To determine the effect of IM on colon injury, we administered IM rectally to mice with DSS-induced colitis. Mice with DSS-induced colitis showed 10% body weight loss, diarrhea, and gross anal bleeding with an increased DAI score that peaked at day 8 (Fig. 1a). The clinical symptoms were ameliorated thereafter, with decreasing DAI. At around day 14, visible fecal blood could no longer be detected, although stool consistency remained loose. In contrast, rectal administration of IM suppressed decrease of body weight less than 5%, and clearly ameliorated diarrhea and anal bleeding, shown by significant decrease of DAI score (Fig. 1a). Rectal administration of vehicle or IM had no effect on DAI score of normal mice (Fig. 1a).

Effects of IM on colon length, colon weight, and the ratio of colon weight to colon length

Shortening of colon length and increase of colon weight reflect the extent of colon damage of chronic DSS-induced colitis, and the ratio of colon weight to colon length is well correlated with thickening of the colon wall.[23] Rectal administration of IM did not show any significant change for decrease of colon length, increase of colon weight, or ratio of colon weight to colon length of mice with DSS colitis (Fig. 1b) at day 19. Rectal administration of vehicle or IM had no effects on these parameters of normal mice (Fig. 1b).

Rectal administration of IM ameliorated histological findings of chronic DSS-induced colitis

As we reported recently, the colon of DSS-induced colitis showed minimal change at day 3.[23] The histological changes were prominent at day 6 in acute phase with mucosal ulceration and degeneration, decrease of goblet cells, inflammatory cellular infiltration, and submucosal edema. In contrast to the amelioration of clinical symptom after cessation of DSS, histological colitis progressed with increasing inflammatory cellular infiltration into mucosal and submucosal layers, at day 10 and thereafter in chronic phase.[22,23] The histological changes of chronic intestinal inflammation and fibrosis with thickening colonic wall width peaked at day 19 as previously reported[23]; therefore, we evaluated the effects

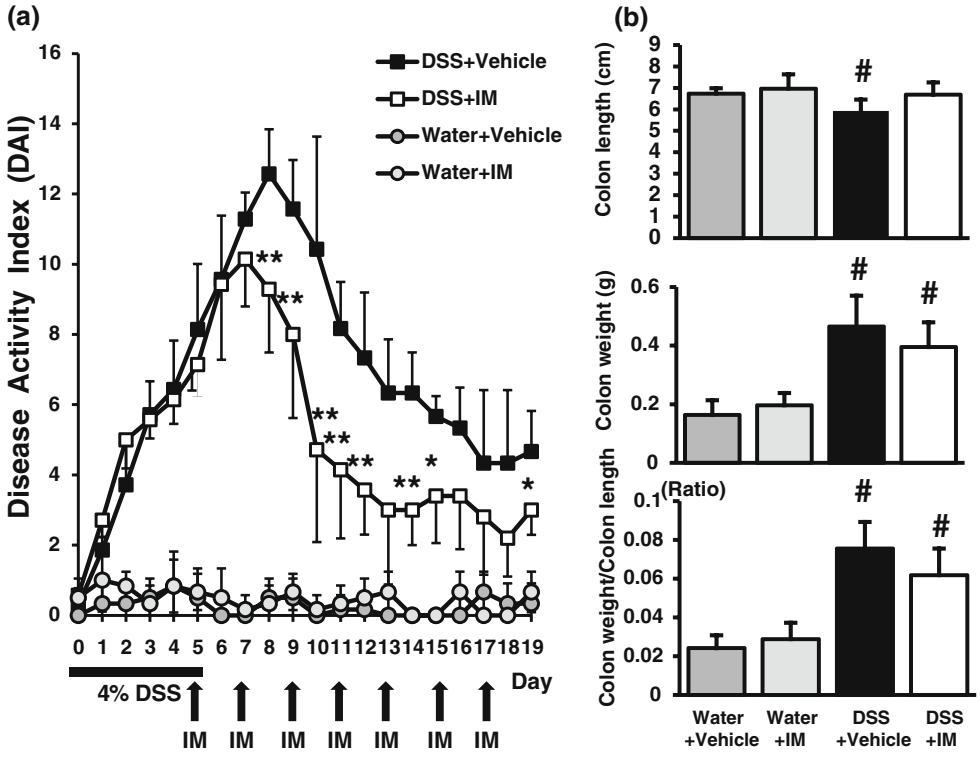


Fig. 1. **a** Irsogladine maleate (*IM*) enema ameliorated clinical symptoms of mice with dextran sulfate sodium (DSS)-induced chronic colitis with decreased disease activity index (*DAI*). C57BL/6 (B6) female mice were given *4% DSS* orally in distilled water ad libitum from day 0 to day 5; thereafter they received regular drinking water. IM was administered by enema on the days indicated by *arrows* labeled *IM*. **b** Colon length, colon weight, and colon weight/colon length of mice with DSS-induced chronic colitis. Data are expressed as means ± SD (n = 10 in each group). There was no statistical significant difference between parameters among groups

of IM at day 19. Rectal administration of IM significantly ameliorated these histological findings of DSS-induced colitis (Fig. 2c–f). An administration of vehicle or IM showed insignificant histological effects on the colon of normal mice (Fig. 2a,b,e–h). The number of mucus-containing cells was decreased among colonic crypt epithelial cells in chronic DSS colitis mice treated with vehicle enema when compared with that of normal mice with vehicle or IM enema (see Fig. 2). Interestingly, the numbers of mucus-containing cells were increased among colonic crypt epithelial cells in chronic DSS colitis mice treated with IM enema (Fig. 2). A thickening of colon wall was induced not only by these pathological changes in mucosal and submucosal layers but also by the increase in width of muscularis mucosa and muscularis propria compared with those of normal mice (Fig. 2a–d). IM treatment significantly decreased the width of both muscularis mucosa and muscularis propria of mice with chronic DSS colitis (Fig. 2g,h).

Rectal administration of IM ameliorated collagen deposition and mRNA expression for collagen 1 in chronic DSS colitis

As we reported recently, Masson's trichrome staining of the colon sections of chronic DSS-induced colitis clearly revealed gradual collagen deposition in the mucosal and submucosal layers (shown as blue-stained area), which peaked at day 19 (Fig. 3a–d).[23] In muscularis mucosa and muscularis propria, blue staining was also detected, and the intensity and area in these muscular layers increased, peaking at day 19. IM treatment apparently decreased the area of fibrosis in both mucosal and submucosal layers of the colon of chronic DSS-induced colitis. The treatment also decreased the intensity and area of blue staining in the muscular layers (Fig. 3c,d).

To measure the area of collagen deposition in mucosal and submucosal layers, we performed digital image analysis with the computer software Image-Pro Plus at day 19. Image analysis clearly revealed that fibrosis area was markedly increased in both muscular layers of the colon of mice with chronic DSS colitis, and IM treatment significantly reduced the area of fibrosis compared with those of DSS colitis (Fig. 3e).

Next, we analyzed mRNA expression levels for collagen 1 by real-time PCR. At day 19, mRNA expression levels for collagen 1 were significantly elevated in chronic DSS colitis compared with those of control mice (Fig. 3f). IM treatment significantly reduced mRNA expression levels for collagen 1 of mice with chronic DSS-induced colitis (Fig. 3f).

Effects of IM on mRNA expressions of cytokines, MMP, and TIMP by real-time polymerase chain reaction (RT-PCR) analysis

To reveal the immune response in the colon of mice with DSS-induced colitis, we investigated the mRNA expressions of several cytokines. At day 19 after induction of colitis, levels of expression of mRNA for proinflammatory cytokines such as TNF-α and IFN-γ were increased (Fig. 4a,b). Administration of IM clearly decreased the expression of mRNA for IFN-γ but not for TNF-α (Fig. 4a, b).

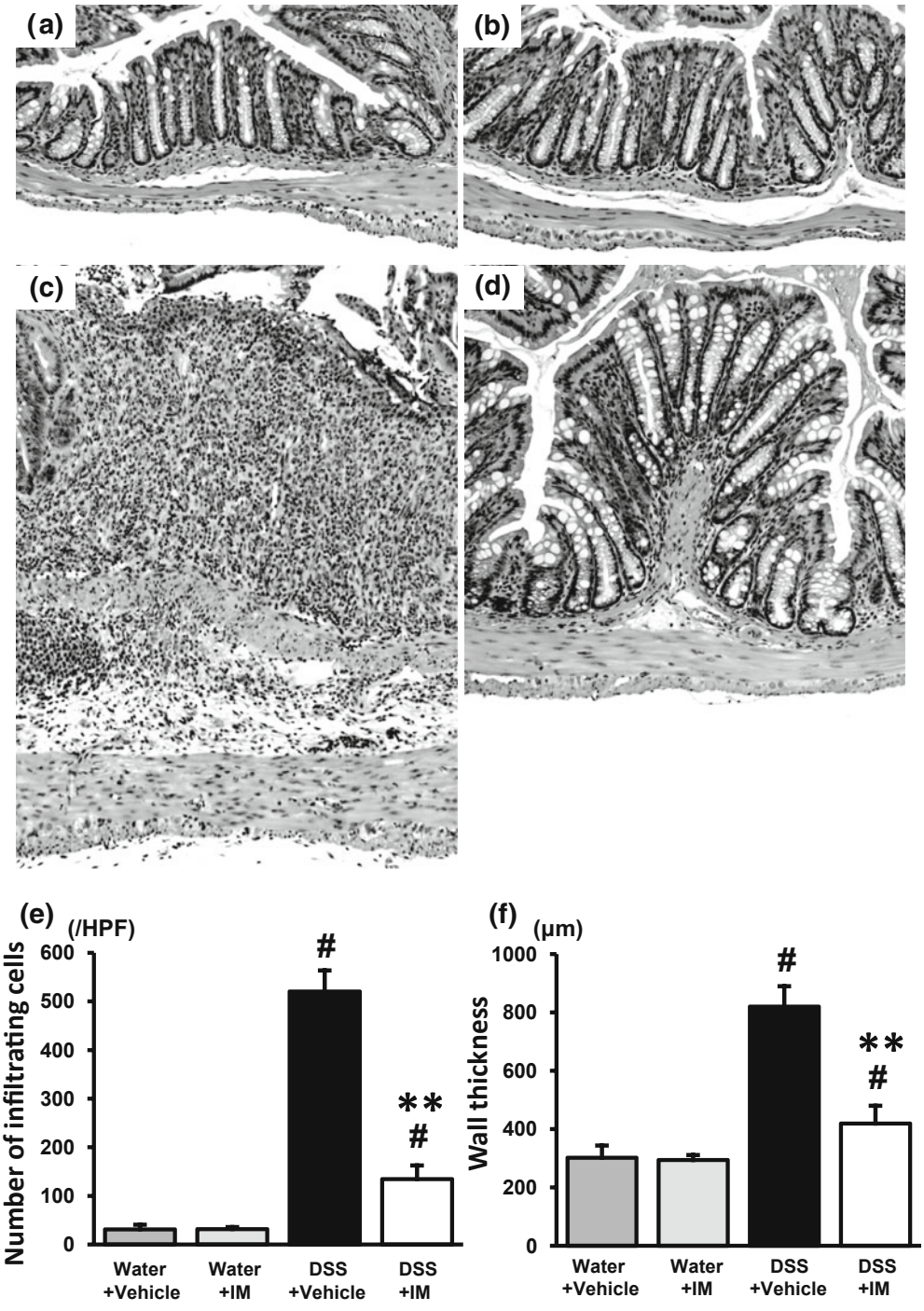


Fig. 2. Irsogladine maleate (IM) enema ameliorated histological findings in mice with dextran sulfate sodium (DSS)-induced colitis. Sections of the distal colon were stained with hematoxylin and eosin (H&E) at day 19 from a normal mouse treated with vehicle enema (**a**); a normal mouse treated with IM enema (**b**); a mouse with DSS-induced colitis treated with vehicle enema (**c**); and a mouse with DSS-induced colitis treated with IM enema (**d**). **e** Number of cells infiltrating colonic tissues in mice with DSS-induced colitis. *HPF*, high-power field. **f** Wall thickness of colon of mice with DSS-induced colitis. **g** Thickness of muscularis mucosae. **h** Thickness of muscularis propria. Data are expressed as means ± SD ($n = 5$ in each group). *HPF*, high-power field. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with normal mice; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with DSS-induced colitis mice treated with vehicle enema. **i** Number of goblet cells per crypt. Data are expressed as means ± SD ($n = 5$ in each group). $^{\#\#}P < 0.01$ compared with normal mice; $^{**}P < 0.01$ compared with DSS-induced colitis mice treated with vehicle enema. *DSS*, dextran sulfate sodium. **a–d** ×200

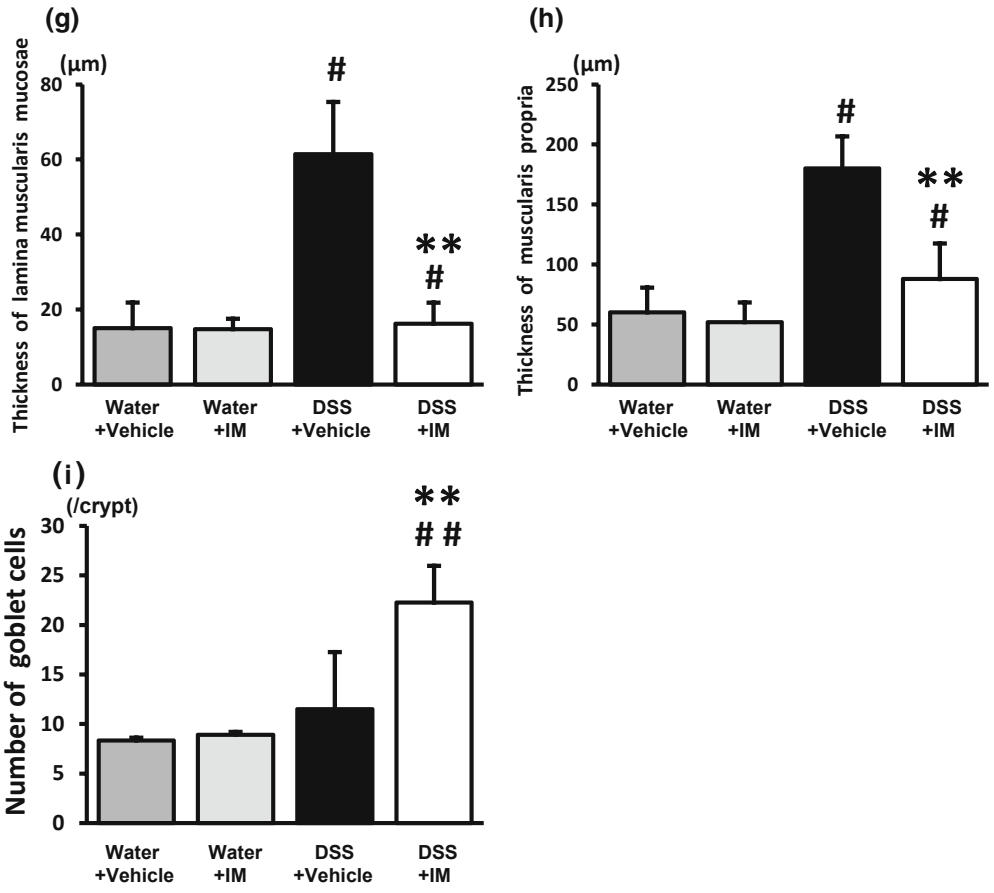
IL-10, an antiinflammatory cytokine known to be increased in mRNA expression in the colon of humans and other animals, was increased at day 19 in the colon of mice with DSS-induced colitis (Fig. 4c). IM treatment significantly decreased the enhanced mRNA expression level of IL-10 in the colon of chronic DSS colitis mice (Fig. 4c).

We investigated the mRNA expression levels of fibrogenic master cytokine (TGF-β), fibrolytic marker (MMP-2), and tissue inhibitor of metalloproteinase (TIMP)-1. At day 19, mRNA expression levels of these factors were markedly increased in chronic DSS-induced colitis compared with those of control mice, and IM treatment significantly reduced those of mice with chronic DSS colitis (Fig. 4d–f).

Effects of IM on fibroblasts and myofibroblasts in vivo by immunofluorescent analysis

Profibrogenic mesenchymal cells such as fibroblasts and myofibroblasts play an important role in intestinal fibrosis

Fig. 2. *Continued*



as well as chronic DSS colitis, as we reported previously.[23] To evaluate the effect of IM on the localization of fibroblasts (vimentin$^+$ and α-SMA$^-$) and myofibroblasts (vimentin$^+$ and α-SMA$^+$) in mucosa and submucosa, immunofluorescent analysis was performed for vimentin and α-SMA in chronic DSS-induced colitis at day 19.

In the mucosa of the colon of normal mice, irrespective of IM administration, fibroblasts (vimentin$^+$, α-SMA$^-$) and myofibroblasts (vimentin$^+$, α-SMA$^+$) were detected, and fibroblasts exceeded myofibroblasts in number (Fig. 5a,b,e,f). There was no significant difference in the number of vimentin$^+$ and α-SMA$^-$ fibroblasts in the mucosa of the colon of chronic DSS-induced colitis with vehicle administration when compared with that of normal control mice, irrespective of IM administration (Fig. 5e). IM treatment showed no significant effect on the number of the fibroblasts in the mucosa of the DSS colitis mice (Fig. 5e). In contrast, the number of vimentin$^+$ and α-SMA$^+$ myofibroblasts was significantly increased in the mucosa of the colon of chronic DSS-induced colitis with vehicle administration when compared with that of normal control mice, irrespective of IM treatment (Fig. 5f). Administration of IM significantly decreased the number of myofibroblasts in the mucosa of colitis mice (Fig. 5f).

In the submucosa of the colon of normal mice, irrespective of IM treatment, fibroblasts (vimentin$^+$, α-SMA$^-$) were detected, but myofibroblasts (vimentin$^+$, α-SMA$^+$) were rarely observed, as we have reported[23] (Fig. 6a,b,e,f). The number of vimentin$^+$ and α-SMA$^-$ fibroblasts was increased in the submucosa of the colon of chronic DSS-induced colitis with vehicle administration when compared with that of normal control mice, irrespective of IM administration (Fig. 6a,b,c,e). In contrast, IM treatment significantly decreased the number of fibroblasts in submucosa of colitis mice (Fig. 6e).

Discussion

Key factors for intestinal fibrosis in patients with CD as well as prerequisites for ideal animal models of intestinal fibrosis are excessive deposition of ECM, proliferation of profibrogenic mesenchymal cells in the colon, thickening of all layers of the gut wall, overgrowth of muscular layers of the intestine, enhanced Th-1 type immune response in the intestine, and overexpression of profibrogenic cytokines and growth factors.[27–31] As we have reported recently, chronic fibrosing colitis induced by single-cycle administration of DSS, which we used in this study, fulfills all the features previously described.[23] In this study, we showed that IM administration by enema could ameliorate chronic fibrosing DSS-induced colitis clinically and pathologically (see Figs. 1–3) through inhibiting the production of several cytokines

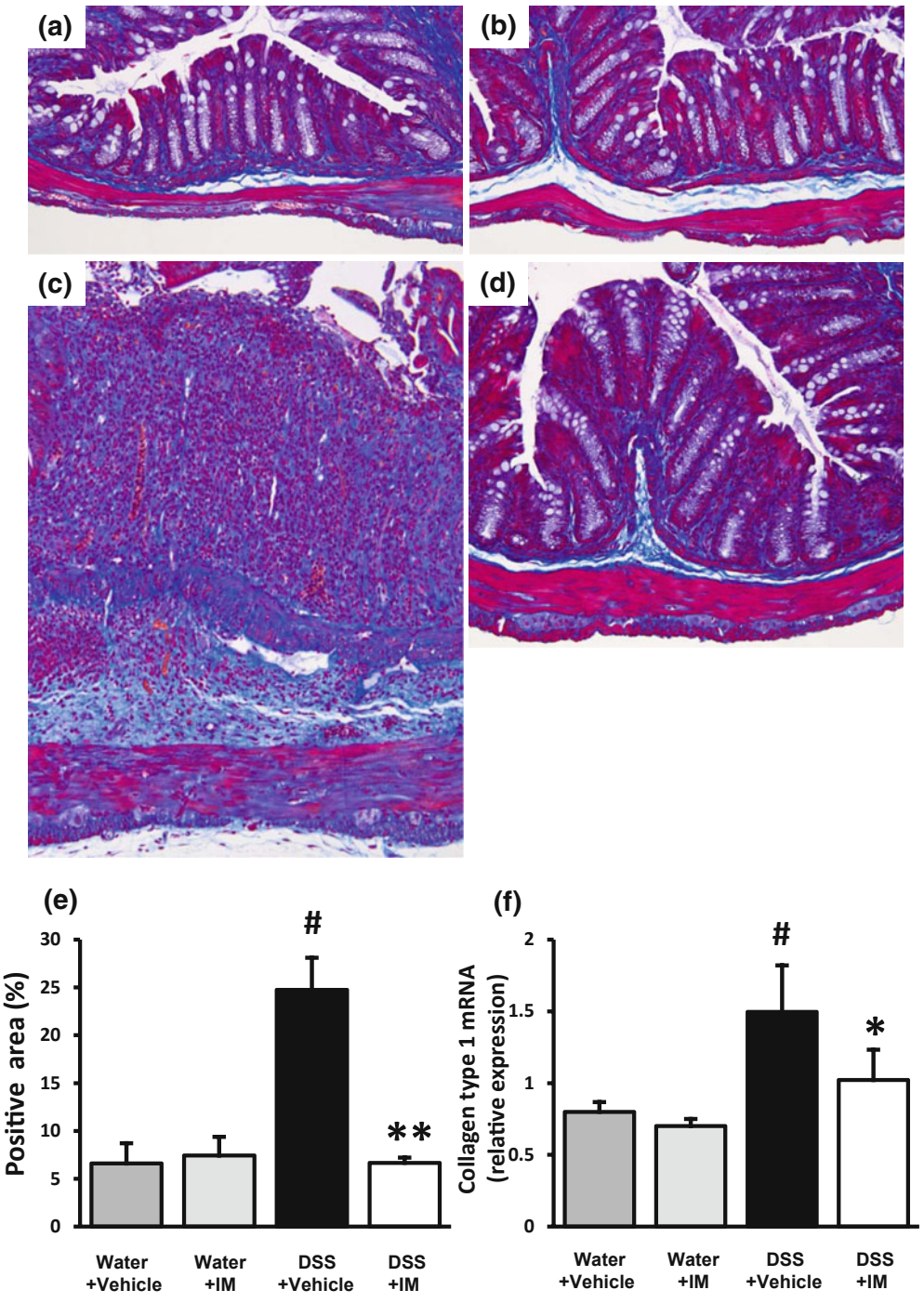


Fig. 3. Irsogladine maleate (IM) enema decreased the area of extracellular matrix deposition in the colon of mice with dextran sulfate sodium (DSS)-induced colitis. Masson's trichrome staining of colonic tissues in mice with dextran sulfate sodium (DSS)-induced colitis at day 19 from a normal mouse treated with vehicle enema (**a**); a normal mouse treated with IM enema (**b**); a mouse with DSS-induced colitis treated with vehicle enema (**c**); and a mouse with DSS-induced colitis treated with IM enema (**d**). Extracellular matrix is *blue*. **e** Image analysis of area of extracellular matrix deposition in mucosal and submucosal layers in the colon of mice with DSS-induced colitis. **f** mRNA expression levels of collagen type 1. $^{\#}P < 0.05$ compared with normal mice treated with or without vehicle enema; $^{*}P < 0.05$ compared with DSS colitis mice with vehicle enema; $^{**}P < 0.01$ compared with chronic DSS colitis mice treated with vehicle enema. **a–d** ×200

(see Fig. 4) and regulating profibrogenic mesenchymal cells such as fibroblasts and myofibroblasts in the colon (see Figs. 5,6).

IM, a widely used anti-ulcer drug in Japan, is known to protect the gastric mucosa by enhancing the mucosal integrity of the stomach through several mechanisms including facilitating gap junctional intercellular communication (GJIC) with enhanced expression of connexin.[32,33] IM also works for amelioration of indomethacin-induced intestinal lesions and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats, at least by its mucosal protecting activity.[17,34] Fibrosis in CD is the result of transmural chronic inflammation with repeated episode of immune-mediated damage and repair.[3,4] The precise mechanism of fibrosis in chronic fibrosing DSS colitis remains largely unknown. However, to stop a series of progression of inflammation from acute to chronic phase is a theoretically ideal approach for the amelioration of intestinal fibrosis including CD as well as the chronic DSS colitis model. In DSS colitis, the first event of DSS injury is colonic epithelial damage following the influx of luminal bacterial components into the gut.[21,22,24] These exogenous factors induce activation of innate

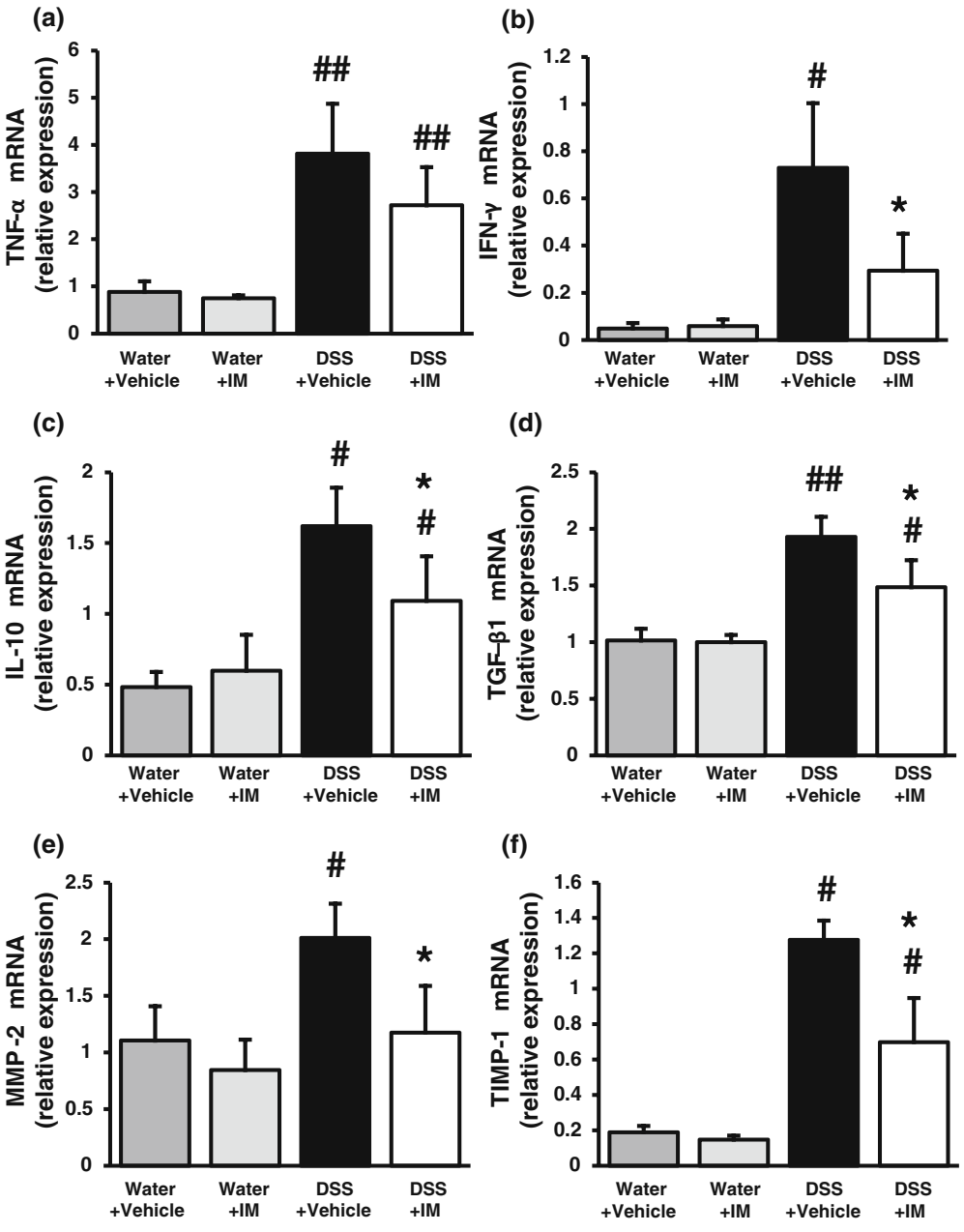


Fig. 4. Irsogladine maleate (IM) enema decreased the mRNA expression levels of cytokines in the colonic tissues of mice with dextran sulfate sodium (DSS)-induced colitis. **a–c** mRNA expression levels of proinflammatory cytokines such as tumor necrosis factor-α (*TNF*-α), interferon-γ (*IFN*-γ), and anti-inflammatory cytokine of interleukin-10 (*IL-10*) in the colonic tissues of mice. Data are expressed as means ± SD ($n = 5$ in each group). $^{*}P < 0.05$ compared with normal mice. **d–f** mRNA expression levels of profibrogenic cytokine transforming growth factor-β (*TGF*-β), matrix metalloproteinase-2 (*MMP-2*), and tissue inhibitor of matrix metalloproteinase-1 (*TIMP-1*) in the colonic tissues of mice. Data are expressed as means ± SD ($n = 5$ in each group). $^{\#}P < 0.05$ compared with normal mice with or without vehicle enema; $^{\#\#}P < 0.01$ compared with normal mice with or without vehicle enema; $^{*}P < 0.05$ compared with DSS colitis mice treated with vehicle enema

immunity, leading to acute colitis, followed by chronic colitis in several conditions.[22–25] Therefore, to maintain colonic epithelial integrity is considered a useful therapeutic approach that might block the progression of acute inflammation into chronic and into intestinal fibrosis in chronic DSS-induced colitis. Thus, the therapeutic effects, especially the antifibrosis effect of IM in this study at least, might be attributed to its intestinal mucosal protective effect on DSS-induced epithelial injury (see Figs. 1–3). In fact, IM treatment significantly decreased the DAI score from day 7 to day 19 (Fig. 1a), indicating a clinical inflammation-ameliorating effect of IM. The other gross indicators for disease activities such as colon length, colon weight, and their ratio did not show any significant difference among DSS colitis with vehicle treatment and DSS colitis with IM treatment (Fig. 1b). However, the colon length of mice with DSS colitis was already recovered by IM treatment at day 19. Although colon weight remained higher at day 19, this was caused by IM-induced increased epithelial masses, and not by cellular inflammation, as shown by histological analysis (see Fig. 2a–e). Kamei et al.[17] have reported that IM protects the small intestine against indomethacin-induced lesions in rats, probably by increased mucous secretion through facilitating GJIC. They speculated that this effect of IM is induced by the inhibitory action of phosphodiesterase (PDE), resulting in the suppression of enterobacterial invasion and inducible nitrous oxide (iNOS) expression.[15–17] In this report, we observed an increased number of mucus-secreting cells (goblet cells) in the colonic epithelial cells of chronic DSS colitis mice treated with IM enema (Fig. 2d,i). In IBD, goblet cell deple-

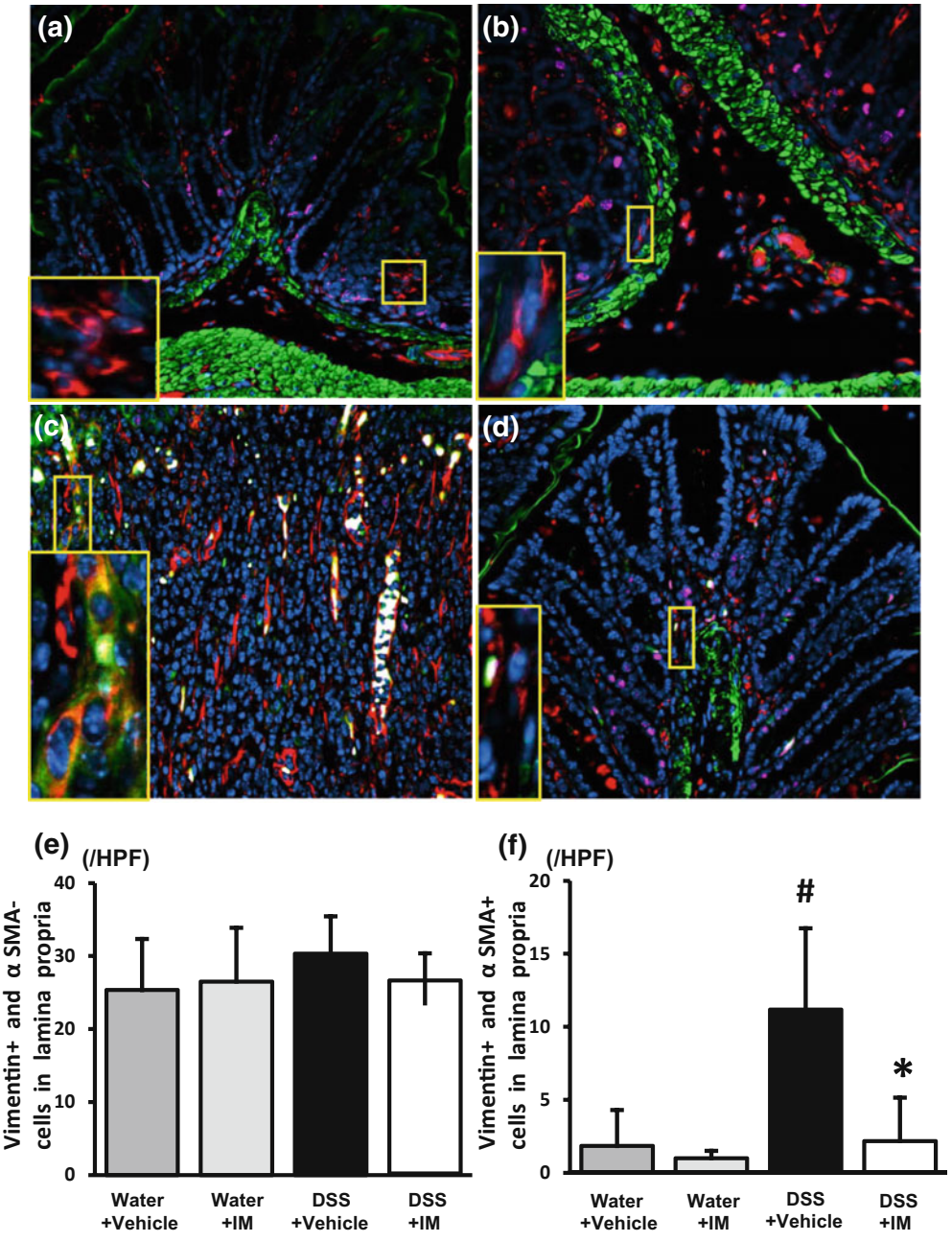


Fig. 5. **a–d** Immunofluorescence analysis of the effect of irsogladine maleate (IM) enema on the distribution of mesenchymal cell types in the colonic mucosa of chronic dextran sulfate sodium (DSS)-induced colitis at day 19. **a**, **b** Fibrogenic mesenchymal cells such as fibroblasts (vimentin$^+$, α-SMA$^-$) and myofibroblasts (vimentin$^+$, α-SMA$^+$) were increased in the mucosa of DSS colitis mice with vehicle enema (**c**). **d** IM administration decreased their number; some were proliferating (PCNA$^+$) (**c**, **d**). Vimentin (*red*); α-smooth muscle actin (α-SMA) (*green*); proliferating cell nuclear antigen (PCNA) (*pink*); 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). **e** Number of vimentin$^+$ and α-SMA$^-$ cells (fibroblasts) in mucosa of chronic DSS-induced colitis. Data are expressed as mean ± SD ($n = 5$ in each group). **f** Number of vimentin$^+$ and α-SMA$^+$ cells (myofibroblasts) in mucosa of chronic DSS-induced colitis. Data are expressed as mean ± SD ($n = 5$ in each group). #$P < 0.05$ compared with normal mice with or without vehicle enema; *$P < 0.05$ compared with DSS colitis mice treated with vehicle enema. *Bars* **a–d** ×200; *insets* ×400

tion is a well-known histopathological feature, and successful treatments recover the number of goblet cells among colonic crypt epithelial cells. Thus, the number of goblet cells reflects the integrity of colonic crypts, and IM exerts a colonic mucosal protecting effect by increasing goblet cells and mucous secretion, leading to inhibition of intestinal fibrosis. Further, we should analyze the precise mechanism between the antifibrosis effect and intestinal mucosal protecting effect of IM on the colonic epithelial cells and mesenchymal fibrogenic cells, with special relevance of molecules including iNOS, PDE, and connexin.

In this study, we showed that IM administration could significantly decrease the area of ECM deposition (see Fig. 3) in the colon of chronic DSS colitis. In addition, the IM enema also decreased the expression of inflammatory cytokines of TNF-α and IFN-γ in the colon of colitis mice (see Fig. 4). Melgar et al. and other investigators speculated that the intensity of Th-1 immune response together with several chemokines, such as monocyte chemoattractant protein-1 (MCP-1) expression, in the colon of the chronic phase of the DSS-induced colitis could determine whether chronic cellular infiltration with ECM deposition might continue in the colon of the DSS-induced colitis model.[22,35,36] Based on the previous studies, IM could downregulate cytokine production by ameliorating colonic mucosal integrity, as well as by blocking the progression and perpetuation of inflammation in the colon. Therefore, as a next step we should analyze whether IM treatment could directly regulate the cytokine

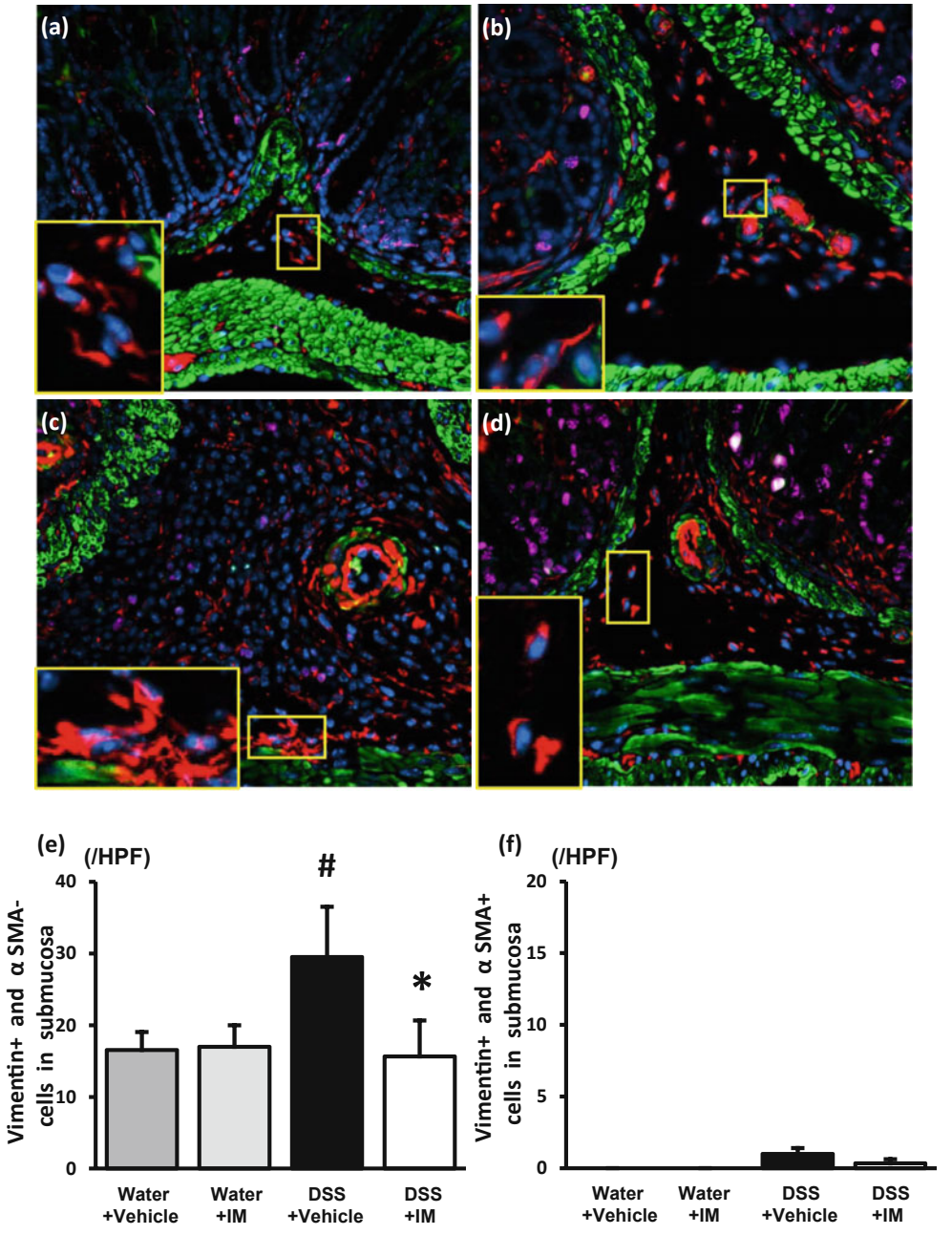


Fig. 6. a–d Immunofluorescence analysis of the effect of irsogladine maleate (IM) enema on the distribution of mesenchymal cell types in the colonic submucosa of chronic dextran sulfate sodium (DSS)-induced colitis at day 19. In the submucosa of colon of normal mice irrespective of IM enema, fibroblasts (vimentin$^+$, α-SMA$^-$) were detected, but not myofibroblasts (vimentin$^+$, α-SMA$^+$) (**a**, **b**). Fibroblasts (vimentin$^+$, α-SMA$^-$), but not myofibroblasts (vimentin$^+$, α-SMA$^+$) were increased in the submucosa of DSS colitis mice with vehicle enema (**c**). IM administration decreased their number (**d**); some were proliferating (PCNA$^+$) (data not shown). Vimentin (*red*); α-SMA (*green*); PCNA (*pink*); DAPI (*blue*). **e** Number of vimentin$^+$ and α-SMA$^-$ cells (fibroblasts) in the submucosa of chronic DSS-induced colitis. Data are expressed as mean ± SD ($n = 5$ in each group). #$P < 0.05$ compared with normal mice with or without vehicle enema; *$P < 0.05$ compared with DSS colitis mice treated with vehicle enema. **f** Number of vimentin$^+$ and α-SMA$^+$ cells (myofibroblasts) in the submucosa of chronic DSS-induced colitis. Data are expressed as mean ± SD ($n = 5$ in each group). **a–d** ×200; *insets* ×400

production using an in vitro system as well as in vivo in a future study.

In intestinal fibrosis, TGF-β has been regarded as the master cytokine, and the regulation of TGF-β in the colon is an ideal therapeutic strategy for the diseases including CD.[28,37,38] The present study showed that IM could downregulate the expression of mRNA of TGF-β in the colon of chronic DSS colitis mice (see Fig. 4d). To the best of our knowledge, there is no previous report that IM treatment could downregulate TGF-β expression. Hence, we are now investigating the direct effect of IM on regulation of TGF-β expression using a fibroblast cell culture system.

In this study, we revealed that the fibrotic area of the colon of mice with colitis was decreased by IM treatment, with decreased numbers of fibroblasts and myofibroblasts in the colon. As we reported recently, profibrogenic mesenchymal cells such as vimentin$^+$ and α-SMA$^+$ myofibroblasts and vimentin$^+$ and α-SMA$^-$ fibroblasts were increased in the colon of chronic DSS colitis mice.[23] These mesenchymal cells were considered to have profibrogenic character and actively produce ECM, leading to intestinal fibrosis.[39,40] Exogenously added TGF-β could transform inactive fibroblasts into active profibrogenic fibroblasts and myofibroblasts with increased production of collagen 1 in vitro.[23] The present in vivo study showed that IM treatment could regulate the activity of these profibrogenic mesenchymal cells in the colon of chronic DSS colitis. Therefore, to reveal the precise mechanism of IM for the amelioration of intestinal

fibrosis, we should reveal whether IM could directly regulate transformation of inactive fibroblasts into active profibrogenic mesenchymal cells, or if IM could exert its function by regulating TGF-β expression using a fibroblast in vitro system.

In conclusion, IM ameliorated chronic DSS colitis and fibrosis in the colon, suggesting a potential adjunctive therapy for intestinal fibrosis. IM could consequently reduce fibrosis in DSS colitis by direct or indirect effects on profibrogenic factors or fibroblasts. The exact effect of IM on intestinal fibrosis is still unclear, and thus we should further reveal the time course of the effects of IM on the DSS colitis parameters, and the direct effects of IM on inflammatory cells or fibroblasts as well.

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