Platelet interaction with lymphatics aggravates intestinal inflammation by suppressing lymphangiogenesis.

Running head: Platelets suppress lymphangiogenesis in colitis

Hirokazu Sato¹, Masaaki Higashiya¹, Hideaki Hozumi¹, Shingo Sato¹, Hirotaka Furuhashi¹, Takeshi Takajyo¹, Koji Maruta¹, Yuichi Yasutake¹, Kazuyuki Narimatsu¹, Kenichi Yoshikawa¹, Chie Kurihara¹, Yoshikiyo Okada¹, Chikako Watanabe¹, Shunsuke Komoto¹, Kengo Tomita¹, Shigeaki Nagao¹, Soichiro Miura², Ryota Hokari¹.

¹ Department of Internal medicine, National Defense Medical College, Saitama, Japan
² National Defense Medical College, Saitama, Japan

Corresponding author: Ryota Hokari
e-mail: ryota@ndmc.ac.jp

Department of Internal Medicine, National Defense Medical College, 3-2, Namiki, Tokorozawa, Saitama, Japan. 359-8513

Tel: +81-4-2995-1609, FAX: +81-4-2996-5201

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Abstract

Lymphatic failure is a histopathological feature of IBD. Recent studies show that the interaction between platelets and podoplanin on the lymphatic endothelial cells (LECs) suppresses lymphangiogenesis. We aimed to investigate the role of platelets in the inflammatory process of colitis, which is likely to be through modulating lymphangiogenesis. Lymphangiogenesis in the colonic mucosal specimens from IBD patients was investigated by studying the mRNA expression of lymphangiogenic factors and lymphatic vessel (LV) densities histologically. The involvement of lymphangiogenesis in intestinal inflammation was studied by administering VEGFR-3 inhibitors to the mouse model of DSS colitis and evaluating platelet migration to LV. Inhibitory effect of platelets on lymphangiogenesis was investigated in vivo by administering anti-platelet antibody to the colitis mouse model and in vitro by co-culturing platelets with LECs. Although mRNA expressions of lymphangiogenic factors such as VEGF-R3 and podoplanin increased significantly in the inflamed mucosa of IBD patients compared to those in the quiescent mucosa, there was no difference in LV densities between them. In the colitis model, VEGF-R3 inhibition aggravated colitis, decreased the lymphatic density, and increased platelet migration to LV. Administration of anti-platelet antibody increased the LV densities and
significantly ameliorated colitis. Co-culture with platelets inhibited proliferation of LECs in vitro. Our data suggest that, in spite of elevated lymphangiogenic factors during colonic inflammation, platelet migration to LV suppressed lymphangiogenesis, leading to the aggravation of colitis by blocking the clearance of inflammatory cells. Modulating the interaction between platelets and LV could be a new therapeutic means for treating IBD.
Introduction

The major roles of lymphatics are the drainage of interstitial fluid and transport of immune cells to the lymph nodes. Blocking lymphangiogenesis by inhibiting VEGF-R3 signaling resulted in aggravation of murine colitis (23). Enhancing lymphangiogenesis through the VEGF-C/VEGF-R3 pathway reduces murine colitis (13). These findings suggest that promoting lymphatic function may be a potential therapeutic strategy for treating inflammatory bowel diseases (IBD). IBD is associated with angiogenesis and lymphangiogenesis from the existing vascular network (14, 17). The LV density has been reported to increase in the intestinal mucosa of IBD patients (30). Significant elevation of lymphatic and blood vessel (BV) density was observed in mouse colitis model (16). This increase in lymphatics may be an adaptation against tissue edema and accumulation of immune cells. A recent study showed that lymphangiogenic factors such as VEGF-C/VEGF-D/VEGF-R3 and angiopoietin-1/2 were detected in colonic mucosa of IBD patients and were increased in severe lesions (2).

Lymphatic failure and obstruction, recognized as a histopathological features of IBD (18, 29, 36), are inconsistent with the increase in the lymphatics and lymphangiogenic factors in the intestinal mucosa of IBD patients. It is reported that lymph edema of intestinal wall of IBD results from obstructive lymphocytic
lymphangitis (2). The failure to collect accumulating filtered fluid including immune
cells and some types of antigens aggravates lymphocytic lymphangitis in IBD.
Therefore, improving the lymphatic function may be important for managing IBD (2).
Moreover, a relatively low LV density was reported to be a significant risk factor for
recurrence after surgery in Crohn’s disease (CD) patients, suggesting that LV
obstruction is one of the causes for IBD being an intractable disease (31). In the colitis
model, the increase in LV density in inflamed colonic mucosa was less than that of BV
density (1). These results suggest that although lymphangiogenic factors increased with
disease activity, increase of LV density was insufficient for some reason, resulting in
lymphatic failure and sustained inflammation in IBD. However, there has been no study
that elucidates the relationship between LV density and lymphangiogenic factors and
disease activity in IBD.

Mechanisms of lymphatic formation are extensively studied in the neonatal stage
but not in the adult. In the neonatal stage, lymphatic endothelial cells (LECs) are
differentiated from blood endothelial cells. Lymphatic vessel endothelial hyaluronan
receptor-1 (LYVE-1) is the first indicator of lymphatic endothelial commitment, and
LECs expressing LYVE-1 can differentiate into the LVs via the signaling pathways
involving transcription factors such as Prox1, and VEGF-C/VEGF-R3 (25, 35). BV/LV
divergence is brought about by the interaction between activated platelets and podoplanin (9), leading to the extension of LVs. C-type lectin-like receptor 2 (CLEC-2) is a platelet activating receptor and podoplanin expressed on LECs is an endogenous ligand for CLEC-2. The interaction between CLEC-2 and podoplanin activates platelets and induces platelet aggregation. It is reported that platelet or CLEC-2 deficient mice showed blood filled lymphatic vessels at the fetal stage, indicating that platelets play a crucial role in BV/LV divergence (25, 35). On the other hand, in the adult colonic inflamed site, several factors such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 stimulate inflammatory cells such as macrophages, which promote the formation of LVs by secreting VEGF-C/D (3, 22, 34).

The mechanisms of generation and extension of LV are well known, whereas mechanism that suppresses LV formation is poorly understood. Recently, it was reported that the binding of activated platelets to podoplanin on LECs induced the formation of thrombus in LV (6, 15, 28). In addition, it was reported that platelets inhibit the proliferation of human LECs (28). These mechanisms might cause the lymphatic vessels to become obstructed, leading to the dysfunction of lymphatic system. Although these are novel findings, it remains unknown whether the same processes occur in inflamed intestinal tissue.
Therefore, we first studied the changes in LV densities and the expressions of lymphangiogenesis-related molecules in the inflamed mucosa of IBD patients and correlated the findings with endoscopic observations. Next, we studied the involvement of lymphangiogenesis in the disease processes in mice colitis model using VEGF-R3 inhibitor. We also investigated the migration of platelets to the intestinal LVs in DSS colitis and acute rat intestinal inflammation models. Finally, we investigated inhibitory effect of platelets on lymphangiogenesis in vivo by administering anti-platelet antibody to the mouse colitis model and in vitro by co-culturing platelets with LECs to see if platelets suppress lymphatic endothelial cell proliferation under inflammatory conditions.

Materials and Methods

Patients

Tissue samples were obtained by colonic biopsies from 42 patients with ulcerative colitis (UC, 19 men, 23 women; mean age: 38.5 years, range: 17–69 years), 18 patients with CD (11 men, 7 women; mean age: 36.8 years; range: 19–70 years), and 8 patients with colonic polyps (5 men, 3 women; mean age: 59.3 years; range: 40–73 years) as controls. We classified obtained tissues from IBD patients into two categories, quiescent
mucosa and inflamed mucosa, depending on the endoscopic findings of redness, erosion
and ulcer.

Each tissue sample was fixed with periodate-lysine-paraformaldehyde (PLP) for
immunohistochemistry, and RNA was extracted from these tissues for real-time reverse
transcriptase (RT)-polymerase chain reaction (PCR).

We investigated whether the lymphangiogenic factors in colonic mucosa of IBD
patients by real-time RT-PCR were correlated with Matt’s score evaluated by endoscopy
(27), Crohn’s disease endoscopic index of severity (CDEIS) (26) and inflammatory
cytokines by real-time RT-PCR.

Informed consent was obtained from all patients. The protocols of the research
were approved by the Ethical Committee of the National Defense Medical College
(NDMC, No. 970).

Animals

Male C57BL/6J mice (weight: 20 g) and male Wistar rats (weight: 140g) were fed
with standard laboratory chow (SLC, Tokyo, Japan). The care and use of laboratory
animals were performed in accordance with the guidelines of the animal facility in
NDMC.
Murine colitis model, inhibition of angiogenesis and inhibition of platelet activation

The experimental protocol was approved by Animal Research Committee of NDMC (No, 13002). Dextran sulfate sodium (DSS, ICN Biochemicals, Cleveland, OH, USA) was adjusted to a concentration of 3% (w/v). Mice were provided DSS mixed drinking water for 6 days, followed by a 3-day interval period with only drinking water. In this experiment, DSS mice were subcutaneously treated daily with 10mg.kg$^{-1}$ MAZ51 (VEGF-R3 kinase inhibitor, Millipore, Billerica, MA, USA) or phosphate-buffered saline (PBS) containing dimethyl sulfoxide (DMSO), as described in a previous study (5). In other experiments, mice were injected intraperitoneally with rat monoclonal antibodies directed against GP Ib alpha (CD42b, EMFRET Analytics, Wurzburg, Germany) dissolved by PBS with 80μg/200μl at day1, 5 (19). For control groups, mice were treated with isotype matched IgG (rat IgG2a kappa Isotype Control, BD Biosciences, NJ, USA).

Histological score assessment of colitis

Hematoxylin and eosin (H&E) stained sections of mice distal colonic mucosa were assessed based on the scoring system with 3 parameters; severity of inflammation,
crypt damage and ulceration (24).

Quantitative real-time RT-PCR

Total RNA was isolated from the mucosal tissue by RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Assays-on-Demand Gene Expression Probes included VEGF-C; Hs01099203, FIGF(VEGF-D); Hs01128657, FLT4(VEGFR3); Hs01047677, Podoplanin; Hs00366766, LYVE-1; Hs00272659, Prox-1; Hs00896294, TNF-a; Hs00174128, IL-1b; Hs01555410, and IL-6; Hs00985639 (Applied Biosystems, Waltham, MA, USA). Target messenger RNA (mRNA) levels were determined by the comparative cycle threshold method for relative quantification. To standardize, beta-glucuronidase (GUS) was quantified.

Immunohistochemistry

Human colonic tissue samples were fixed in PLP and frozen. The following antibodies and working dilutions were used: LYVE-1 (1:500; Abcam, Cambridge, UK) and CD34 (1:100; Nichirei Biosciences Inc, Tokyo, Japan). Isotype-matched IgG was used as a negative control. After blocking by 1% bovine serum albumin and treatment with each antibody, the samples were treated with avidin-biotinylated peroxidase
complex for 0.5 h and visualized under the Zeiss Laser Scanning Microscope (510LSM; Carl Zeiss, Jena, Germany).

For immunohistochemistry of mice distal colonic mucosa, the same procedure was performed using the following antibody: LYVE-1 (1:500; Abcam, Cambridge, UK) and CD62P (1:100; BD Biosciences Pharmingen, USA).

**Quantification of LVs and BVs by immunohistochemistry**

LYVE1-stained sections were used to assess the LV density in colonic mucosa. The LVs were considered countable only when LYVE1-stained microvessels showed visible lumen. In the mucosa, counting was performed above the muscularis mucosa with LSM image browser (Carl Zeiss, Jena, Germany). In the mucosal layers, quantification of LV was performed in 3–4 randomly selected consecutive fields. The LV density was defined as the ratio of the area surrounded by LEC to the total tissue surface area.

In addition, quantification of BVs was performed in randomly selected field in the mucosal layer. BVs were considered countable only when CD34-stained microvessels showed visible lumen.
Enumeration of platelets in the intestinal lymph after induction of intestinal injury

To analyze the number of platelets in the intestinal lymph, Wistar rats were subjected to mesenteric lymphadenectomy (8). After 6 weeks, the thoracic duct was cannulated, and the lymph drained from the intestine was collected (7, 20). The number of platelets migrating to the lymph was examined every 6 hours. The rats were treated with 7mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS) and 0.2 mL of 35% ethanol solution containing a final concentration of 0.1 M TNBS to induce ileitis (32). The numbers of platelets and red blood cells (RBCs) were counted by a hemocytometer (Burker-Turk).

Cells

Human dermal lymphatic endothelial cells (HDLECs) were purchased from Promo cell (Heidelberg, Germany) and incubated in culture dishes in endothelial cell growth medium MV2 (Promo cell, Heidelberg, Germany). Cultures were maintained at 37°C, 5% CO₂.

Cell proliferation Assay
Cell proliferation was investigated following the manufacturer’s protocol of cell proliferation ELISA kit with Bromodeoxyuridine (BrdU) chemiluminescence (Roche, Indiana, USA). Briefly, 100 µl HDLECs were put in 96 well black plate at the concentration of 1×10^6/ml and incubated for 6 h. Then, BrdU was added with TNF-α 5ng/ml and platelets (1×10^8/ml) and incubated for 16h (10, 28). Platelets were isolated from blood of mice as described previously (19). Blood of mice was collected from the heart and platelets were isolated by centrifugation method at 600 g with 0.1 mL acidcitrate dextrose buffer. BrdU incorporation was measured with Gemini EM microplate reader (Molecular devices, CA, USA).

**Statistical Analysis**

Statistical analysis was performed by JMP version 11 (GraphPad Software, Inc. Sandiego, CA, USA). Data were represented as mean ± standard error. Differences in the quantitative measurements were tested by Mann–Whitney U test. In vitro study, differences were evaluated by Student’s t test. The correlation was studied by using Spearman’s nonparametric correlation coefficient test. P <0.05 was considered statistically significant.
Results

mRNA expressions of lymphangiogenesis-related molecules in the colonic mucosa of IBD patients

The expressions of VEGF-C, VEGF-R3, podoplanin and LYVE1 significantly increased in the inflamed colonic mucosa of UC patients than in that of control patients and quiescent mucosa of UC patients (Figure 1A). Furthermore, the expression of VEGF-C and VEGF-R3 was significantly higher in the quiescent colonic mucosa of UC patients than in that of control subjects. On the contrary, there was no change in the expressions of VEGF-D and Prox1. Similarly, the expressions of VEGF-C, VEGF-R3, podoplanin, and LYVE1 significantly increased in the inflamed colonic mucosa of CD patients than in that of control subjects and quiescent mucosa of CD patients (Figure 1B). In addition, the expressions of VEGF-C and VEGF-R3 significantly increased in the quiescent colonic mucosa of CD patients than in that of control subjects. However, the expressions of VEGF-D and Prox1 did not increase.

mRNA expressions of lymphangiogenesis-related molecules relative to the endoscopic activity in IBD patients

The expressions of VEGF-R3, podoplanin, and LYVE1 were well correlated to
endoscopic Matt’s score \((r=0.42, 0.64, 0.5; p<0.01, 0.01, 0.01)\) in UC patients (Figure 2).

In addition, the expressions of VEGF-C, VEGF-R3, podoplanin, and LYVE1 were significantly increased as endoscopic inflammatory score advanced. However, the expression of lymphangiogenic factors did not correlate with CDEIS in CD patients.

Correlation between the expression of lymphangiogenesis-related molecules and Proinflammatory cytokines in the colonic mucosa of IBD patients

The results of these experiments are shown in Table 1. In UC patients, the expressions of TNF-\(\alpha\) and IL-6 showed a high degree of correlation with VEGF-C, VEGF-D, VEGF-R3, podoplanin, and LYVE1. Similarly, the expression of IL-1\(\beta\) was correlated with that of podoplanin. In CD patients, the expressions of TNF-\(\alpha\) and IL-6 was correlated with that of VEGF-C, VEGF-R3, podoplanin, and LYVE1. In addition, IL-1\(\beta\) expression was correlated with that of VEGF-C, VEGF-D, VEGF-R3, podoplanin, and LYVE1.

LV density in the colonic mucosa of IBD patients as determined by immunohistochemistry

We used anti-LYVE1 antibodies for the immunohistochemical localization of
LEC in the colonic mucosa of IBD patients (Figure 3A). There was no
immunoreactivity in the negative control group. In UC patients, the LV density in the
quiescent lamina propria was significantly higher than in that of control subjects (3.4%
vs. 0.6%). Interestingly, in contrast to the positive correlation between the expression of
proinflammatory cytokines and lymphangiogenic factors, the LV density of the
inflamed mucosa was not higher than that of quiescent mucosa (1.9% vs. 3.4%) (Figure
3B). In terms of the morphology, the LVs were mainly located beneath the muscularis
mucosa in control subjects and quiescent mucosa of IBD patients. On the other hand, in
the inflamed mucosa, LVs were irregularly distributed in the mucosal layer. In addition,
in contrast to LVs of the control or quiescent mucosa, the LVs in inflamed mucosa were
irregularly shaped and appeared large and dilated. In other words, the formation of the
LV seemed incomplete under inflamed condition. In the colonic tissues of CD, the LV
density in the quiescent lamina propria was higher compared with that in control
patients (1.6% vs. 0.6%), congruent with the increase in the expression of
lymphangiogenesis-related molecules. However, the LV density did not increase in
inflamed mucosa (0.9%) and was less than that in the quiescent sections (Figure 3B).

The BV density in quiescent mucosa was same as that in control mucosa and
tended to increase in actively inflamed mucosa, although the increase was not
significant in either UC or CD patients (Figures 3C).

Confirmation of the involvement of lymphangiogenesis in DSS-induced murine colitis

To confirm the suitability of murine colitis model for evaluating the role of lymphatics in colitis activity, we first studied the effect of blocking lymphangiogenesis using VEGFR3 inhibitor. After 9 days of DSS treatment, lymphatic vessels were stained with anti-LYVE1 antibody (Figure 4A). The LV density was higher in DSS-induced colitis mice. The LV density in mice administered with VEGF-R3 inhibitor was lower compared to that in DSS-induced mice (Figure 4B). Figure 4C shows a representative image of H&E staining of colon in each group. Reduction in colonic length and increase in the ratio of colon weight to colon length were observed in the group that received VEGF-R3 inhibitor (Fig 4D, 4E), suggesting that VEGF-R3 inhibition aggravates DSS-induced colitis. Inhibition of VEGFR-3 also led to an increase in the number of mononuclear cells infiltrating into the submucosal layer and histological score in DSS colitis (Figures 4F, 4G). These findings suggest that the suppression of lymphangiogenesis during active colonic inflammation exacerbates the inflammation by retaining the lymphocytes in the colonic mucosa.
Infiltration of activated platelets into the LV of murine inflamed colonic mucosa

Based on the finding that LV density in actively inflamed mucosa of IBD patients did not increase in spite of the increase in lymphangiogenic factors, we hypothesized that a different mechanism might be blocking lymphangiogenesis. In fetal development, activated platelets migrating from the BVs block lymphatic development by interacting with podoplanin on LECs (28). Therefore, we investigated whether platelets infiltrate the intestinal lymph during intestinal inflammation. The presence/absence of platelets in the LVs in the inflamed intestinal mucosa was studied immunohistochemically. Mice colonic mucosa with DSS-induced inflammation was double stained with anti-CD62P (P-selectin) antibody for detecting activated platelets and with anti-LYVE1 antibody for the LVs. Our results confirmed the presence of activated platelets in the LVs, suggesting that the platelets migrate to the LVs during colonic inflammation (Figure 5A).

Next, we studied whether platelets migrate into the intestinal lymph from the inflamed intestine. For this purpose, rats with acute intestinal inflammation were subjected to mesenteric lymphadenectomy, and the intestinal lymph was collected by cannulating the thoracic duct (7, 20). In sham-operated rats, few platelets were observed
in the lymph. However, in the TNBS ileitis model, platelet influx into the intestinal
lymph was observed 18 h after TNBS treatment. The number of platelets at 24 h after
the treatment significantly increased compared with that of control animals (Figure 5B).
The number of RBCs did not increase throughout the experimental period.

Anti-platelet treatment ameliorates DSS induced colitis

Our next set of experiments examined the ameliorative effect of anti-platelet
treatment on DSS induced colitis. After 9 days of DSS treatment, reduction in the body
weights of mice administered with GPCb inhibitor was significantly smaller than that of
DSS + control IgG group (Figure 6A). Reduction in the colonic length and increase in
the ratio of body weight to colon length were relatively smaller in the group treated with
the GPIb inhibitor (Figure 6B, 6C). As shown in Figure 6D, GPIb inhibitor treatment of
DSS colitis mice significantly suppressed colitis. Submucosal LV density in GPIb
inhibitor treated group increased compared to that in DSS alone group (Figure 6E).
Thickness of submucosal layer, number of mononuclear cells infiltrating the
submucosal layer and histological disease score were all reduced by the GPIb inhibitor
treatment (Figures 6F, 6G, 6H). These findings suggested that platelets exacerbate
colitis through the suppression of lymphangiogenesis in actively inflamed colonic
inflammation and that the anti-platelet therapies do have an ameliorative effect on the disease pathology.

Effect of platelets for cell proliferation of HDLEC under the inflammatory condition

An earlier report showed that LECs proliferation is suppressed by co-culturing with platelets (28). Therefore, we investigated if platelets suppressed LECs proliferation under inflammatory conditions. Cell proliferation of HDLEC stimulated by TNF-α was significantly inhibited by co-culturing of platelets (Figure7).

Discussion

In this study, we show that platelets migrate to the lymphatics, interact with LECs, and suppress lymphangiogensis, leading to the aggravation of colitis, perhaps by blocking the clearance of inflammatory cells.

The pathological features of IBD have been linked to the hyper-activation of platelets and coagulation (37). It has also been reported that apheresis of myeloid lineage cells including platelets can contribute to amelioration of IBD (33), suggesting that activated platelets play an inflammatory role in IBD and hence, activated platelets
can be a target for the treatment of IBD patients. In addition, it has been reported that platelets play a critical role in the inhibition of lymphangiogenesis by binding to podoplanin on LECs in the fetal period and inhibiting the proliferation of LECs (28). Therefore, we hypothesized that platelets had a suppressive effect on lymphangiogenesis in colitis and aggravated inflammation.

The mechanisms of inhibition of lymphangiogenesis in the fetal period were studied extensively and the involvement of CLEC-2, bone morphogenetic protein (BMP)-9, and transforming growth factor-β (TGF-β) derived from activated platelets was described (29). However, it is unknown whether platelets interact with lymphatic endothelial cells in intestinal inflammation in the adult. In this study, we immunohistochemically detected the presence of platelets in the LV in the mouse colitis model. We collected the intestinal lymph of the TNBS-induced ileitis rats and confirmed that platelets were drained from the inflamed intestinal tissue and the number of platelets migrating to intestinal lymph is more than that of the normal intestinal tissue. RBCs did not increase in the intestinal lymph, suggesting that intestinal inflammation did not induce lymphatic-venous anastomosis.

There has been no comprehensive study that examined LV density, lymphangiogenic factors, and proinflammatory cytokine expressions in IBD patients
relative to those in control subjects. In this study, we showed that the increased
eexpression of multiple lymphangiogenic factors was well correlated with the disease
activity as assessed by endoscopy in UC patients. In an earlier report, endoscopic
investigation showed an increase in several lymphangiogenic factors in IBD patients
(2), and our results are similar. In addition, the expression of lymphangiogenic factors
correlated well with the expression of proinflammatory cytokines such as TNF-α,
IL-1β, and IL-6. Lymphangiogenic factors such as VEGF-C and VEGF-D were reported
to be produced from macrophages stimulated by proinflammatory cytokines (4, 12, 21).
In our study, some lymphangiogenic factors were significantly correlated with TNF-α,
IL-1β, and IL-6. Among them, podoplanin showed the strongest correlation with all the
proinflammatory cytokines and endoscopic Matts score. Although our study was
observational research using human material, these results suggest that podoplanin is
important for lymphangiogenesis in IBD patients. In spite of the increase in
lymphangiogenic factors, LV density was not significantly higher in actively inflamed
mucosa compared to that in quiescent mucosa, which was also reported previously (30).
Taken together, our results imply that the expression of lymphangiogenic factors
increases in inflamed mucosa, probably for clearance of the inflammatory cells.
However, as inflammation increases, platelets become highly activated and
lymphangiogenesis is suppressed by platelet migration into LVs, leading to exacerbation of colitis.

Recently, it is reported that VEGF-C/VEGFR3 axis but not VEGF-D regulates lymphatic function and inflammatory activity in two different animal models (13). In our study too, VEGF-D was not well correlated with lymphangiogenesis in IBD. In addition, our results suggest that not only VEGF-C, but also multiple lymphangiogenic factors were involved in the pathological increase of lymphangiogenesis in IBD patients. In the inflammatory site, proinflammatory cytokines stimulate inflammatory cells such as macrophages, which promote the formation of LVs by secreting VEGF-C/D (2, 21, 34). However, the expression of Prox1, which is a crucial factor in the lymphatic development in the fetal period, did not increase in our study. It is reported that BMP-9, which is present in activated platelets and released upon activation, inhibit expression of Prox-1 (38). It is possible that some molecules released from platelets might block increased expression of Prox-1, although we did not elucidate them in this study.

There are limitations in this study. We used two different animal models of intestinal inflammation, DSS model in mice and TNBS model in rats. The rationale for choosing each of these models was technical as well as ethical, such as the need for
animals larger than mice to insert tubes to collect intestinal lymph and the short duration ethically allowed to induce inflammation and collect lymph while keeping the animals restrained. In addition, although TNBS and DSS induced-intestinal inflammation might involve different inflammatory mechanisms, increased density of lymphatics was found in both models (11). For these reasons, we thought that TNBS-induced ileitis model was acceptable for collecting lymph ethically and experimentally instead of the mouse DSS colitis model. We could indeed confirm the increase in platelets in lymphatics. Our results suggest that modulation of lymphangiogenesis by platelets interaction with lymphatics during inflammation could be a generalized mechanism in intestinal inflammation, which needs to be confirmed using different approaches. Second, in the human study, the control group is about 20 years older on average to the UC and CD groups. However, there has been no report about the effect of aging on normal lymphatic density as far as we searched. In fact, there was no obvious difference between younger controls and older controls at least in our study. Third limitation is that the sample numbers of inflamed mucosa in immunohistochemistry of human colonic mucosa are small. We obtained minimum amount necessary samples by endoscopy from patients with active disease in order not to influence the disease. However, some samples were inappropriate for evaluation
because of too small sample size for analysis. This is due to method to obtain samples by endoscopy. Rahier et al reported relationship between progressive disease course and decreased lymphatic vessel density in patients with CD. They solved this problem by using ileicolonic resection specimens. In spite of these problems, our data is consistent with the previous report (30).

Our study suggests that one of the causes of insufficient lymphangiogenesis could be via platelets suppressing the lymphatic endothelial cell proliferation and lymphangiogenesis stimulated by inflammation. Therefore, this suppressive mechanism of lymphangiogenesis by platelets might be responsible for prolonged inflammation in IBD. Modulating the interaction between platelets and LV could be a new therapeutic target for managing IBD.

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There is no personal conflict of interest to disclose for any of the author listed.


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Figure captions

Figure 1

Lymphangiogenic factors are increased in colonic mucosa of IBD patients.

mRNA quantification of lymphangiogenic factors in the colonic mucosa of control patients (n=8), in the quiescent and inflamed mucosa of (A) UC (inflamed: n=47, quiescent: n=14) and (B) CD (inflamed: n=19, quiescent: n=13) patients. The expressions of VEGF-C, VEGF-R3, podoplanin and LYVE1 increased in the inflamed colonic mucosa of both UC and CD. Data are expressed as means ± SE,*P<0.05, **P<0.01, ***P<0.001.

Figure 2

mRNA expressions of lymphangiogenesis-related molecules, relative to endoscopic Matt’s score.

The expressions of VEGF-R3, podoplanin, and LYVE1 were well correlated to Matt’s score in UC patients. In addition, the expressions of VEGF-C, VEGFR-3, podoplanin, and LYVE1 were significantly increased as endoscopic inflammatory score advanced.
Figure 3

**Evaluation of the density of lymphatic vessels (LV) in colonic mucosa of IBD patients.**

(A) LV of the colon were stained by LYVE-1 antibodies. Representative images in each group are shown. Lower pictures are magnified ones of the upper. (B) The density of LV in the mucosal layer of control, UC and CD patients. LV density increased in the quiescent mucosa of both UC and CD. (C) The density of the blood vessels in the mucosal layer of control, UC and CD patients. BV density was not significantly different among three groups. Data are expressed as means ± SE, (UC; inflamed: n=4, quiescent: n=5, CD; inflamed: n=3, quiescent: n=5, control: n=6), *P < 0.05.

Figure 4

**The effects of VEGF-R3 inhibitor on DSS-induced colitis.**

(A) Representative immunohistological image of the colon in each group are shown. Lymphatic vessels were stained by LYVE-1 antibodies. (B) The density of LV in the
submucosal layer. LV density increased in the colonic mucosa of DSS mice. VEGF-R3 inhibitor suppressed the increase of LV induced by DSS. (C) Hematoxylin and eosin (H&E) staining of the colonic mucosa. The colonic inflammation worsened by the administration of VEGF-R3 inhibitor. (D) The colon length of each group. (E) The ratio of colon weight to colon length. (F) The number of lymphocytes infiltrating in the submucosal layer. (G) The histological score in the mice colon. Data are expressed as means ± SE, (control: n=5, DSS: n=8, DSS+VEGF-R3 inhibitor: n=8), *P < 0.05, **P<0.01.

Figure 5

Platelet infiltration into lymphatic in inflamed intestine.

(A) Representative image of double immunostaining with P-selectin (green) and LYVE1 (red) in the inflamed distal colon of DSS-induced colitis mice. Activated platelet (arrow) infiltrated into the lymph vessels. These images show the infiltration of activated platelets into lymph vessel. (B) The number of platelets in the intestinal lymph significantly increased after 24 h of TNBS administration. Data are expressed as means ± SE, (TNBS: n=6, control: n=6), *P < 0.05.
The effects of GPIb antibody on DSS-induced colitis

(A) The change of body weight in each group. (B) The colon length of each group. (C) The ratio of body weight to colon length. (D) Representative images of H&E staining of the colonic mucosa. Bars indicate 100 μm. (E) LV density in the submucosal layer in each group. (F, G, H) Histological evaluation in each group. The increased thickness, number of lymphocytes infiltrating in the submucosal layer and histological score by DSS treatment were improved by GPIb antibody. Data are expressed as means ± SE, (control: n=6, DSS+IgG: n=6, DSS+GPIb antibody: n=5), †P< 0.05 vs control, ‡P<0.01 vs control, §P< 0.05 vs DSS+IgG, *P < 0.05, **P<0.01. anti-GPIb in figures means anti-GPIb antibody.

Co-culturing platelets and HDLEC under the inflammatory condition

Cell proliferation of HDLEC was significantly suppressed by co-culturing with platelets under TNF-α stimulation. Data are expressed as means ± SE, (control: n=5, TNF-α: n=5, TNF-α+platelet: n=5), *P < 0.05.
Table 1 Correlation between the expression of lymphangiogenesis-related molecules and proinflammatory cytokines in the colonic mucosa of IBD patients

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control 3% DSS 3% DSS+VEGF-R3 inhibitor

B

lymph vessel density in submucosal layer (%)

control DSS DSS+VEGF-R3 inhibitor

C

control 3% DSS 3% DSS+VEGF-R3 inhibitor

D

colon length (cm)

control DSS DSS+VEGF-R3 inhibitor

E

colon weight / colon length (g/cm)

control DSS DSS+VEGF-R3 inhibitor

F

lymphocytes in submucosal layer (LPF)

control DSS DSS+VEGF-R3 inhibitor

G

histological score

control DSS DSS+VEGF-R3 inhibitor
**A**

- **P-selectin**
- **LYVE1**
- **Merge**

**B**

<table>
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<td>number of platelets ($\times 10^4/\mu l$)</td>
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* indicates significant differences.
A control  DSS+IgG  DSS+anti GPIb

B

colon length (cm)

0 1 2 3 4 5 6 7 8 9

control  DSS+IgG  DSS+anti GPIb

C

colon weight/colon length (g/cm)

0 5 10 15 20 25 30 35 40

control  DSS+IgG  DSS+anti GPIb

D control  3% DSS+IgG  3% DSS+ anti-GPIb antibody

E

lymph density in submucosal layer (%)

0 4 8 12 16

control  DSS+IgG  DSS+anti GPIb

F

thick of submucosal layer (μm)

0 20 40 60 80 100 120 140 160

control  DSS+IgG  DSS+anti GPIb

G

lymphocytes in submucosal layer (HPF)

0 10 20 30 40 50

control  DSS+IgG  DSS+anti GPIb

H

histological score

0 1 2 3 4 5 6 7

control  DSS+IgG  DSS+anti GPIb
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