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Evaluation of the degree and distribution of lymphangiectasia in full-thickness canine small intestinal specimens diagnosed with lymphoplasmacytic enteritis and granulomatous lymphangitis

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ABSTRACT. Intestinal lymphangiectasia (IL) is often observed in dogs with chronic small intestinal diseases. Hypoplasia of the lymphatic vessel due to decreased lymphangiogenesis, which has been suggested in human idiopathic IL, may contribute to the pathogenesis of canine IL. This study aimed to evaluate the diameter and number of lymphatic vessels in full-thickness small intestinal specimens of dogs with IL. Immunohistochemical labeling of lymphatic endothelial cell markers was performed on retrospectively retrieved full-thickness small intestinal specimens. Sixteen dogs with histologically confirmed IL were included, of which 10 had lymphoplasmacytic enteritis (LPE), and six had granulomatous lymphangitis (GL). Nine dogs that died from non-gastrointestinal disorders and with little or no abnormalities in the small intestine were used as controls. Lymphatic vessel diameters in dogs with IL were significantly increased in all layers of the small intestine, including the villus lacteal, lamina propria, submucosa, muscularis, and mesentery, compared with controls (all $P<0.01$). There was no significant difference in the lymphatic vessel diameters between dogs with LPE and GL (all $P>0.05$). There was no significant difference in the number of lymphatic vessels between dogs with IL and the controls in all layers of the small intestine (all $P>0.05$). This study demonstrated that IL was observed in all layers of the small intestine, including the submucosa, muscularis, and mesentery, independent of the underlying disease. Factors other than reduced lymphatic vessels would contribute to the pathogenesis of IL in dogs.

KEYWORDS: chronic small intestinal disease, dog, immunohistochemistry, lymphangiectasia

Intestinal lymphangiectasia (IL) is a disorder that leads to the dilation of lymphatic vessels in the small intestine. Although direct causes cannot be found in dogs with primary IL, chronic small intestinal diseases such as lymphoplasmacytic enteritis (LPE), granulomatous lymphangitis (GL), and neoplastic diseases, including gastrointestinal lymphomas, can induce secondary IL [6, 16, 17]. In several cases of LPE, the gastrointestinal mucosa is often diffusely affected [8, 19]. In contrast, GL forms a focal mass lesion, which is considered as an inflammatory response to the chronic leakage of lipid-rich chyle and lymphatic vessel rupture [15, 22]. However, the relationship between concurrent disease and the features of IL in the full-thickness small intestines of dogs has not been elucidated.

In dogs with IL, the leakage of lymph into the small intestinal lumen leads to protein-losing enteropathy. Previous histopathological studies have shown an association between protein-losing enteropathy in dogs and IL in the villus and lamina propria compared with dogs without protein-losing enteropathy [23, 24]. In addition, the width of the villus lacteal was negatively

correlated with serum albumin concentrations [23, 24]. In the serosa and mesentery of dogs with chronic small intestinal disease, IL and/or GL caused due to lymph leakage were observed [15, 22]. Another study that used full-thickness biopsy samples from the gastrointestinal tract observed transmural IL in some dogs with gastrointestinal disease [14]. Since intestinal tissue collected via endoscopic biopsy is typically limited to the mucosal layer with variable visualization of the submucosa, full-thickness intestinal specimens are required to evaluate the accurate features of the deeper layers in the small intestine with IL.

Lymphangiogenesis is a process in which lymphatic endothelial cells migrate and proliferate to form new lymphatic vessels. Lymphangiogenesis is essential for maintaining the structure and function of lymphatic vessels. In human idiopathic IL, a decrease in vascular endothelial growth factors- C and -D, which stimulate lymphangiogenesis, has been suggested [10]. Hypoplasia of the lymphatic vessel due to decreased lymphangiogenesis can result in the dilation of the peripheral lymphatic vessel. Although there are no reports on lymphangiogenesis or the number of lymphatic vessels in dogs with IL, a decreased number of lymphatic vessels may contribute to the pathophysiology of IL in dogs.

Lymphatic endothelial cells express various proteins that distinguish lymphatic endothelial cells from blood endothelial cells [2, 11]. Prospero-related homeobox-1 (Prox-1) is a highly conserved nuclear transcription factor required for the development of lymphatic endothelial cells [25]. Lymphatic vascular endothelial receptor-1 (LYVE-1) is a cell surface receptor for the large extracellular matrix hyaluronan [2] and seems to be associated with hyaluronan clearance and degradation in the lymphatics and lymph nodes. Both Prox-1 and LYVE-1 have been used as lymphatic endothelial cell markers to evaluate the diameter of the lymphatics in the small intestine of dogs [24] or differentiate lymphangiosarcoma from hemangiosarcoma in dogs [9].

This study aimed to evaluate the distribution and degree of dilation of lymphatic vessels in full-thickness small intestinal specimens of dogs with IL using lymphatic endothelial cell markers. In addition, we evaluated the number of lymphatic vessels. We hypothesized that the distribution of IL differs according to the concurrent disease in dogs and that a decrease in the number of lymphatic vessels would contribute to the pathogenesis of IL in dogs.

MATERIALS AND METHODS

Inclusion criteria

The database of the Laboratory of Veterinary Pathology at the University of Tokyo was retrospectively reviewed to identify cases with full-thickness small intestinal specimens. First, we set the reference range to diagnose IL in the present study because there is no consensus on the standard for dilation of the lymphatic vessel in layers other than the villus lacteal. As a control group, full-thickness small intestinal specimens from dogs that died from non-gastrointestinal disorders were selected as candidates. After careful review of hematoxylin and eosin-stained sections by one veterinarian, dogs with little or no abnormalities in the small intestinal tissues were included as controls. Using the samples of the controls, we calculated the mean value of the mean lymphatic vessel diameter for the villus lacteal, lamina propria, submucosa, muscularis, and mesentery. The specimen was considered IL-positive when the lymphatic vessel diameter was above the reference range, the mean value + 2 standard deviations of the controls. Next, dogs with IL-related descriptions in the histopathological record were selected as candidates, and full-thickness small intestinal specimens confirmed as IL by immunohistochemistry in any part of the small intestine were finally included as IL dogs. Dogs with neoplastic disease, severe necrosis, or parasitic infectious diseases in the small intestine were excluded.

Histopathologic evaluation

Samples from the small intestine were fixed in 10% neutral buffered formalin and processed for routine histopathological analysis after paraffin embedding. Hematoxylin and eosin-stained sections were used for the histopathological diagnosis, performed by a single pathologist who was not blinded to the dogs' information. We diagnosed LPE when significant infiltration of lymphocytes and plasma cells was observed. Also, GL was diagnosed when granulomas around the lymphatic vessels in any layer of the small intestine were observed.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues were sectioned with 4μm thickness and used for immunohistochemical analysis. Heat-induced antigen retrieval was performed by autoclaving the samples for 20 min at 121°C in ethylenediaminetetraacetic acid buffer (pH 9.0) for Prox-1 and sodium citrate buffer (pH 6.0) for LYVE-1. For LYVE-1, endogenous peroxidase was blocked using 3% H_2O_2 in methanol for 3 min at room temperature. Next, the sections were blocked with 10% goat serum in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 30 min at room temperature and incubated overnight at 4°C with primary antibodies. Anti-Prox-1 antibody (rabbit polyclonal, 1:500; Abcam, cat. no. ab199359) and anti-LYVE-1 antibody (rabbit polyclonal, 1:2,500; Abcam, cat no. ab33682) were used in this study. After washing with TBS-T, the samples were incubated for 30 min at room temperature with alkaline phosphatase-labeled anti-rabbit IgG (Histofine Simple Stain AP (R), Nichirei Biosciences, Tokyo, Japan) for Prox-1 and with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (EnVision + System HRP Labeled Polymer Anti-Rabbit, DAKO, Glostrup, Denmark) for LYVE-1. For Prox-1, color was developed using a Histofine New Fuchsin Substrate Kit (Nichrei Biosciences, Tokyo, Japan) for 10 min. For LYVE-1, color was developed using 3,3-diaminobenzidine for 3 min. Counterstaining was performed with hematoxylin.

Immunohistochemical analysis

Slides stained with Prox-1 and LYVE-1 were used to evaluate the lymphatic vessel diameter and staining intensity. The tissues

were first observed using a light microscope with a 4× objective lens and a 10× eyepiece lens. In cases with several intestinal specimens, the specimen with the most significant lymphatic vessel dilation was chosen. A randomly chosen specimen was subjected to evaluation when multiple tissues showed a similar degree of lymphatic vessel dilation. The small intestine was divided into five areas: the villus lacteal, lamina propria, submucosa, muscularis, and mesentery. For evaluating the diameter and density of lymphatic vessels, five regions were chosen from each area using a 20× objective lens. The objective lens was changed to 10× when the lymphatic vessel was severely dilated beyond the screen. Digitized images were transferred to a computer for evaluation of the lymphatic diameter and number using imaging software (ImageJ, National Institute of Mental Health, Bethesda, MD, USA). All lymphatic vessels in each picture were evaluated, and the mean diameter was calculated for each layer. The number of lymphatic vessels was assessed as the density of the lymphatic vessels per 1 mm^2 in the lamina propria, submucosa, and muscularis.

The staining intensities of LYVE-1 and Prox-1 in the villus lacteal were assessed subjectively by one veterinarian and graded as positive (entire expression), weak (partial expression), or negative (no expression). In addition, the degree of dilation of the villus lacteal was assessed based on the width of the villus lacteal (mm) and the percentage of villus width (%). On average, 10 images of the villus lacteal were obtained from each dog, and the villus lacteals in each image were evaluated.

Statistical analysis

Analyses were performed using RStudio v.1.1.463 (RStudio, Boston, MA, USA). The age and body weight of the controls and dogs with IL-positive LPE or GL were compared using the Kruskal–Wallis test. The Mann–Whitney *U* test was used to compare the diameter and density of the lymphatic vessels between the control dogs and dogs with IL. The diameter and density of the lymphatic vessels were compared among the controls, IL-positive LPE, and IL-positive GL groups using the Dunn–Bonferroni test. Moreover, the relationship between the staining intensities of the lymphatic endothelial markers and the degree of dilation of the villus lacteal were also compared using the Dunn–Bonferroni test. In addition, the number of dogs with weak LYVE-1 staining or negative staining was compared between LPE and GL groups using Fisher's exact test. Finally, the correlation between plasma albumin concentration and lymphatic vessel diameter was evaluated using Pearson's correlation coefficient. Statistical significance was set at a *P*-value of <0.05.

RESULTS

Dogs

Sixteen dogs with IL were included in the study. Specimens from the small intestine were collected by autopsy in six dogs and full-thickness biopsy by laparotomy in ten dogs. The jejunal and ileal specimens were collected from all 16 dogs, while duodenal samples were available from only three of the six dogs that underwent autopsy. Therefore, immunohistochemical analysis was conducted using jejunal and ileal specimens. Based on the histopathological examination, 10 and six dogs were diagnosed with LPE and GL, respectively.

Nine dogs were included as controls. The causes of death in the control dogs were bronchopneumonia (2), mammary gland tumor (2), thromboembolism (1), acute myeloid leukemia (1), chronic kidney disease (1), and undiagnosed (1). In addition, one experimental dog euthanized for another study approved by the Animal Use and Care Committee of The University of Tokyo (P17-074) was also included as a control. The clinical information, including breed, sex, age, and body weight of the control, LPE, and GL groups, is summarized in Table 1. There was no significant difference in the age and body weight between the control dogs and dogs with LPE or GL (Kruskal–Wallis test, *P*>0.05). In addition, plasma albumin concentration was compared between dogs with LPE and dogs with GL, showing no significant difference (Mann–Whitney *U* test, *P*=0.058).

Table 1. Clinical information of the dogs included in the present study

	Control (9)	Lymphoplasmacytic enteritis (10)	Granulomatous lymphangitis (6)
Breeds (number of dogs)	Miniature dachshund (3) Beagle (1) Labrador Retriever (1) Pug (1) Toy Poodle (1) Yorkshire Terrier (1) Mixed breed (1)	German Shepherd Dog (2) Cavalier King Charles Spaniel (1) Chihuahua (1) Dalmatian (1) Labrador Retriever (1) Maltese (1) Pomeranian (1) Toy Poodle (1) Yorkshire Terrier (1)	Toy Poodle (2) American Cocker Spaniel (1) Boston terrier (1) Pomeranian (1) Toy Manchester Terrier (1)
Sex (number of dogs)	CM (2), SF (4), F (3)	CM (1), M (3), SF (5), F (1)	CM (5), F (1)
Age (years)	12.0 (2.0–16.0)	9.5 (4.9–14.0)	8.3 (3.8–13.0)
Body weight (kg)	6.9 (1.0–12.0)	10.2 (2.0–34.0)	4.9 (3.6–8.9)
Plasma albumin concentration (g/dl)	NA	2.1 (1.3–2.9)	2.9 (2.1–3.5)

Data are presented as the median and range in parentheses for age, body weight and plasma albumin concentration. CM, castrated male; M, male; SF, spayed female ; F, female; NA, not available.

Analysis of the lymphatic vessel diameter and density using Prox-1 staining

Representative images of slides stained with Prox-1 are shown in Fig. 1. As in previous reports, the nuclei were positively stained by Prox-1 in enteroendocrine epithelial cells in the crypts, venous valves, and lymphatic endothelial cells [3, 18, 25]. Therefore, we considered tubular structures positive for Prox-1 as the lymphatic vessels. All villus lacteals evaluated in the present study were positive for Prox-1 staining.

Since there are no defined diagnostic criteria for IL in the lamina propria, submucosa, muscularis, and mesentery according to the guidelines for histopathological evaluation [21], we determined IL by comparing the lymphatic vessel diameter with the reference ranges calculated from the values of control dogs. The upper limit of the reference range of the lymphatic vessel diameter in each layer was as follows: villus lacteal, 0.060 mm; lamina propria, 0.029 mm; submucosa, 0.028 mm; muscularis, 0.092 mm; and mesentery, 0.143 mm. In dogs with LPE, the lymphatic vessel diameter was above the reference range in all 10 dogs in the villus lacteal, 9/10 in the lamina propria, 9/10 in the submucosa, 7/10 in the muscularis, and 2/8 in the mesentery. As for dogs with GL, 5/6 in the villus lacteal, all six dogs in the lamina propria, submucosa, and muscularis, and 3/4 in the mesentery showed lymphatic vessel diameters above the reference range. The mesentery could not be evaluated in two of the 10 dogs with LPE and two of the six dogs with GL because the small intestinal specimens contained only the villus lacteal to muscularis layers.

Through the evaluation of the jejunal and ileal specimens in the 16 dogs with IL, the median lymphatic diameters in the villus lacteal, lamina propria, submucosa, muscularis, and mesentery were found to be 0.258 mm (range: 0.048–0.710 mm), 0.064 mm (range: 0.020–0.254 mm), 0.081 mm (range: 0.020–0.423 mm), 0.121 mm (range: 0.038–0.326 mm), and 0.122 mm (range: 0.065–0.298 mm), respectively. These were significantly larger than the 0.024 mm (range: 0.018–0.061 mm, $P<0.001$), 0.020 mm (range: 0.016–0.027 mm, $P<0.001$), 0.021 mm (range: 0.018–0.028 mm, $P<0.001$), 0.036 mm (range: 0.026–0.094 mm, $P<0.001$), and 0.066 mm (range: 0.030–0.146 mm, $P=0.004$), respectively, observed in the control dogs. In contrast, there was no significant difference in the number of lymphatic vessels between the controls and dogs with IL in any layer of the small intestine (all $P>0.05$).

Lymphatic vessel diameters were assessed in 10 dogs with LPE. The median lymphatic vessel diameters in the villus lacteal, lamina propria, submucosa, and muscularis were 0.276 mm (range: 0.095–0.542 mm), 0.065 mm (range: 0.020–0.179 mm), 0.069 mm (range: 0.020–0.423 mm), and 0.111 mm (range: 0.038–0.326 mm), respectively, which were significantly larger than those in control dogs (villus lacteal, $P<0.001$; lamina propria, $P=0.001$; submucosa, $P=0.004$; muscularis, $P=0.004$) (Fig. 2). The increasing tendency of the lymphatic vessel diameter was observed in the mesentery compared with the control specimens, although the difference was statistically insignificant ($P=0.058$). In addition, the lymphatic vessel diameters of six dogs with GL were evaluated. The median lymphatic vessel diameters in the villus lacteal, lamina propria, submucosa, muscularis, and mesentery were 0.233 mm (range: 0.048–0.710 mm), 0.061 mm (range: 0.030–0.254 mm), 0.164 mm (range: 0.065–0.299 mm), 0.126 mm (range: 0.093–0.228 mm), and 0.236 mm (range: 0.087–0.298 mm), respectively, which were significantly larger than those in control dogs (villus lacteal, $P=0.005$; lamina propria, $P=0.002$; submucosa, $P=0.001$; muscularis, $P=0.002$; mesentery, $P=0.007$) (Fig. 2). There was no significant difference in the diameter of the lymphatic vessels in each layer between dogs with LPE and GL. Moreover, there was no significant difference in the number of lymphatic vessels assessed as the lymphatic vessel density in the jejunum and ileum between the controls and dogs with LPE or GL.

The correlation between the plasma albumin concentration and lymphatic vessel diameter in the villus lacteal, lamina propria, submucosa, muscularis,
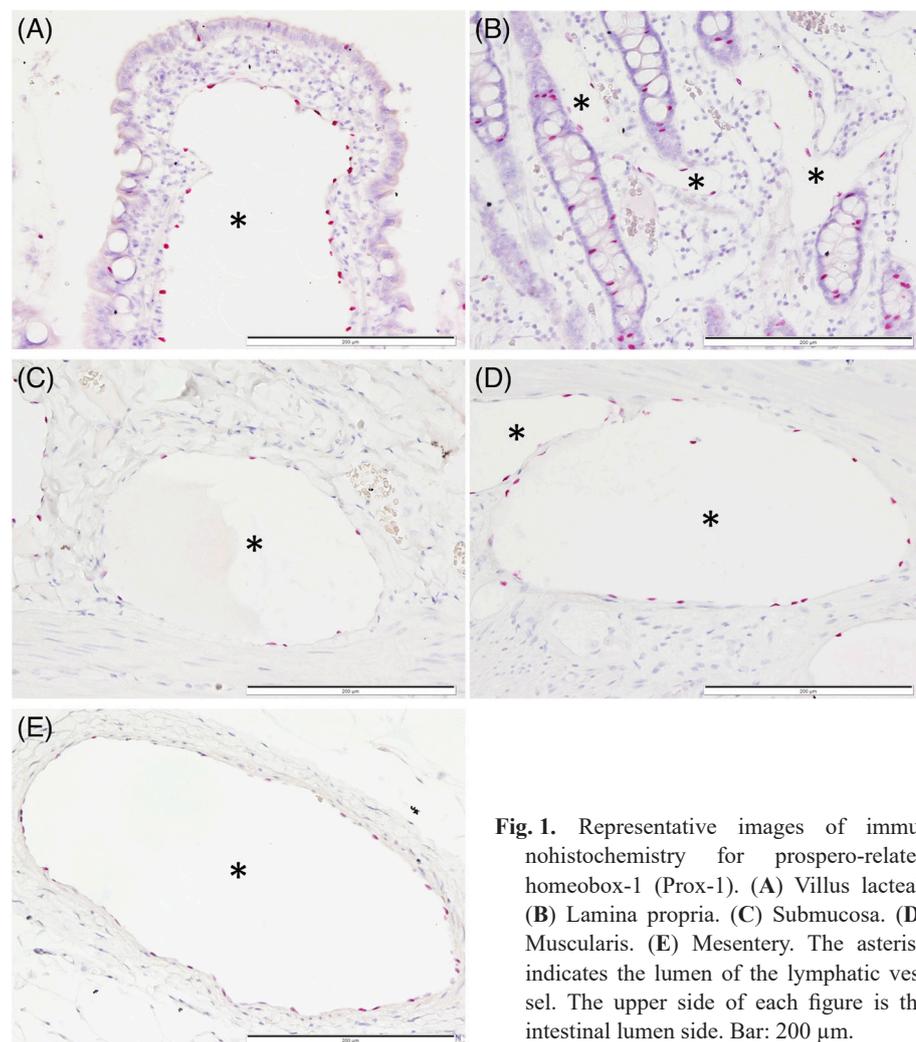

Fig. 1. Representative images of immunohistochemistry for prospero-related homeobox-1 (Prox-1). (**A**) Villus lacteal. (**B**) Lamina propria. (**C**) Submucosa. (**D**) Muscularis. (**E**) Mesentery. The asterisk indicates the lumen of the lymphatic vessel. The upper side of each figure is the intestinal lumen side. Bar: 200 µm.

and mesentery were also assessed. There was no significant correlation between the plasma albumin concentration and lymphatic vessel diameter in dogs with LPE (villus lacteal, $P=0.639$, r=0.246; lamina propria, $P=0.151$, r=0.664; submucosa, $P=0.159$, r=0.653; muscularis, $P=0.655$, r= −0.234; mesentery, $P=0.670$, r= −0.330). In dogs with GL, a significant negative correlation between the plasma albumin concentration and lymphatic vessel diameter was observed in the submucosa ($P=0.002$, r= −0.984) and muscularis ($P=0.029$, r= −0.916), whereas no significant correlation was detected in the villus lacteal ($P=0.919$, r= −0.063) and lamina propria ($P=0.101$, r= −0.793). The correlation could not be evaluated in the mesenteric layer because of the small number of dogs with GL.

Immunohistochemical analysis of LYVE-1 staining

Representative images of the slides stained with LYVE-1 are shown in Fig. 3. Lymphatic endothelial cells from the villus lacteal in which the cytoplasm was entirely stained, as shown in Fig. 3 (A), were defined as LYVE-1-positive. As shown in Fig. 3 (B and C), lymphatic endothelial cells in the villus lacteal that were partially- and not stained by LYVE-1 were defined as weakly LYVE-1 positive (B) and LYVE-1 negative (C), respectively. Figure 3 (D) shows the same villus lacteal location as in Fig. 3 (C) is positively stained with Prox-1, that is, Prox-1-positive/LYVE-1-negative staining. We evaluated the relationship between the degree of lymphatic vessel dilation and LYVE-1 staining intensity in villus lacteal. Lymphatic vessels unstained by LYVE-1 were identified based on anatomical findings. In the jejunum and ileum, 436 villus lacteal samples were evaluated, including 348 with positive staining, 60 with weak staining, and 28 with negative staining intensities. Weakly LYVE-1 positive or negative villus
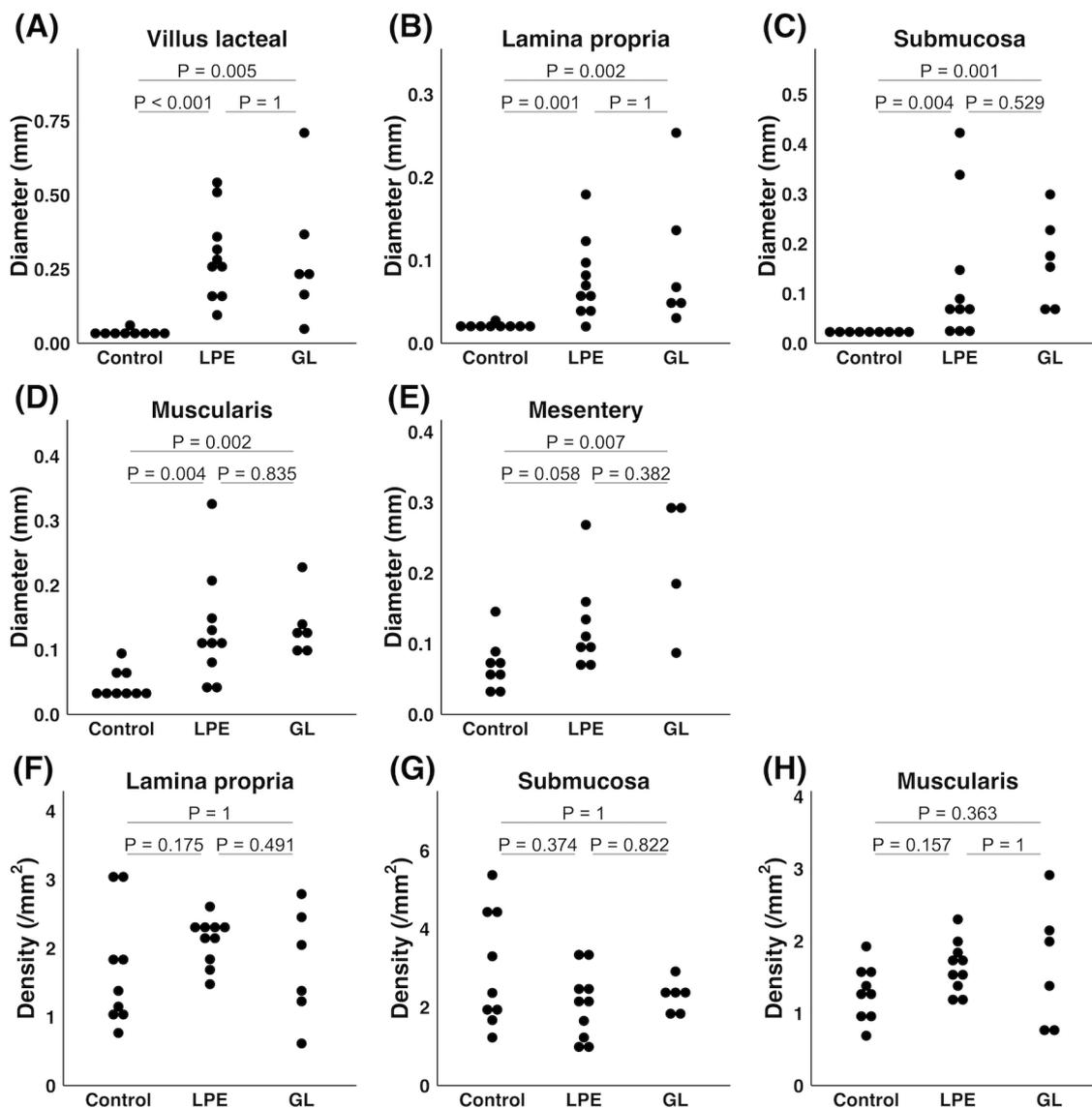

Fig. 2. Lymphatic vessel diameter in the villus lacteal (**A**), lamina propria (**B**), submucosa (**C**), muscularis (**D**), and mesentery (**E**) in the jejunum and ileum. The lymphatic vessel diameter was compared among control dogs, dogs with lymphoplasmacytic enteritis (LPE), and dogs with granulomatous lymphangitis (GL). The density of lymphatic vessels in the lamina propria (**F**), submucosa (**G**), and muscularis (**H**) were also compared. Statistical analysis was performed using the Dunn-Bonferroni test.

lacteals were found in six of the 10 dogs with LPE and four of the six dogs with GL. There was no significant difference in the prevalence of weakly LYVE-1 positive or negative villus lacteals between dogs with LPE and GL (P=1.0). The relationships between villus lacteal dilation and LYVE-1 staining intensity are summarized in Fig. 4. The median diameters of the weakly LYVE-1 positive and LYVE-1-negative villus lacteal specimens were 0.362 mm (range: 0.103–1.744 mm) and 0.475 mm (range: 0.177–1.076 mm), respectively. Both were significantly dilated compared with the 0.046 mm (range: 0.006–0.896 mm) observed in LYVE-1-positive villus lacteal samples (both P<0.001). The median percentages of villus lacteal in the villus width of weakly positive LYVE-1 and LYVE-1-negative specimens were 69% (range: 29–98%) and 84% (range: 43–97%), respectively, both of which were significantly dilated compared with 15% (range: 4–91%) observed in LYVE-1-positive villus lacteal specimens (both P<0.001). There were no significant differences in the villus lacteal width and percentage of the villus width between the weakly positive LYVE-1 and LYVE-1-negative villus lacteal (P>0.05).

DISCUSSION

The present results suggest that the lymphatic vessels in the deep layer of the small intestine, that is, the submucosa, muscularis, and mesentery, are dilated in dogs with IL. In contrast, there was no significant difference in the number of lymphatic vessels between the controls and dogs with IL. In addition, the differences between LPE and GL were not noticeable in terms of the lymphatic vessel diameter and density. Moreover, the LYVE-1 staining intensity and degree of villus lacteal dilation revealed severe dilation in villus lacteal specimens with a decreased LYVE-1 staining intensity.
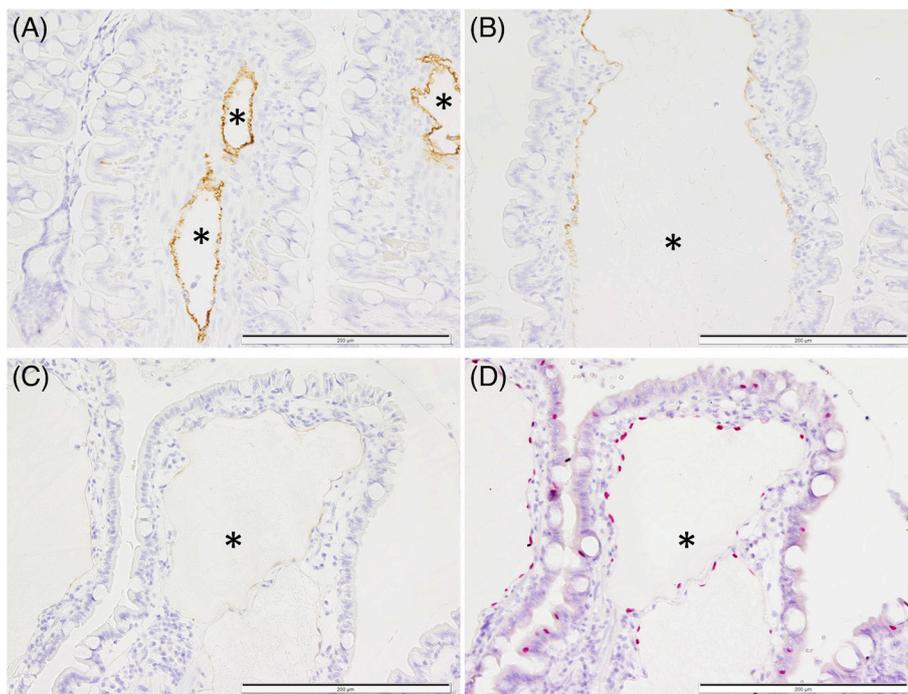

Fig. 3. Representative images of immunohistochemistry for lymphatic vascular endothelial receptor-1 (LYVE-1). (A) LYVE-1-positive villus lacteal sample. (B) Weak LYVE-1 signal in the villus lacteal. (C) LYVE-1-negative villus lacteal. (D) The same region as in (C) but stained with prospero-related homeobox-1 (Prox-1), showing Prox-1-positive and LYVE-1-negative staining in the villus lacteal. The asterisk indicates the lumen of the villus lacteal. The upper side of each figure is the intestinal lumen side. Bar: 200 µm.
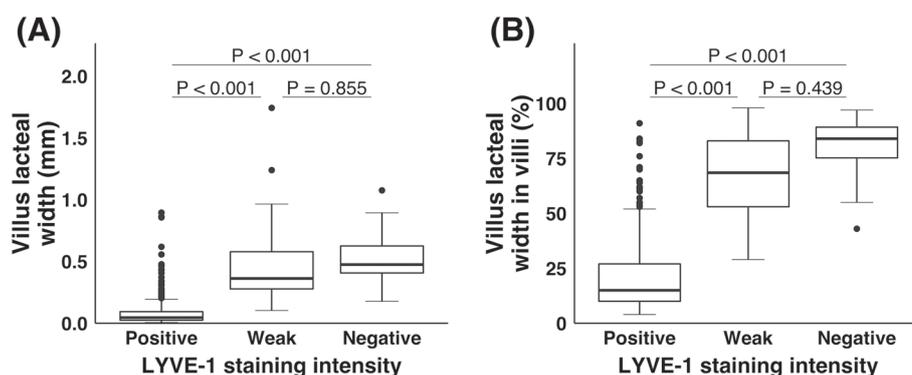

Fig. 4. Box-and-whisker plots of the relationship between the dilation of the villus lacteal and lymphatic vascular endothelial receptor-1 (LYVE-1) staining intensity in the jejunum and ileum. Dilation of the villus lacteal was evaluated according to the villus lacteal diameter (A) and percentage of the villus lacteal in villus width (B). The villus lacteals with weak and negative LYVE-1 staining showed significantly increased lymphatic diameter and percentages of the villus lacteal in the villus width compared to those with positive LYVE-1 staining. The statistical analysis was performed using the Dunn-Bonferroni test.

Contrary to our hypothesis, there was no significant difference in the number of lymphatic vessels. Microstructural abnormalities of lymphatic vessels can cause lymphatic vessel dilation in the small intestine. Lymphangiogenesis involves the growth, migration, and survival of lymphatic endothelial cells to regenerate lymphatic vessels and maintain the lymphatic structure [20]. Deficiency in lymphangiogenesis is a candidate pathogenetic factor in canine IL [10]. However, since the lymphatic density was not decreased in dogs with IL in this study, lymphatic vessel hypoplasia was not likely a cause of IL in our study dogs.

Lymphatic vessel diameter was significantly larger in dogs with IL than in control dogs. There are several explanations for the presence of IL in the small intestine and mesentery, including obstruction of lymphatic vessels and dysfunction in lymphatic contraction. Obstruction of lymphatic vessels is a possible explanation for canine IL in intestinal granulomatous or neoplastic diseases. In the present study, physical obstruction of lymphatic vessels may have occurred in the six dogs diagnosed with GL. Alternatively, dysfunction in lymph transport caused by decreased lymphatic smooth muscle contraction and lymphatic valve defects can also cause IL. A previous report observed decreased lymphatic pumping by increased inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β, through nitric oxide-mediated mechanisms [1, 4]. Therefore, the inflammatory response in the small intestine may cause dysfunction in lymphatic transportation due to decreased lymphatic vessel pumping in dogs with IL.

In contrast, there was no significant difference in the lymphatic vessel diameters between dogs with LPE and GL. In LPE, the lesions are observed mainly on the mucosal surfaces [8], whereas in GL, the lesions are primarily found in the muscularis, serous surfaces, and mesentery [15, 22]. We expected that there would be a difference in the distribution of IL between LPE and GL due to the difference in the vertical lesion sites. However, dilation of lymphatic vessels was observed in all layers of the small intestine in both disease groups with no significant difference. We hypothesized that the underlying mechanism of lymphatic vessel dilation was different between LPE and GL, resulting in diverse clinical features. However, it might be possible that a shared pathological condition exists, given that the distribution of IL was not different. Alternatively, the difference in the anatomical location of the lesion might be related to the underlying mechanism. When focused on the macroscopic distribution of the lesion, the gastrointestinal mucosa is often diffusely affected over a large area of the small intestine tissue in LPE [8, 19]. In contrast, GL forms focal mass lesions limited to a particular area, that is, the lower ileum [15, 22]. However, we could not evaluate the horizontal distribution of IL in the present study. Future studies are necessary to assess the distribution of IL over the small intestine in LPE and GL.

The results of the present study are consistent with those of previous reports that IL is observed not only in the villus lacteal but also in the lamina propria [24] or transmural [14] in dogs with chronic small intestinal diseases. Therefore, full-thickness intestinal specimens labeled with lymphatic endothelial cell markers may identify more lymphatic vessel abnormalities than endoscopic biopsy samples. However, an endoscopic biopsy is deemed safer and preferable compared to full-thickness biopsy in dogs with hypoalbuminemia, which often accompanies IL [5]. In this study, except for one GL case without IL in the villus lacteal, dilated lymphatic vessels were observed in both the villus lacteal and deep layers of the small intestine. Therefore, an endoscopic biopsy would be sufficient for IL diagnosis, although it cannot evaluate the muscularis or mesentery. However, it should be of note that dilation of lymphatic vessels also exists in the deep small intestine of dogs with IL.

There was no significant correlation between the plasma albumin concentration and lymphatic vessel diameter in dogs with LPE. In contrast, strong negative correlations between the plasma albumin concentration and lymphatic vessel diameter in the submucosa and muscularis were observed in dogs with GL. Therefore, it is possible that factors other than the IL-related lymph leakage, such as increased intestinal permeability secondary to mucosal inflammation and mucosal erosion or ulcer [5], are also involved in hypoalbuminemia in dogs with LPE. In contrast, dilation of lymphatic vessels is possibly the direct cause of hypoalbuminemia in dogs with GL.

Evaluation of villus lacteal dilation and LYVE-1 staining intensity suggested that the decreased LYVE-1 expression was related to severe dilation of the villus lacteal. However, no apparent relationship between the clinical parameters and reduced LYVE-1 expression was observed in the present study. Combined with the results of a previous study that reported no abnormalities in the lymphatic vessel structure and function in LYVE-1 knockout mice [7], a decrease in LYVE-1 expression in lymphatic endothelial cells might not be the cause of the dilation of the villus lacteal. A previous report suggested that inflammatory cytokines, including TNF-α or TNF-β, decrease LYVE-1 expression in lymphatic endothelial cells [12]. Also, TNF-α decreases the frequency of lymphatic vessel pumping [4], which may contribute to the dilation of lymphatic vessels. Thus, the overproduction of inflammatory cytokines such as TNF-α in the small intestine can cause both decreased LYVE-1 expression and dilation of the villus lacteal. Further studies are needed to investigate the physiological roles of LYVE-1 in lymphatic vessels and lymphatic endothelial cells. The villus lacteal with decreased LYVE-1 staining intensity maintained positive staining with Prox-1. A previous report showed that Prox-1 is a transcription factor essential for developing and maintaining lymphatic endothelial cell identity [13, 25]. Furthermore, downregulation of Prox-1 is sufficient for reprogramming lymphatic endothelial cells into blood endothelial cells [13]. The villus lacteals are anatomically evident and would not be missed; however, using LYVE-1 as a lymphatic endothelial marker would require some cautions. We assume that Prox-1 is superior to LYVE-1 in describing lymphatic vessels, especially lymphatic vessels with severe dilation.

The present study has some limitations. First, the sample size was small. Second, clinical information such as plasma biochemistry, clinical signs, medication, and treatment response was not evaluated. Finally, our study had a bias in that one-third of the dogs had small intestinal samples collected post-mortem.

In conclusion, the lymphatic vessels were dilated in the mucosal surface and deep layer of the small intestine, including the submucosa, muscularis, and mesentery, in dogs with IL. On the other hand, there was no significant difference in the lymphatic vessel diameter between dogs with LPE and GL. Furthermore, there was no significant difference in the number of lymphatic

vessels when compared to controls. Therefore, factors other than a decreased number of lymphatic vessels may contribute to the pathogenesis of IL in dogs. Moreover, the decreased staining intensity of LYVE-1 was associated with severe villus lacteal dilation. Further studies are required to clarify the cause and clinical importance of villus lacteal dilation and decreased LYVE-1 staining intensity, and to further identify the role of lymphatic abnormalities in the pathophysiology of chronic small intestinal disease in dogs.

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