J Toxicol Pathol 2025; 38: 37–42

Original Article

Lymphangiomas with the presence of erythrocytes in mesenteric lymph nodes of Wistar Hannover rats

Yuumi Awazuhara[1], Yuki Tomonari[1*], Hiroko Kokoshima[1,2], Yumi Wako[1], and Takuya Doi[1]

[1] Pathology Department, Kashima Laboratories, Non-clinical Business Segment, Mediford Corporation, Kamisu-shi, Ibaraki 314-0255, Japan
[2] Current affiliation: Pathology Department, Drug Safety Research Laboratories, Shin Nippon Biomedical Laboratories, Ltd., Kagoshima-shi, Kagoshima 891-1394, Japan

Abstract: We performed morphological and immunohistochemical analyses of erythrocyte-rich vascular proliferative lesions of mesenteric lymph nodes in six male and one female Wistar Hannover rats. These lesions are conventionally diagnosed as hemangiomas due to abundant erythrocytes. Immunostaining was positive for prospero-related homeobox 1 (Prox-1) and/or vascular endothelial growth factor receptor 3 (VEGFR3) in all lesions, suggesting a lymphangitic origin. In 6 of 7 lesions, von Willebrand factor (vWF) immunostaining was negative, suggesting a non-blood vascular origin. These results demonstrated that almost all hemangiomas in rat mesenteric lymph nodes were lymphangiomas. To the best of our knowledge, this is the first report highlighting the lymphatic origin of vascular proliferative lesions in the mesenteric lymph nodes of rats. (DOI: 10.1293/tox.2024-0007; J Toxicol Pathol 2025; 38: 37–42)

Key words: rat, lymphangioma, hemangioma, mesenteric lymph node, vascular endothelial growth factor receptor 3 (VEGFR3), prospero-related homeobox 1 (Prox-1)

Introduction

Vascular proliferative lesions in the rat mesenteric lymph nodes have occasionally been observed in long-term toxicity studies[1–5]. Vascular proliferative lesions in the lymph nodes are conventionally diagnosed as hemangiomas owing to the abundance of erythrocytes in the vessel, which has been considered to be a point of differentiation from lymphangiomas[4, 6, 7]. However, angiomatous hyperplasia in the rats that shows similar morphology with hemangiomas have been described as vascular transformation of lymphatic sinuses, which includes plexiform vasculopathy in cats and angiomatosis in humans[2, 3, 6]. No reports have examined the origin of vascular lesions in the rat mesenteric lymph nodes using immunostaining. Because blood vessels and lymphatic vessels have very similar morphologies, immunostaining using a vascular epithelial cell marker (CD31), blood vessel endothelial marker von Willebrand factor (vWF), and lymphatic endothelial markers prospero-related homeobox 1 (Prox-1) and vascular endothelial growth factor receptor 3 (VEGFR3) were used to distinguish between them[8–11]. In this report, we describe in detail the morphological and immunohistochemical characteristics of vascular proliferative lesions that were morphologically diagnosed as hemangiomas using hematoxylin-eosin (HE)-stained specimens.

Received: 25 January 2024, Accepted: 21 August 2024
Published online in J-STAGE: 13 September 2024
*Corresponding author: Y Tomonari
(e-mail: yuki.tomonari@mediford.com)

Materials and Methods

Eight vascular proliferative lesions in the mesenteric lymph nodes of seven males and one female were observed in a 104-week background-collecting study (200 males and 200 females) using Wistar Hannover rats (Crl:WI (Han), Charles River Laboratories Japan Inc., (Yokohama, Japan). Animals, including post-mortem or moribund necropsy, ranged from 64 to 110 weeks of age. No mesenteric lymph node lesions caused death or moribundity. Organs collected at necropsy were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 4 µm, and stained with HE. The original diagnosis of these lesions using HE-stained specimens was hemangioma. The lesions of six males and one female were subjected to immunohistochemical investigations. In one male (No. 8), the immunohistochemical investigation could not be performed because the lesion was too small to be sectioned. Information on the animals with vascular proliferative lesions is shown in Table 1.

Slides of lesions diagnosed as hemangioma were re-examined microscopically and analyzed immunohistochemically using antibodies against vWF, Prox-1, VEGFR3, and

CD31 to identify vascular epithelial cells, and further against α-smooth muscle action (α-SMA) to identify vascular structure and interstitial components[8–10]. In addition, silver staining (Watanabe's method for the reticulum) was performed to identify interstitial cellular components. Immunostaining with an anti-proliferative cell nuclear antigen (PCNA) antibody was performed to examine cell proliferative activity. Normal lymphatics (Prox-1, VEGFR3, and CD31), blood vessels (vWF and CD31), and intestinal tract (PCNA) on the same slides of the mesenteric lymph node lesions were used as positive controls[11]. Lymph node sections were deparaffinized and rehydrated in a descending ethanol series. Endogenous peroxidase activity was blocked using 3% H_2O_2. Subsequently, antigen retrieval was performed using one of the following methods: protease (Code: 415231, Nichirei, Tokyo, Japan) (vWF), heat induction (121°C, 10 min) in pH9 antigen retrieval solution (Code: 415211, Nichirei) (Prox-1), or microwave heating (10 to 20 min) in pH9 antigen retrieval solution (Code: 415211, Nichirei) (VEGFR3 and CD31) or pure water (PCNA). Immunohistochemistry (IHC) was performed using the reagents listed in Table 2. Histofine Simple Stain Rat MAX PO (MULTI) (Code:414191, Nichirei) or Histofine Simple Stain Rat MAX PO(G) (Code:414331, Nichirei) were used as secondary antibodies. Liquid DAB+ Substrate Chromogen System (Code: 3468, Agilent (Dako), Santa Clara, CA, USA) was used as the chromogen. Hematoxylin (Tissue-Tek) was used for nuclear staining. The intestinal epithelium was PCNA-positive, and the intestinal smooth muscle and its vascular smooth muscle were α-SMA-positive.

In the background-collecting study, rats were orally administered drinking water daily, and animals that died during the administration period were necropsied upon discovery; those found in moribund conditions were anesthetized and euthanized by exsanguination from the abdominal aorta and then subjected to necropsy. Surviving animals were euthanized under anesthesia (in the same manner as the moribund animals) and subjected to necropsy at 110 weeks of age. The animals were cared for and euthanized according to the principles outlined in the Guidelines for the Care and Use of Laboratory Animals of the Japanese Association of Laboratory Animal Science and the guidelines of our institution.

Table 1. Animal Information

Animal no.	Sex	Weeks old	Fate
1	Male	64 w	Moribund
2	Male	91 w	Death
3	Male	110 w	Scheduled sacrifice
4	Male	110 w	Scheduled sacrifice
5	Male	97 w	Death
6	Male	110 w	Scheduled sacrifice
7	Female	110 w	Scheduled sacrifice
8	Male	110 w	Scheduled sacrifice

Results

Macroscopic findings

At necropsy, an enlarged mesenteric lymph node with a dark reddish cut surface was found as corresponded to a vascular proliferative lesion in only one case (No. 5), whereas there were no evident findings in the remaining seven cases.

Microscopic findings

Microscopically, nodular lesions of various sizes were observed within the lymph nodes in all the cases. Proliferating cells were eosinophilic and spindle-shaped with small, round to oval nuclei, and formed vascular structures (Fig. 1A, 1B, 1D and 1E). In six cases, these vascular structures included abundant erythrocytes with very few white blood cells (Fig. 1B and 1E). The proliferative cells were slightly larger than the endothelium lining normal lymphatic sinuses, although no cellular and/or nuclear atypia was observed. Interstitial connective tissue was found between the vascular structures. In two male cases with large nodules (Nos. 1 and 5) and the female case (No. 7), interstitial components, including spindle cells, were predominant, in which vascular formation was unremarkable (No. 1). Silver staining revealed the presence of microvascular structures (Fig. 1C). In only one case, smooth muscle-like cells were observed around some vessels in a limited area (No. 5). In all cases, blood absorption was observed in the medullary lymphatic sinuses surrounding the nodules (Fig. 1F). Blood absorption included white blood cells as well as erythrocytes, which were different from the proliferative vascular structures filled with almost only erythrocytes (Fig. 1B and 1E).

Immunohistochemical analysis

The immunohistochemical results are shown in Table 3.

In the lesions, proliferative spindle-shaped cells showed as CD31- (Fig. 2A), Prox-1- (Fig. 2B), and VEGFR3-positive (Fig. 2C) in all cases except for two (Nos. 2 and 5) that were not stained for Prox-1. In both cases, Prox-1

Table 2. Reagents Used for Immunohistochemistry

Antibody	Abbreviation	Supplier	Host	Clonality	Cat No.	Dilution
Alpha smooth muscle actin	α-SMA	Abcam	Rabbit	Poly	ab5694	1/1000
Prospero-related homeobox1	Prox1	AngioBio Co.	Rabbit	Poly	11-002P	1/200
Vascular endothelial growth factor receptor 3	VEGFR3	R&D	Goat	Poly	AF743	1/200
Platelet endothelial cell adhesion molecule-1	CD31	Abcam	Rabbit	Mono	ab182981	1/400
von Willebrand factor	vWF	DAKO	Rabbit	Poly	A0082	1/2000
Proliferating cell nuclear antigen	PCNA	DAKO	Mouse	Mono	M0879	1/50

were designated "not determinable" because normal lymphatics were not stained for Prox-1, suggesting the effects of post-mortem changes and/or long-term formalin fixation. In No. 1, a small number of proliferative cells at the marginal area of the lesion were VEGFR3-positive, and the staining was weaker than that in the other rats. In No. 5, the vascular structure area was α-SMA-negative (Fig. 3A), vWF-negative (Fig. 3B), and VEGFR3-positive (Fig. 3C), suggesting lymphangiogenesis. vWF was positive at the limited proliferative cells in the abundant interstitial components

Table 3. Immunohistochemistry

Animal no.	CD31	Prox-1	VEGFR3	vWF	α-SMA
1	+	+	+	−	−
2	+	ND	+	−	−
3	+	+	+	−	−
4	+	+	+	−	−
5	+	ND	+	+[a]	+[a]
6	+	+	+	−	−
7	+	+	+	−	−

+: Positive; −: Negative; ND: Not determined. a: The positivity was limited to part of the lesion.

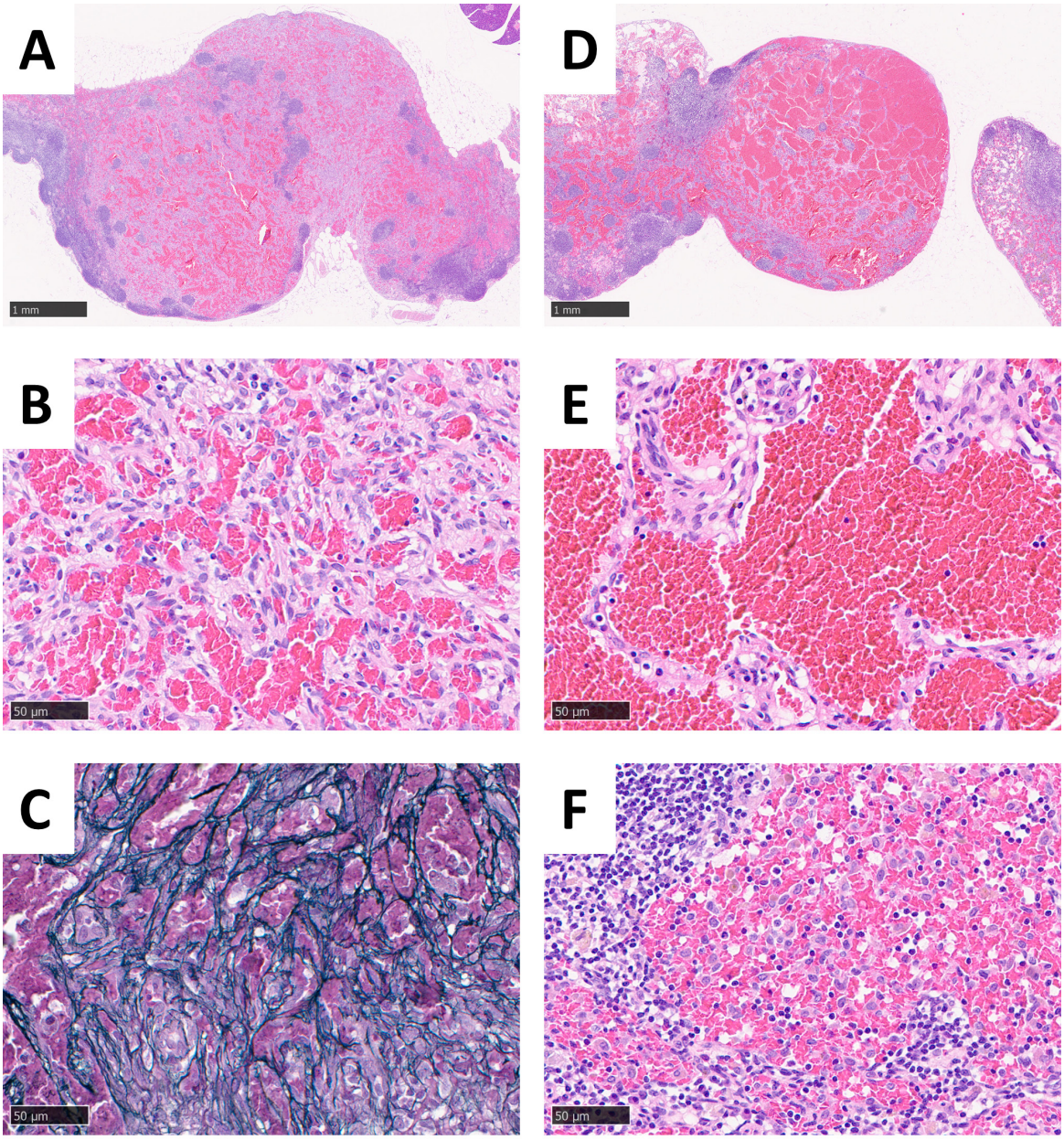

Fig. 1. HE stain, No. 1 and No. 3. No. 1 (A, B, C, F). No. 3 (D, E). Large nodules were seen within the lymph nodes (A, D). Vascular structures were formed by eosinophilic spindle-shaped cells with small round to oval nuclei (B, E). Interstitial connective tissues were found between the vessels, with No. 1 having a greater amount of interstitial components than No. 3, with spindle cells present between the vessels. Silver stain showed the presence of micro-vascular structures (C). Blood absorption was observed at the medullary lymphatic sinuses surrounding the nodule including white blood cells as well as erythrocytes (F). In the proliferative vascular structures, almost all blood absorption was only erythrocytes (B, E). A and D: Bar=1 mm. B, C, E, and F: Bar=50 µm.

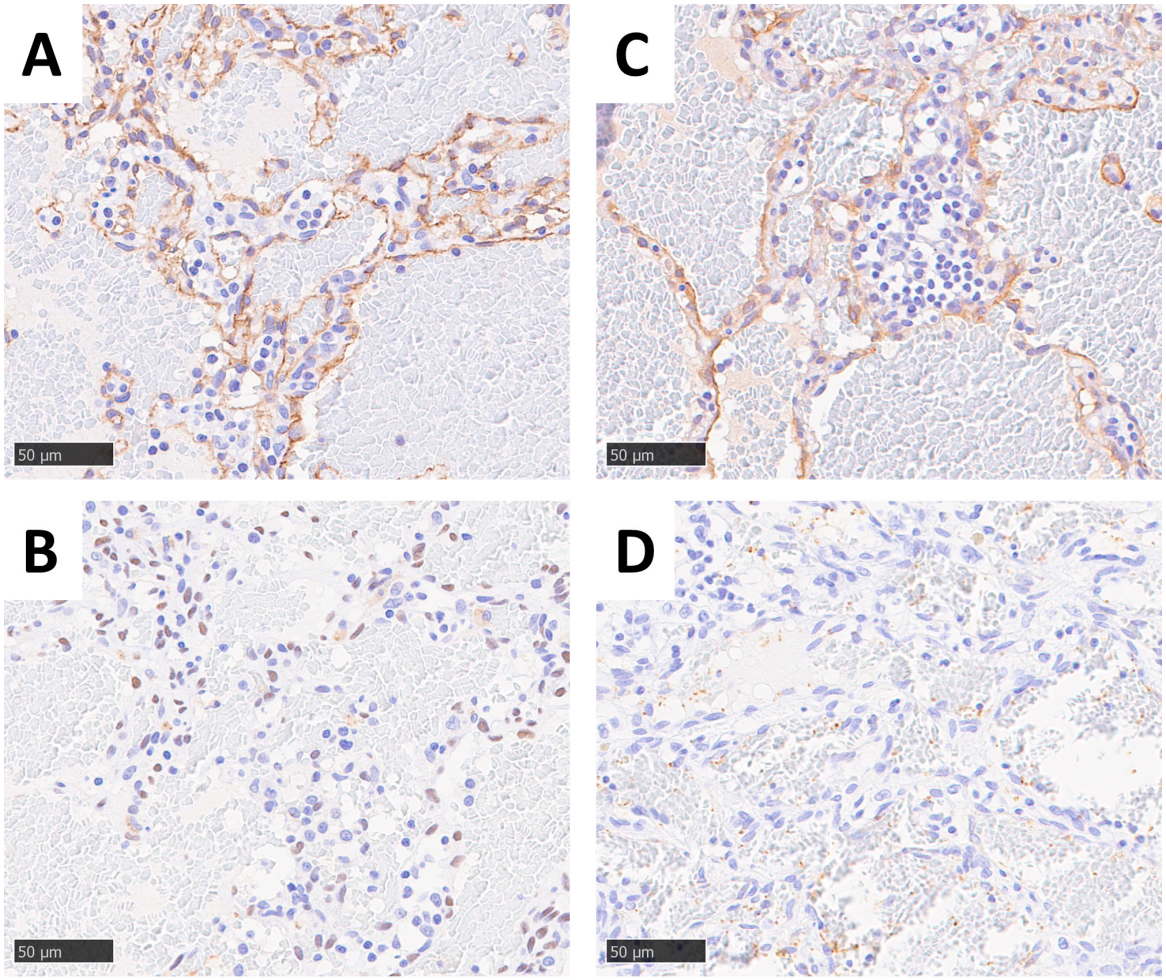

Fig. 2. Immunohistochemical features of case No. 3. Spindle cells with small, round to oval nuclei were proliferating and forming vessels. Spindle cells were positive for CD31 (A), Prox-1 (B), and VEGFR3 (C), and negative for vWF (D). Bar=50 µm.

(Fig. 3E). The vWF-positive and VEGFR3-positive (Fig. 3F) vessels were surrounded by α-SMA-positive smooth muscle (Fig. 3D). A few vessels included both vWF- and VEGFR3-positive cells, but each positivity was observed in a different region of one vessel (Fig. 4A and 4B). PCNA-positive cells were observed in all cases (range: 13 to 124/20 high power fields).

Discussion

In long-term toxicity studies, dilated vessels filled with erythrocytes were occasionally observed as hyperplastic lesions in rat mesenteric lymph nodes[1, 4, 5, 7]. Based on histological features, a diagnosis of hemangioma, hemangiosarcoma, or angiomatous hyperplasia was made. Since the detection of VEGFR3 specifically expressed in the lymphatic vessel endothelium in 1995[12, 13], it has become possible to identify lymphatic and blood vessels, and various projects have made advances[12–14]. Jungwirth *et al.* reported a rare plexiform vasculopathy in the cervical lymph nodes of cats, which was considered as lymphatic origin[2].

In the seven cases examined immunohistochemically, vascular proliferative lesions in rat mesenteric lymph nodes were positive for lymphatic markers (Prox-1 and/or VEGFR3) and negative for blood vessel marker (vWF), except for a limited area in No. 5. In No. 5, the positivity of vWF was limited to the α-SMA-positive area which has the presence of vascular smooth muscle-like structures. This suggests differentiation into blood and lymphatic vessels. Furthermore, some vessels were positive for vWF and VEGFR3. Each positivity was observed in a different region of one vessel and the VEGFR3-positive area was predominant, suggesting that most may differentiate into lymphatic vessels. In all vascular lesions in the mesenteric lymph nodes, immunohistochemical results suggested that the origin of the proliferative cells was considered to be lymphatic vessels regardless of the presence of erythrocytes in the vessel.

Lymphangiomas have been considered not contain erythrocytes within the vessel[4, 6]. The origin of the erythrocytes observed in the present study is unclear. Blood absorption was observed in the tissues surrounding the lesions; however, the relationship was unclear because the erythrocyte composition differed between the surrounding area and within the lesions.

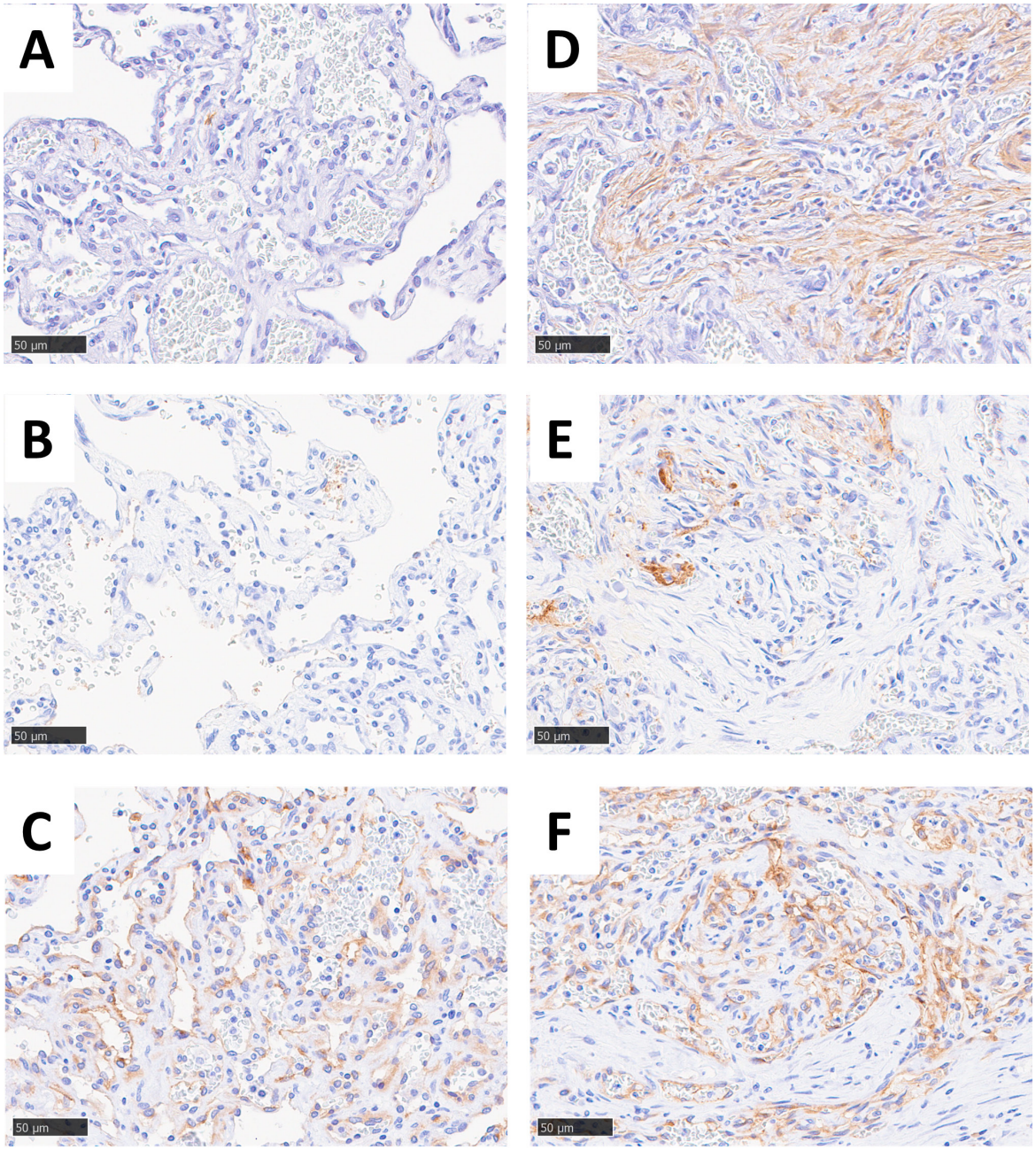

Fig. 3. Immunohistochemical features of case No. 5. The vascular structure area (A–C) was α-SMA-negative (A), vWF-negative (B), and VEGFR3-positive (C), suggesting lymphangiogenesis, similar to the other rats. In the limited area of the interstitial components (D–F), the vWF-positive (E) and VEGFR3-positive (F) vessels were surrounded by α-SMA-positive smooth muscle (D), suggesting blood vessel proliferation. The positivity of vWF was limited to the α-SMA-positive area. Bar=50 µm.

In a report on cats, proliferative endothelial cells expressed CD31 and factor VIII antigens, and 80% of the proliferative endothelial cell nuclei were Prox-1-positive, suggesting that the proliferative cells originated from lymphatic vessels[2]. Despite a clear expression of vWF was observed in cat plexiform vasculopathy[2], vessels were negative for vWF in rats in our results, and it may be necessary to search for a relationship with the lymphangioma reported here.

In conclusion, immunohistochemical analysis of vascular proliferative lesions in the mesenteric lymph nodes of seven rats showed Prox-1 and/or VEGFR3 positivity in all cases and vWF negativity in all cases except for one case, suggesting that most vascular proliferative lesions in rat mesenteric lymph nodes are likely to be lymphangiogenic in origin, even in the presence of erythrocytes. However, because it is difficult to differentiate morphologically between blood and lymphatic vessels, it is recommended to give a diagnosis of angioma (hemangioma/lymphangioma) when diagnosing by morphology without immunohistochemical staining.

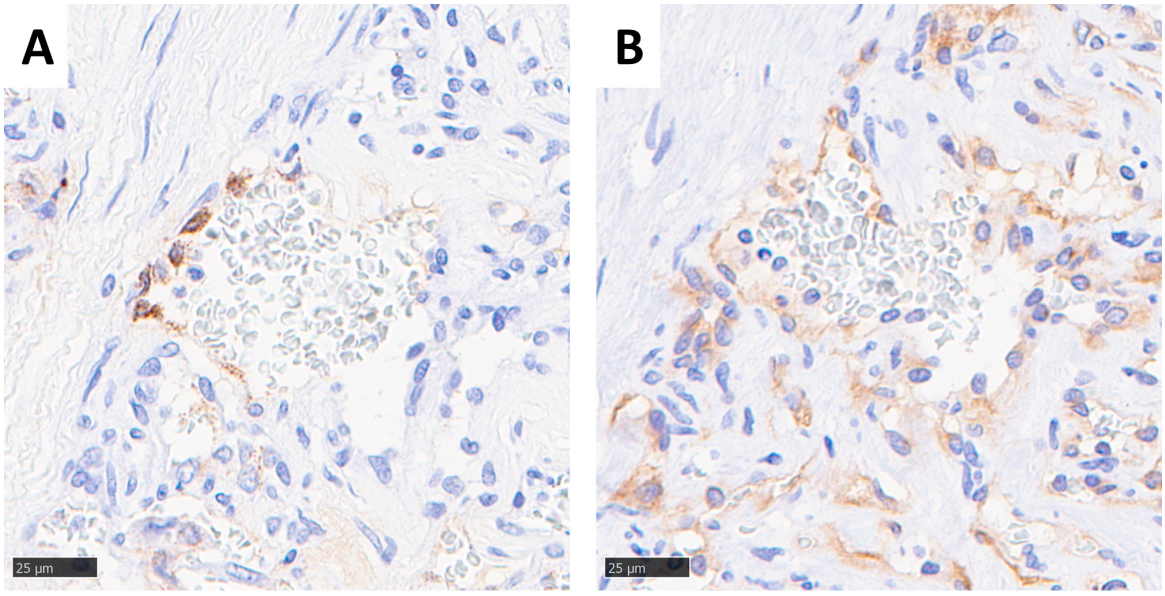

Fig. 4. Immunohistochemical features of case No. 5. Positive staining for vWF (A) and VEGFR3 (B) was observed in different regions within one vessel. Bar=25 µm.

Funding: The authors received no financial support for the research, authorship, or publication of this article.

Disclosure of Potential Conflicts of Interest: The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

Acknowledgment: We are grateful to Dr. Takeshi Izawa (Osaka Metropolitan University) for assistance with this study. We thank Mr. Takayoshi Ito, Ms. Chie Shiroumaru, and Mr. Hideyuki Watanabe for their technical assistance.

References

1. Reindel JF, Dominick MA, and Gough AW. Mesenteric lymph node hemangiomas of Wistar rats. Toxicol Pathol. **20**: 268–273. 1992. [Medline] [CrossRef]
2. Jungwirth N, Junginger J, Andrijczuk C, Baumgärtner W, and Wohlsein P. Plexiform vasculopathy in feline cervical lymph nodes. Vet Pathol. **55**: 453–456. 2018. [Medline] [CrossRef]
3. Nelissen S, and Chamanza R. An Angiomyomatous hamartoma with features of vascular transformation of sinuses in the mediastinal lymph node of a beagle dog. Toxicol Pathol. **48**: 1017–1024. 2020. [Medline] [CrossRef]
4. Willard-Mack CL, Elmore SA, Hall WC, Harleman J, Kuper CF, Losco P, Rehg JE, Rühl-Fehlert C, Ward JM, Weinstock D, Bradley A, Hosokawa S, Pearse G, Mahler BW, Herbert RA, and Keenan CM. Nonproliferative and proliferative lesions of the rat and mouse hematolymphoid system. Toxicol Pathol. **47**: 665–783. 2019. [Medline] [CrossRef]
5. Piccicuto V, Dincer Z, Walker S, and Gray C. Angiomatous hyperplasia in the heart of a young rat. J Toxicol Pathol. **33**: 29–32. 2020. [Medline] [CrossRef]
6. Radi ZA, and Morton D. Human safety risk assessment of lymph node angiomas observed in 2-year carcinogenicity studies in rats. Regul Toxicol Pharmacol. **64**: 435–441. 2012. [Medline] [CrossRef]
7. Hendrick MJ. Lymphangioma and lymphangiosarcoma. In: Tumor in Domestic Animals, 5th ed. DJ Meuten (ed). Washington, D.C.: John Wiley & Sons, Inc.; 2017. p. 142–175.
8. Ratajska A, Jankowska-Steifer E, Czarnowska E, Flaht A, and Radomska-Leśniewska D. [Morphogenesis, structure and properties of lymphatic vessels]. Postepy Hig Med Dosw. **66**: 901–912. 2012 (in Polish). [Medline] [CrossRef]
9. Bridenbaugh EA, Nizamutdinova IT, Jupiter D, Nagai T, Thangaswamy S, Chatterjee V, and Gashev AA. Lymphatic muscle cells in rat mesenteric lymphatic vessels of various ages. Lymphat Res Biol. **11**: 35–42. 2013. [Medline] [CrossRef]
10. Furukawa S, Nagaike M, and Ozaki K. Databases for technical aspects of immunohistochemistry. J Toxicol Pathol. **30**: 79–107. 2017. [Medline] [CrossRef]
11. Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, Skobe M, and Skobe M. Molecular characterization of lymphatic endothelial cells. Proc Natl Acad Sci USA. **99**: 16069–16074. 2002. [Medline] [CrossRef]
12. Tammela T, and Alitalo K. Lymphangiogenesis: Molecular mechanisms and future promise. Cell. **140**: 460–476. 2010. [Medline] [CrossRef]
13. Sato J, Doi T, Wako Y, Hamamura M, Kanno T, Tsuchitani M, and Narama I. Histopathology of incidental findings in beagles used in toxicity studies. J Toxicol Pathol. **25**: 103–134. 2012. [Medline] [CrossRef]
14. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, and Alitalo K. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA. **92**: 3566–3570. 1995. [Medline] [CrossRef]