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Histological similarity of primo vascular systems derived from three internal organs of rats

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ABSTRACT

Background: The primo vascular system has been suggested as the third circulatory system. Although primo tissues have been observed in various tissues isolated from a number of animal species, including mice, rats, and rabbits, very few reports on the histological characteristics of primo tissues from different body parts are available. This study was designed to compare the histological characteristics of primo tissues isolated from different body parts of rats.

Methods: Rats were anesthetized and operated on, to locate primo tissues. The primo tissues were searched at the all organs' surface and in lymphatic vessels using suitable finding methods. The tissues found were then separated and observed by histological test methods.

Results: This histological study revealed that there was no difference between the histological characteristics of the organ-surface primo tissues, the primo tissues inside lymphatic vessels, and the primo tissues on the falciform ligament. Moreover, primo tissues could be differentiated from those with a similar structure, such as lymphatic vessels and blood vessels, by immunostaining against a-LYVE-1 and a-CD31, and can be observed by specific immunostaining against the Von Willebrand factor (vWF).

Conclusion: The results of this study support the fact that primo tissues could possibly constitute a third circulatory system in the whole-body network.

1. Introduction

The primo vascular system was first suggested by Kim[1] in the 1960s. Since then, it has been established as the third circulatory system along with the meridian pathway and acupuncture points. According to the theory of acupunctural therapy, the meridian system, which is a whole-body network system that is known as the pathway for Qi energy, constitutes the major portion of the human body. Although the Bonghan theory was postulated to support the existence of the meridian pathway, scientific evidence accounting for the meridian pathway is very limited.

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Visualization of primo tissues is critical to finding evidence that will explain the meridian system. However, primo tissues, which consist of very thin, ivory white vessels, are not apparent to the naked eye. Therefore, many efforts have been made to visualize primo tissues using various staining methods. Yoo et al[2] applied trypan blue staining to the abdominal cavity in order to observe the primo vascular system and differentiate it from the lymphatic system in a lung-cancer mouse model. Lee et al[3] used Janus Green B to visualize the primo tissues observed inside the rabbit's lymphatic vessels. The microstructure of the visualized primo tissues was then analyzed using confocal laser scanning microscopy. In addition, Lee et al[4] suggested evidence to account for novel structures (primo vessels and primo nodes) floating in the venous sinuses of rat brains by differentiating the primo tissues from thrombus using a chromium–hematoxylin solution.

For the primo vascular system to function as a network for signal transduction and substance transportation in the body, the primo tissues found in different parts of body should have identical characteristics and should be connected to each other through a close networking system. Therefore, any findings supporting this assumption would provide evidence to account for the existence of a networking structure that connects primo tissues. The questions to be investigated in the next step should include how does the structure of the network system that connects the primo tissues actually looks like, whether the primo tissues are systematically connected, and what the histological characteristics of primo tissues isolated from different body parts are like.

Thus, this study aims to investigate network formation among primo tissues by comparing the histological and immunological characteristics of organ-surface primo tissues, the primo tissues inside lymphatic vessels, and primo tissues on the falciform ligament.

2. Methods

2.1. *Preparation of primo tissues*

Male 8–9-week-old Sprague-Dawley strain rats (Dae Han Bio Link, Chungbuk, Korea) weighing 250–320 g were used for this study. The study animals were anesthetized by an intramuscular injection of 20% urethane (dose: 1.5 mg/kg; Sigma-Aldrich) in the left femoral vein. The organ-surface primo vessels were collected as white translucent masses or striated tissues. The primo tissues observed on the falciform ligament were removed by pulling them with a forceps while lifting up the chest section after checking for the existence of white tissue, which was believed to consist of corpuscles. To collect primo tissues from lymphatic vessels, the entire lymphatic vessel was removed after checking for the existence of the primo duct and the primo node, and the stained part inside the lymphatic vessel was identified exclusively by injecting 0.2% alcian blue in phosphate-buffered saline into the lymph node. The primo tissues inside lymphatic vessels were prepared by either using the whole lymphatic vessel with the primo tissue retained within it or isolating the tissue removed by forceps from the lymphatic vessel. The primo tissues were observed under a stereo microscope (SM1500; Nikon, Japan).

2.2. *Histology and immunohistochemistry*

The primo tissues were fixed in neutral phosphate-buffered formalin (pH 7.4), embedded in an optimal cutting

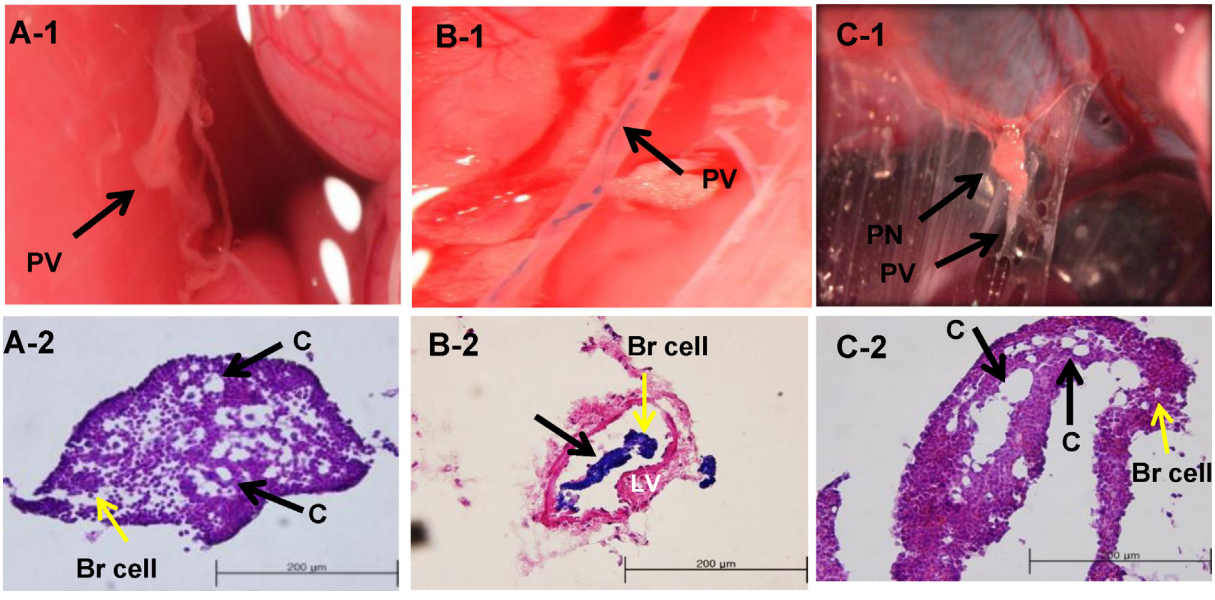

Fig. 1 – Histological study of the primo tissues inside lymphatic vessels, on the organ surfaces, and on the falciform ligament of a rat. (A-1 and A-2) Organ-surface primo tissue found on the left abdominal wall of a rat. (B-1 and B-2) Primo tissue inside a lymphatic vessel visualized using alcian blue dye. Primo tissue stained with alcian blue is observed inside the lymphatic vessel (LV). (C-1 and C-2) Primo tissue attached on the falciform ligament. (A-1, B-1, and C-1) Photos of primo node (PN) and primo vessel (PV) taken with a stereomicroscope. (A-2, B-2, and C-2) Results of hematoxylin and eosin (HE) staining (400 ×). Primo tissues have many cavities (C) and their plasma showed relatively bright staining (Br cell). The HE staining confirmed that all three primo tissues are histologically identical.

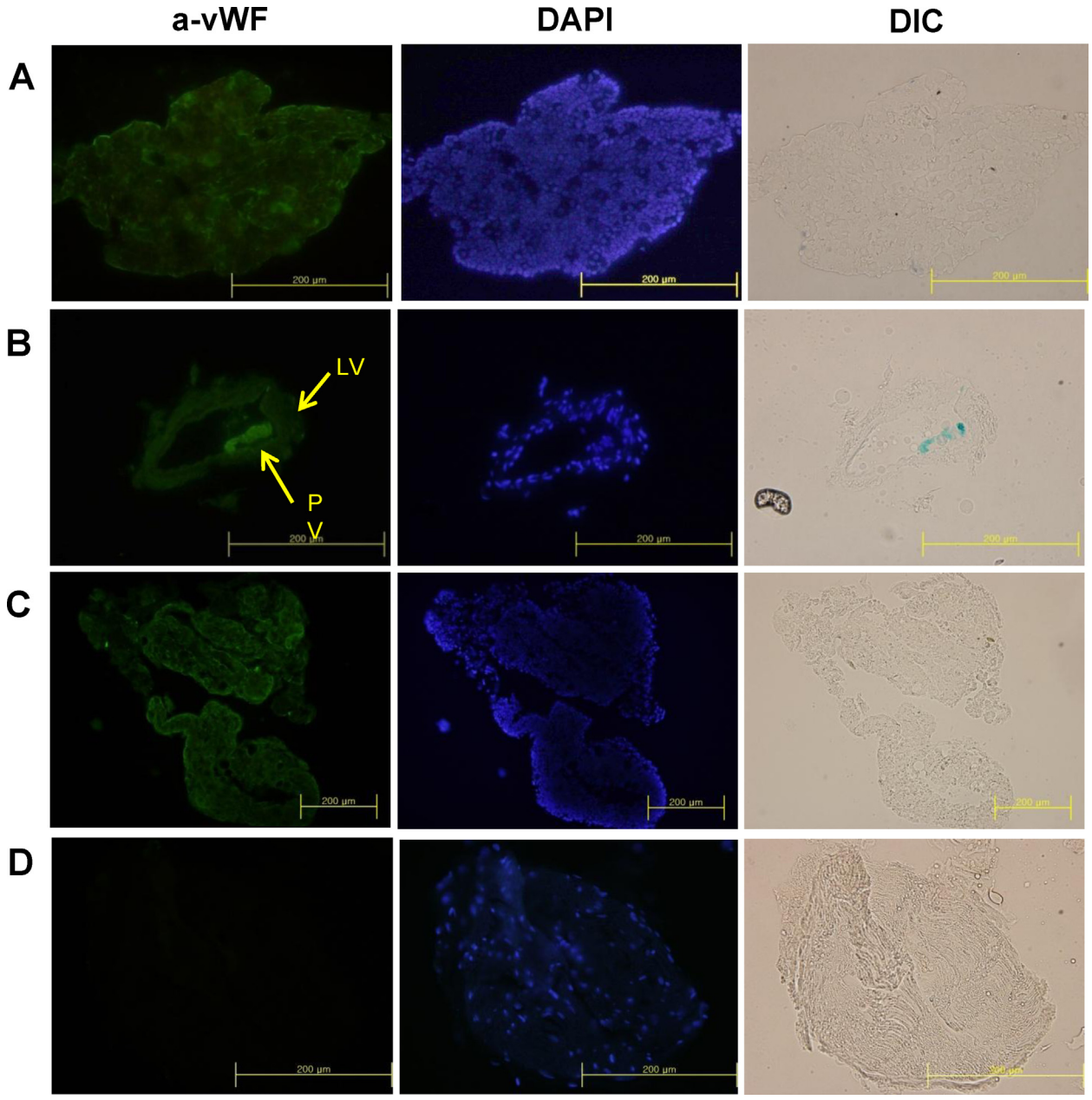

Fig. 2 – Immunofluorescence staining of the primo tissues with Von Willebrand factor (vWF). The a-vWF antibody (green) was used to stain primo tissues. Primo tissues [primo vessel (PV)] strongly responded to a-vWF, whereas lymphatic vessels (LVs) showed a weak response. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei. (A) Organ-surface primo tissue (400 ×); (B) primo tissue inside the LV (400 ×); (C) primo tissue on the falciform ligament (200 ×); (D) nerve (400 ×). DIC, differential interference contrast.

temperature compound, and cut into 8-μm thin sections using a cryotome (CM-3050-S; Leica, Germany). The cell nuclei and basic plasma structure of the primo tissues were observed by hematoxylin and eosin (HE) staining.

The nonspecific binding of antibodies to the sections was blocked with 5% normal goat serum, and endogenous enzyme activities were inhibited by adding a mixture of MeOH and H_2O_2 at a ratio of 5:1. Antibodies against LYVE-1 (Cambridge, United Kingdom, Abcam), CD31 (Cambridge, United Kingdom, Abcam), and Von Willebrand factor (vWF; Cambridge, United Kingdom, Abcam) were used for immunostaining. We used a fluorescein isothiocyanate-conjugated secondary antibody (Alexa Fluor 488 goat antimouse and goat antirabbit, Carlsbad, CA, Invitrogen) as a fluorescent molecular marker for 1–2 hours at room temperature (22–25 °C) in the dark. These steps were performed using the recommended dilutions for the antibodies. The stained tissues were observed under a fluorescent microscope (DP70; Olympus, Japan).

3. Results

The organ-surface primo tissues were observed between the abdominal wall and the small intestine and were found to be

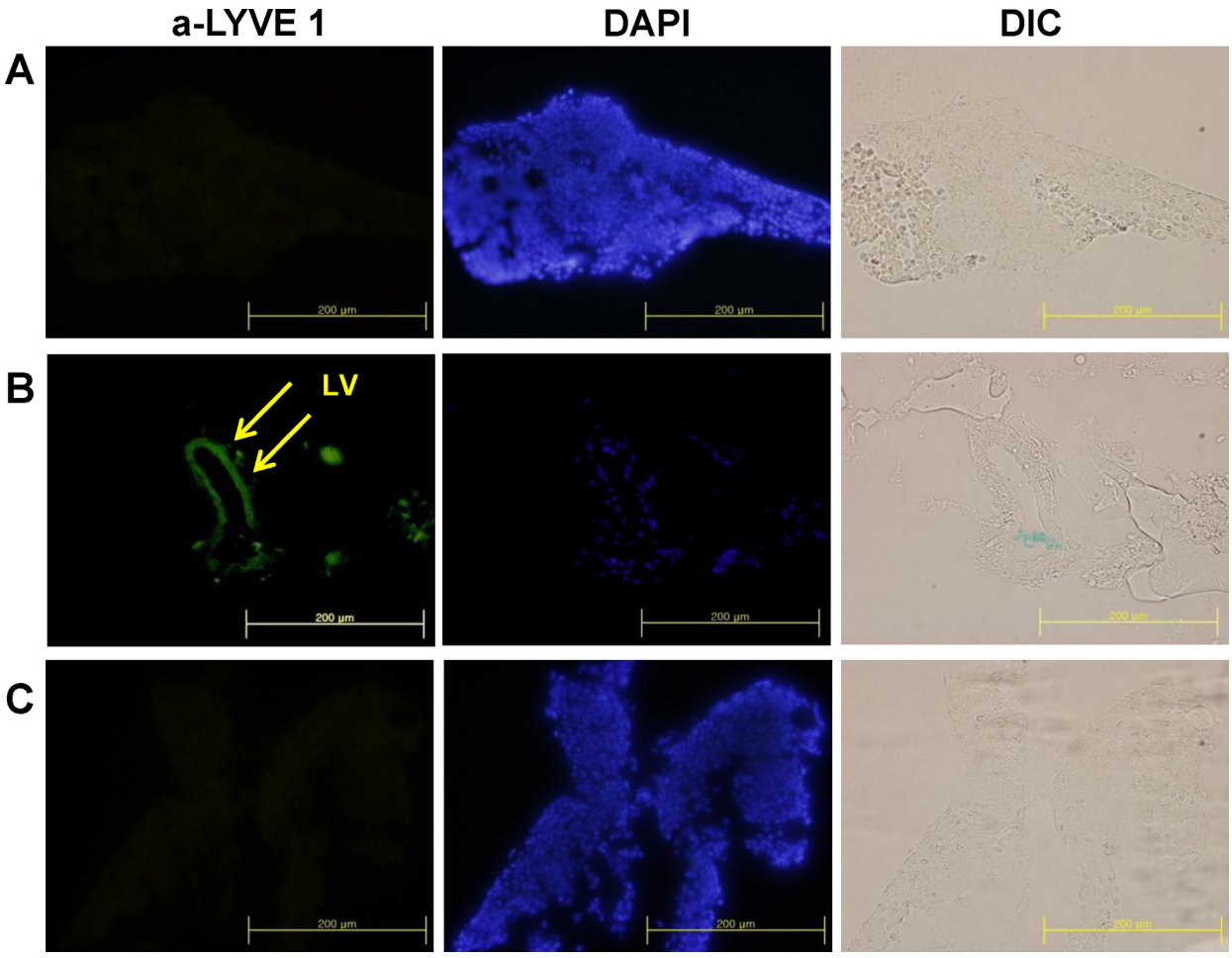

Fig. 3 – Immunofluorescence staining of the primo tissues with LYVE-1. The LYVE-1 was used as a lymphatic vessel (LV) marker to differentiate primo tissues from the LVs. The LVs were stained with LYVE-1 antibody (LV), but no response was detected in all primo tissues. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei. (A) Organ-surface primo tissue; (B) primo tissue inside the LV; (C) primo tissue on the falciform ligament. Image magnification = 400 × . DIC, differential interference contrast.

ivory white and elastic. In addition, they were also found on the organs' surface, and both ends appeared to infiltrate into the interior portion of the organ or mesentery (Fig. 1, A-1).

Primo tissues inside the lymphatic vessel were visualized by injecting 0.1% alcian blue. We could observe the blue-stained primo node and duct moving slightly while floating inside the lymphatic vessel. For the collection of primo tissues inside lymphatic vessels, the pure primo tissue was exposed and then extracted from the inside of the whole lymphatic vessel (Fig. 1, B-1).

With respect to the primo tissues on the falciform ligament, the primo node was attached to the ligament, and the white vessel (primo vessel) was connected to the primo node (Fig. 1, C-1).

Densely patterned cell nuclei and bright cells containing basophilic granules were commonly observed in all the primo tissues obtained from three different body parts. In addition, all of the primo tissues contained relatively large-sized cells, forming a multiple cavity structure with epithelial cells and their plasma showed relatively bright staining (Fig. 1). All of the primo tissue samples were specifically stained against vWF, which is an endothelial cell marker. However, lymphatic vessels were stained very weakly against vWF along with the primo tissue inside lymphatic vessels (Fig. 2).

The HE staining of three different primo tissue types and the lymphatic vessel revealed that lymphatic vessels have distinct duct shapes, with no smaller-sized vessels in their interior walls and a low density of cell nuclei. These histological characteristics helped us to differentiate the primo tissues from lymphatic vessels (Fig. 1, B-2). To clearly differentiate the primo tissues from the organs' surface, inside lymphatic vessels, and on the falciform ligament, LYVE-1 (a lymphatic marker) and CD31 (a blood vessel marker) were used for immunostaining. We used nerve tissues for the vWF-negative control, and blood vessel tissues for the CD31-positive control. The results indicated that no response was observed in the primo tissues derived from the different body parts (Figs. 3 and 4).

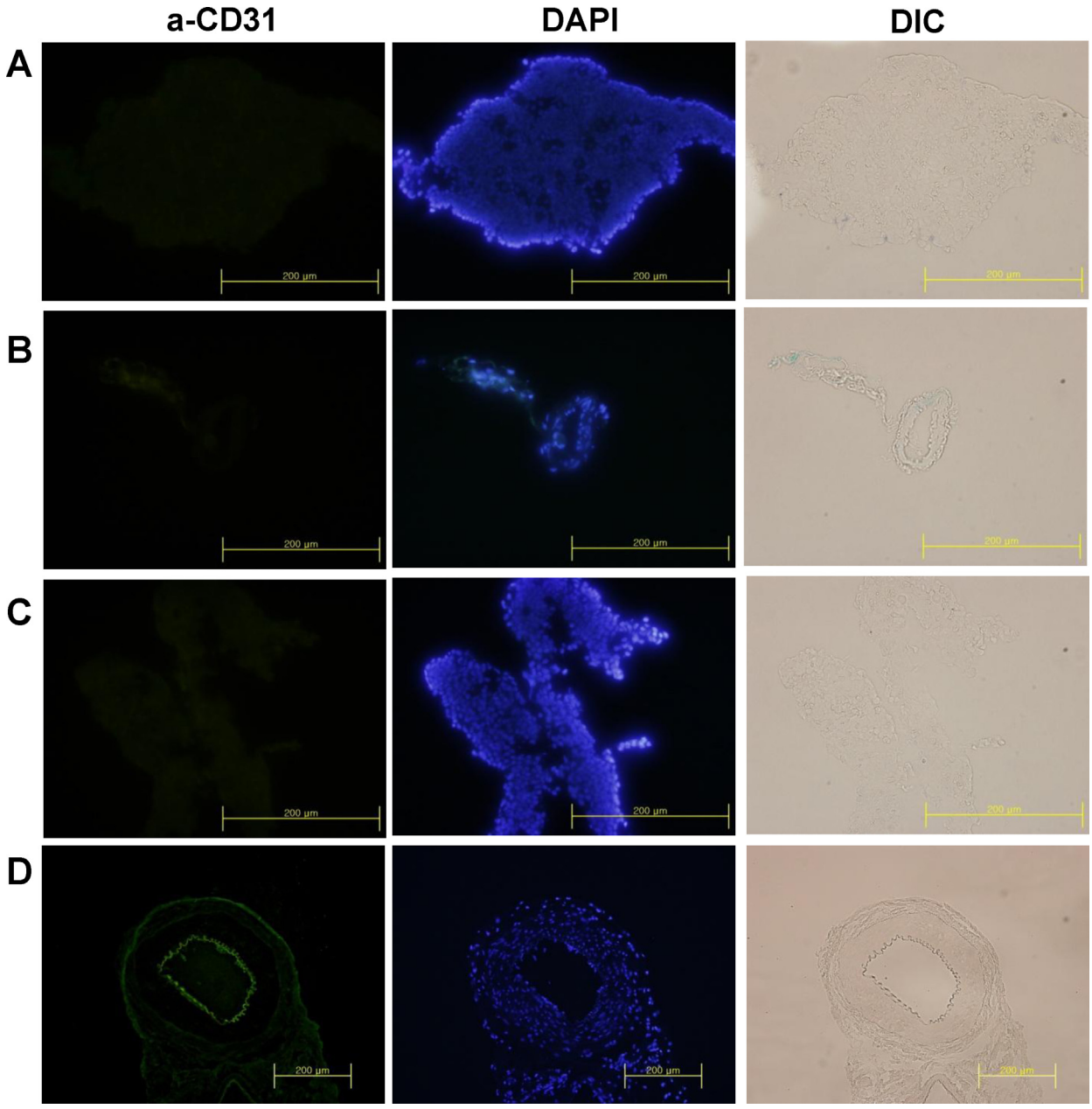

Fig. 4 – Immunofluorescence staining of the primo tissues with CD31. CD31 was used as a blood vessel marker to distinguish between primo tissues and lymphatic vessels. Neither the primo tissues nor the lymphatic vessels responded to CD31 antibody. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei. (A) Organ-surface primo tissue (magnification 400 ×); (B) primo tissue inside a lymphatic vessel (magnification 400 ×); (C) primo tissue on the falciform ligament (magnification 400 ×); (D) blood vessel (magnification 200 ×). DIC, differential interference contrast.

4. Discussion

Primo tissues inside the vessels, brain, and heart[5–7] as well as on the organs' surfaces[8] have been confirmed by a number of investigators with an interest in the primo vascular system. Current investigational interests are focused on revealing the function of the primo vascular system rather than its existence.

To address the function of primo tissues, the distribution profile and histological characteristics of primo tissues have been studied in a lung-cancer mouse model[2] and a melanoma tumor model.[9] However, the histological characteristics and distribution of primo tissues should be preferentially confirmed in a normal animal model before investigating the functional role of primo tissues using a disease model. Lee et al[10] used DiI, a fluorescent carbocyanine dye, and reported that primo tissues exist in a branched form, consisting of two to three branches, with the ends of the primo tissues appearing to infiltrate the organs or fascia. These observations suggested the possibility that primo tissues might form a type of network system and that, if true, the primo tissues may play important roles in the body through this network.

Although primo tissues have been observed in various organs by histological studies and staining methods, the difficulty of clearly separating the primo tissues has limited any investigation of their histological characteristics.

In this study, we tried to investigate the possibility that primo tissues might form a network-like system by studying the histological characteristics of primo tissues obtained from different body parts while elaborating and applying separation methods to obtain primo tissues only. We were able to identify the histological characteristics of primo tissues obtained from different organs, including organ-surface primo tissues and primo tissues on the falciform ligament, which could be separated without any special staining methods, and the primo tissues inside lymphatic vessels, which could be separated from lymphatic vessels by staining with alcian blue.

Organ-surface primo tissue can be found on the organs' surface independently, and both ends appeared to infiltrate the interior of the organ or fascia.[10] Based on this observation, the organ-surface primo tissues in different body parts were assumed to be connected through a network-like system. Because the primo tissues contained in lymphatic vessels are located inside the vessel structure, which is a significantly different environment from the location of the other two types of primo tissues, the primo tissues inside lymphatic vessels might be thought to show different histological characteristics from those of the other two primo tissue types. Conversely, however, if all primo tissues derived from different organs showed common histological characteristics, it would be greatly helpful in predicting the physiological function of primo tissues.

Organ-surface primo tissues were observed as ivory white branches with an independent structure spanning throughout the organs' surfaces. Primo tissue on the falciform ligament was found to be an ivory white mass attached to the ligament itself rather than located inside the falciform ligament. Because primo tissues inside lymphatic vessels are not visible without a staining method, alcian blue was injected to observe the independently floating primo vessel in the lymph.

Organ-surface primo tissues, primo inside lymphatic vessels, and primo tissues on the falciform ligament contained relatively large-sized cells, forming a multiple cavity structure with epithelial cells and their plasma showed relatively bright staining with HE. Consequently, all of the primo tissue types may share a common histological structure.

In addition, these three types of primo tissues showed a positive response when immunostained against vWF, which is consistent with the observation made by Kwon et al.[11] Primo tissues did not show any response against LYVE-1 (a lymphatic vessel marker) and CD31 (a blood vessel marker) immunostaining, which suggested that the primo tissues identified by staining against vWF are neither lymphatic vessels nor blood vessels.

The meridian pathway, a route for energy movement, helps provide nutrition evenly throughout tissues and organs as well as maintaining normal physiological activity. The Bonghan theory was postulated to prove the existence of the meridian pathway and acupuncture points in a scientific manner. Our results, which showed that all three different types of primo tissues have a microvessel structure and share similar histological characteristics, account for the known features of the meridian pathway, which connects the inside and outside of the body and is distributed throughout the body. Therefore, the observation of primo tissues derived from different organs would provide evidence suggesting that primo tissues form a network system in the body.

However, our results do not provide direct evidence for the possibility of a network structure composed of primo tissues. In addition, as our study did not investigate the whole primo system throughout the whole body, further in-depth studies should be performed to address this question. To perform such studies, primo tissues should be isolated from various body parts, and a technical resolution for the whole network observation would be critical.

In conclusion, this study has investigated the possibility that primo tissues might form a network system by studying the histological characteristics of primo tissues obtained from different body parts of rats. Organ-surface primo tissues, primo tissues inside lymphatic vessels, and primo tissues on the falciform ligament contained a multiple cavity structure and a large number of nuclei. These three types of primo tissue were not stained with a-LYVE-1 and a-CD31. Primo tissues showed a positive response when immunostained against vWF. Based on these data, we suggest that the primo vascular system supports the possibility of a primo network throughout the body.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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