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PROTOCOL

Primo Vascular System Floating in Lymph Ducts of Rats

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Abstract
An epoch-making development in the gross anatomy of the lymph system has emerged: the observation of the primo vascular system (PVS), which is a threadlike structure floating in lymph ducts. The PVS, which was proposed as the conduit for the acupuncture Qi, is a complex network distributed throughout an animal's body. The lymph-PVS, which is a subsystem of the PVS, is one of the most convincing visual demonstrations of the PVS. Because its existence is not easily demonstrated, even with a microscope, due to its transparency, in current anatomy its existence is largely unknown despite its potential significance in physiology and medicine. The lymph-PVS has been observed in rabbits, rats, and mice by several independent teams. Because the involved techniques are rather complicated, we provide detailed protocols for surgery, for injection of the staining dye, and for detection, extraction, and identification of the PVS in a rat.

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1. Introduction

The discovery of the specific marker LYVE-1 [1] was the culmination of a series of investigations on lymphatic endothelial cells that had been triggered by the discovery of vascular endothelial growth factors [2,3]. For a long time, the lymphatic system had only played an obscure role as a second circulation system in addition to the blood system. Then, its medical importance became prominent as a key player in cancer metastasis [4] and as a major culprit in inflammation due to the protein podoplanin and its signaling molecule CCL21 [5].

In the current revolutionary period, another astounding development in lymph research has appeared, the discovery of a threadlike structure floating in lymph fluid, whose existence had not been noticed previously due to its optical transparency. It is a subsystem of the primo vascular system (PVS), which was discovered by Bong-Han Kim in the early 1960s [6] and was hypothesized to be a third circulatory system, an anatomical structure corresponding to the acupuncture meridians, the classic Qi flowing path, but had been ignored until its rediscovery by the Seoul National University team [7] in 2002. Since 2002, there have been an increasing number of reports on the PVS, which is composed of threadlike structures called primo vessels (PVs), thickened corpuscle-like parts called primo nodes (PNs), and a fluid flowing in the vessels. The PVS has been observed in various organs, such as the bloodstream [8,9], heart [10], and brain [11], and on the surfaces of internal organs such as the stomach, intestines, bladder, and liver [12,13]. The animals studied most often were mice, rats, and rabbits, and rarely pigs, dogs, and cows. The PVS has also been found in adipose tissue [14], which is a source of pluripotent stem cells [15].

With the aid of staining dyes, the presence of the PVS has been impressively demonstrated as a threadlike structure floating in the lymphatic flow. It was observed in the large-caliber lymph ducts near the caudal vena cava of a rabbit by using the staining dye Janus green B [16] and in those of a rat by using fluorescent nanoparticles [17] and Alcian blue (AB) [18]. The PVS was observed in the thoracic duct of a mouse without the use of a staining dye [19], and other similar works on the PVS in lymphatic flow have subsequently been reported [20–23].

One of the important investigations on the medical significance of the PVS addressed the abundances in PNs of immune cells, such as mast cells (20%), eosinophils (16%), neutrophils (5%), histiocytes (53%), and a few lymphocytes (1%). There were also round immature cells, which were possibly stem cells (3%). Those stemlike cells showed some positive signatures of hematopoietic stem cells [23] and embryonic-like stem cells [24], which is in agreement with the immune function augmentation by acupuncture [25]. Another medically significant finding was the possible implication of cancer metastasis through the PVS that developed on the surface of a tumor [26–28]. The flowing fluids carried hormones, such as adrenaline and noradrenaline [29], and the flow speed was measured to be 0.3 ± 0.1 mm/sec [30].

Although descriptions of the methods used in PVS experiments have been given [16–23], those methods are not easy to use and lack the detailed protocols necessary to obtain, for instance, PVS specimens for molecular-level investigations to find vascular endothelial growth factors and specific markers for the PVS. Here, we provide a detailed protocol for surgery, dye injection, observation, isolation, identification, and histological analysis of the PVS in lymph ducts of a rat so that more researchers can reproduce the experiments to elucidate the physiological and the medical functions of the PVS.

2. Material and methods

2.1. Reagents

2.1.1. Experimental animals

Rats (Sprague–Dawley, male, 9 weeks old, 280–300 g) were purchased from DooYeol Laboratory Animal Company (Seoul, Korea). The animals were housed in a constant temperature-controlled environment (23 °C) with 60% relative humidity. All animals were exposed to a 12-hour light-dark cycle and had *ad libitum* access to food and water. Procedures involving animals and their care conformed to institutional guidelines (approval number: WJIACUC 20130212-1-07), which were in full compliance with current laws and policies [31].

2.1.2. Anesthesia

Zolatil (Virbac Laboratories, Carros, France) and xylazine (Bayer, Korea).

2.1.3. PVS staining

Phosphate-buffered saline solution (PBS). 0.9% saline solution (Choongwae Pharmaceuticals, Korea).

2.1.4. AB staining

Alcian blue (AB) 8GX (Sigma, St. Louis, MO, USA), PBS pH 7.2 (1X; Life Technologies Corp., Grand Island, NY, USA).

2.1.5. Histology

Tissue-Tek optimal cutting temperature (OCT) freezing compound (Sakura Finetek , Tokyo, Japan), base molds (15 mm × 15 mm × 5 mm, Fischer Scientific, Waltham, MA, USA), gel/mount medium (Biomeda Corp., Poster City, CA, USA, no. M01).

2.1.6. DAPI staining

4′,6-diamidino-2-phenylindole (DAPI; Invitrogen Molecular Probes, Grand Island, NY, USA cat. no. D1306; 1:10,000).

2.1.7. Phalloidin staining

Alexa Fluor 488 Phalloidin (Invitrogen, Grand Island, NY, USA).

2.1.8. DiI staining

1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Sigma, St. Louis, MO, USA).

2.1.9. Hematoxylin and eosin (H&E)

Hematoxylin (Sigma, St. Louis, MO, USA), ethanol (Ducksan Chemicals Co. Ltd, Korea), eosin (Sigma, St. Louis, MO, USA), and xylene (Ducksan Chemicals Co, Ltd).