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RESEARCH ARTICLE

Visualization of the Network of Primo Vessels and Primo Nodes Above the Pia Mater of the Brain and Spine of Rats by Using Alcian Blue

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Abstract
By spraying and injecting Alcian blue into the lateral ventricle, we were able to visualize the network of the nerve primo vascular system above the pia mater of the brain and spine of rats. Staining these novel structures above the pia mater with 4′,6-diamidino-2-phenylindole demonstrated that they coexisted in cellular and extracellular DNA forms. The cellular primo node consisted of many cells surrounded by rod-shaped nuclei while the extracellular primo node had a different morphology from that of a general cell in terms of DNA signals, showing granular DNA in a threadlike network of extracellular DNA. Also, differently from F-actin in general cells, the F-actin in the primo vessel was short and rod-shaped. Light and transmission electron microscopic images of the PN showed that the nerve primo vascular system above the pia mater of the brain and spine was a novel dynamic network, suggesting the coexistence of DNA and extracellular DNA. Based on these data, we suggest that a novel dynamic system with a certain function exists above the pia mater of the central nerve system. We also discuss the potential of this novel network system in the brain and spine as related to acupuncture meridians and neural regeneration.

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1. Introduction

Beyond the modern medical point of view, Bonghan Kim published the "Bonghan theory" in the 1960s to establish the acupuncture meridian as a new circulatory system called the primo vascular system (PVS, formerly the Bonghan System) [1]. Bonghan Kim, the founder of the Bonghan theory, suggested that a network of the nerve PVS (NPVS), consisting of primo nodes (PNs) and primo vessels (PVs) existed in the cerebrospinal fluid flowing in the brain and spine [1]. In order to verify his claims, as the first step, we tried to visualize PVs and PNs inside the brain ventricles and central canals of rabbits [2,3]. After trypan blue (TB) staining methods had been developed, we were able to visualize PVs and PNs on the internal organs of rats [4–9], and we applied the method to visualize PVs and PNs in the brains, spinal cords, and sciatic nerves of rats *in situ* and *in vivo* [10].

In previous studies, at first, in order to visualize the NPVS, we had to decapitate the rat for TB injection [10]. In order to overcome the drawbacks caused by decapitation, we used the intraventricular injection method with TB to visualize PVs in the rat brain [11]. For visualization of PVs under the arachnoid mater of the spinal cord, TB was injected into the spinal cord *in situ* and *in vivo* [12]. All previous studies had just implied that a novel system might exist in central nerve systems of rats. Unfortunately, those approaches failed to prove the existence of a whole network of PNs and PVs above the pia mater of the brain and spine.

With two kinds of approaches using Alcian blue, we were able to prove the existence of a network of PVs and PNs above the pia mater of the brain and spine of rats. We also confirmed that the PVs and the PNs were cellular structures or extracellular ones different from the blood vessels and the trabecular fibers above the pia mater of the brain and spine by using 4′,6-diamidino-2-phenylindole (DAPI) and phalloidin staining. In this article, we, for the first time, report the existence of an entire network above the pia mater of the brain and spine of rats.

2. Materials and methods

Female Wistar rats aged 5–6 weeks ($n = 10$; Samtako Bio Korea, Gyunggi-Do, Korea) were housed in a room that was temperature controlled at 24–26°C and light controlled with a 12/12-hour light/dark cycles, and were provided water and commercial rat chow *ad libitum*. The rats were acclimatized for 1 week before the experiment. These experiments were carried out in accordance with the guidelines (KAIST approval number: KA2011-13) of the Laboratory Animal Care Advisory Committee of the Korea Advanced Institute of Science and Technology (KAIST).

The rats were anesthetized by intramuscular injection of a combination of ketamine (45 mg/kg) and xylazine (5 mg/kg) into the right hind femoral limb. In order to visualize the network of the primo vascular system composed of PNs and PVs, we used two staining methods. In order to expose the pia maters of rat brains, we used a bone rongeur to remove crudely the skulls and spinal bones of rats. After rough cutting the bones, we used fine scissors to remove completely the dura mater and arachnoid mater under stereomicroscope (SZ61; Olympus, Tokyo, Japan). One method was to spray 1% Alcian blue in phosphate-buffered saline (PBS) at pH 7.4 into the surface of the pia mater of the rat brain. Spraying of Alcian blue was followed by washing with saline. Later, we developed the other method, which is better for visualizing the nerve primo vascular system above the pia mater of the rat brain. In this new method, we injected about 0.2 mL of 0.1% Alcian blue (about pH 7.4) in saline into the lateral ventricle for 2 to 5 hours. All pH measurements were done by pH paper before the experiment. As shown in Fig. 1, the exact injection point is about 2 mm to the side from the bregma and about 5 mm below the rat skull. However, because Alcian blue was injected manually, the injection point may differ slightly from injection to injection. In order to overcome this shortcoming, we confirmed where the Alcian blue had been injected by dissecting the brain.

After spraying or injecting with Alcian blue, as described above, we examined the surface of the pia mater of the

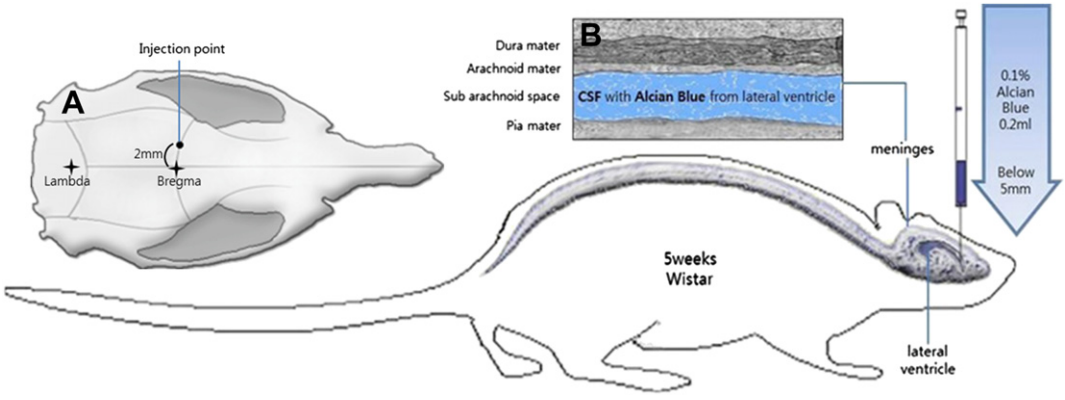

Figure 1 Illustration of the experimental method for Alcian blue injection into a rat's lateral ventricle. Alcian blue in saline, 0.1% for 0.2 mL, was injected into the lateral ventricle. (A) The exact injection point is located about 2 mm to the side from the bregma. The injected Alcian blue mainly flows in the subarachnoid space, blue color drawn in (B), for 2–5 hours. After the experiment, we dissected the brain to verify whether the injected portion was mainly in the lateral ventricle.

brain and spine under a stereomicroscope (SZ61; Olympus) with a CCD camera (ProRes C12plus; Jenoptik, Jena Germany). All data are summarized in Table 1. In order to verify that the visualized structures were significant as biological structures, we stained the specimens with DAPI and phalloidin (100 times dilution of 300-unit TEXAS Red; Molecular Probe, Eugene, Oregon, USA). The images of the structures were obtained by using a fluorescence microscope (AX 70; Olympus, Tokyo, Japan) with a CCD camera (eXcope X3; DIXI, South Korea).

For light microscopic and transmission electron microscopic examination, the specimens were fixed in 2.5% paraformaldehyde and 2.5% glutaraldehyde in a neutral 0.1 M phosphate buffer for 1 hour. The specimens were post-fixed for 1 hour in 1% (w/v) osmic acid dissolved in PBS, dehydrated in graded ethanol, and embedded in Epon812 (EMS, Fort Washington, PA). Semithin sections of 1-μm thickness stained with 1% toluidine blue were observed and photographed under a light microscope (BX 53, Olympus) with a CCD camera (eXcope X3; DIX). Ultrathin sections were cut and mounted on nickel grids and were double-stained with uranyl acetate, followed by staining with lead citrate. The sections were examined with a Technai G2 Spirit TEM (FEI, Hillsboro, Oregon, USA).

3. Results

With the two approaches using Alcian blue, as described above, we were able to prove the existence of a novel network of threadlike structures (PVs) and corpuscle structures (PNs) above the pia mater of the brain and spine of rats. All data for the visualized PVs and PNs are summarized in Table 1. For this experiment, we sacrificed 10 rats. In the first five rats, we visualized the PVs and the PNs by spraying Alcian blue into the pia mater of the rat brain (subject nos. 1—5), and in the last five rats, we visualized the PVs and the PNs by injecting Alcian blue into the lateral ventricle of the rat brain (subject nos. 6—10). In Fig. 1, the locations of PNs and PVs are the places where they are clearly visualized. *L* and *S* indicate the longest and the shortest diameters in mm of a PN. *D* and *d* indicate the diameters (in μm) of the PV at the thickest and thinnest points, respectively.

In Fig. 2, we illustrate the network of PVs and PNs above the pia mater of the brain and spine of a rat with stereoscopic images. All PNs are connected with PVs. Stereomicroscopic images are arranged according to the locations where PVs and PNs were visualized. By spraying 1% Alcian blue into the pia mater of the brain of a rat, stereoscopic

Table 1 Nerve primo node and primo vessel of brain and spine of rat.

Subject no.	Locations of PN and PV found	L (mm × mm × mm)	D (μm)	d (μm)
1	Parietal lobe of cerebrum	0.2 × 0.1 × 0.05	30	20
	Longitudinal cerebral fissure	0.2 × 0.12 × 0.06	35	30
2	Ansiform lobule of cerebellum	0.08 × 0.05 × 0.03	25	25
	Longitudinal cerebral fissure	0.3 × 0.2 × 0.1	40	20
	Parietal lobe of cerebrum	0.25 × 0.12 × 0.06	20	15
	Area of olfactory nerves	0.2 × 0.15 × 0.08	35	15
3	Fundus of Pineal Body	0.15 × 0.12 × 0.06	20	15
	Upper Part of Pineal Body	0.15 × 0.15 × 0.08	30	15
4	ophthalmic division of the trigeminal nerve	0.07 × 0.06 × 0.03	20	15
5	Longitudinal cerebral fissure	0.1 × 0.08 × 0.04	15	10
6	Lobulus simplex of cerebellum	0.2 × 0.15 × 0.08	10	10
	Medulla oblongata	0.07 × 0.04 × 0.02	20	10
7	Posterior lobe of cerebellum	0.11 × 0.09 × 0.05	20	10
	Medulla oblongata	0.12 × 0.08 × 0.04	30	10
	Cervical division of spinal cord	0.11 × 0.1 × 0.05	20	10
8	Posterior lobe of cerebellum	0.13 × 0.08 × 0.04	10	10
	Medulla oblongata	0.1 × 0.07 × 0.04	10	10
9	Posterior lobe of cerebellum	0.04 × 0.03 × 0.02	10	10
	Medulla oblongata	0.2 × 0.1 × 0.05	20	10
	Cervical division of spinal cord	0.12 × 0.09 × 0.05	20	10
10	Posterior lobe of cerebellum	0.15 × 0.1 × 0.05	10	10
	Medulla oblongata	0.08 × 0.04 × 0.03	20	10
	Cervical division of spinal cord	0.11 × 0.08 × 0.04	20	10
11	Longitudinal cerebral fissure	0.11 × 0.03 × 0.02	25	10
	Longitudinal cerebral fissure	0.06 × 0.04 × 0.02	30	10
Average ± SD		0.14 ± 0.064 × 0.09 ± 0.04 × 0.05 ± 0.02	22 ± 8.5	13 ± 5.4

Experimental data to demonstrate the sizes of threadlike structures (PVs) and corpuscles (PNs) visualized above the pia maters of the brains and spines of rats. For this experiment, we sacrificed 11 rats; the first five rats' PVs and PNs were visualized by spraying Alcian blue (subject nos. 1—5), and the last six rats' PVs and PNs were visualized by using the injection method with Alcian blue (subject nos. 6—11). The location of a PN and a PV is the place where the PN and the PV are clearly visualized. *L* indicates the longest, the shortest, and the thickest diameters of a PN in mm as PNs are three-dimensional oval-shaped. *D* and *d* indicate the diameters in μm of a PV at its thickest and thinnest points, respectively. PN = primo nodes; PV = primo vessels.

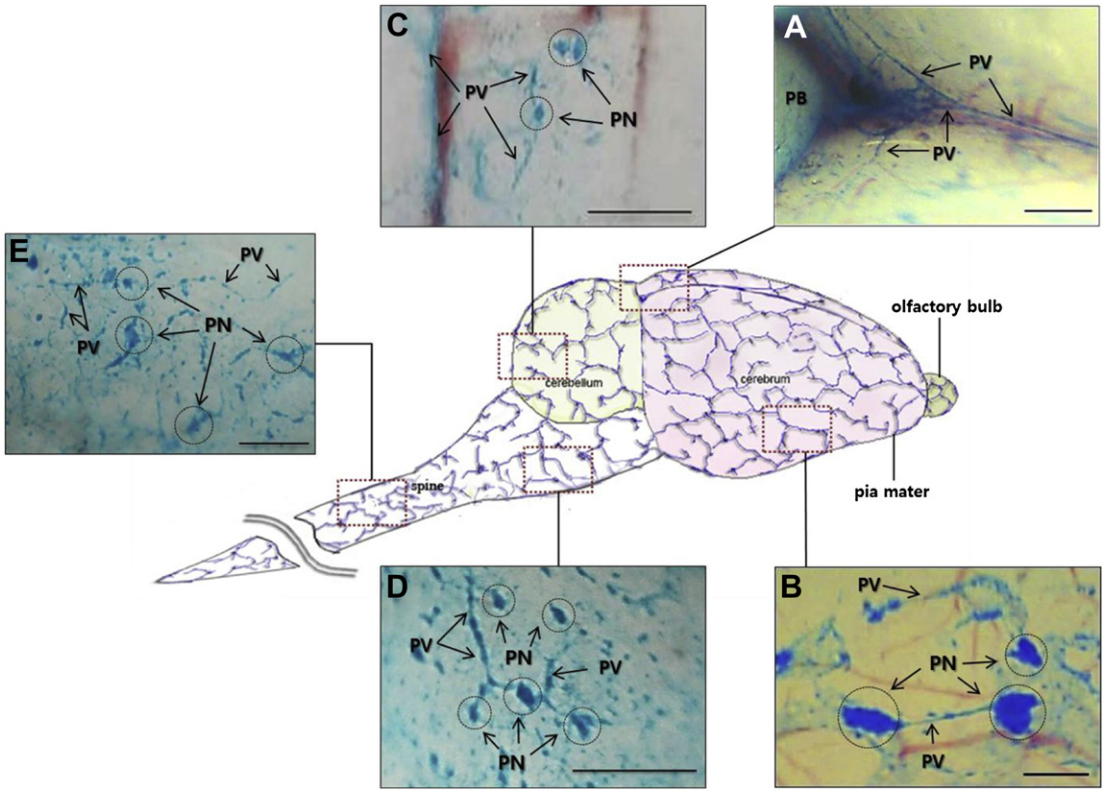

Figure 2 Illustration of a network of threadlike structures (primo vessels, PVs) and corpuscles (primo nodes, PNs) above the pia mater of the brain and spine of rats with stereoscopic images. All PNs are connected with PVs. The stereoscopic images are arranged based on the locations where the PVs and the PNs were visualized. (A, B) Stereoscopic images visualized by spraying 1% Alcian blue in phosphate-buffered saline (PBS) into the pia mater of the brain. PB in stereoscopic image A is the pineal body. (C, D, E) Stereoscopic images were visualized by injecting 0.1% Alcian blue in saline into the lateral ventricles of rats for 2—5 hours. Noticeably, the red-colored blood vessels in stereoscopic images A, B and C images were not stained by Alcian blue. All scale bars are 500 μm.

images A and B were obtained. In particular, image A shows that there is a distinctive network of PVs around the pineal body of the rat brain. As shown for subject no. 3 in Table 1, the pineal body itself also has PVs and PNs above the pia mater of not only its fundus but also its upper area. Stereoscopic images C, D, and E were obtained by injecting Alcian blue into the lateral ventricle. Noticeably, the red-colored blood vessels in stereoscopic images A, B, and C are not stained by Alcian blue.

Fig. 3 shows a merged microscopic image of the phase contrast and fluorescence images of PVs and PNs stained with DAPI. The PN is composed of many cells surrounded by a boundary of rod-shaped nuclei, indicated by arrows, and a PV consisting of rod-shaped nuclei is connected to this PN. However, we found that one PN consisted of cellular structures; the other PN did not. During examinations of the PNs taken from 10 rats, we noticed that three kinds of PNs coexisted above the pia mater of the brains and spines of the rats. The first PN was composed of extracellular DNA (eDNA) with rare nucleus-like structures as shown in Fig. 4A. The second PN had many nucleus-like structures connected with threadlike structures in which eDNA signals were observed. The third PN had many normal nuclei, and its image is the same as the image in Fig. 3. The DNA-signal-containing threadlike structures in Fig. 4B are magnified into the image shown in Fig. 5A, indicating that the DNA is granular or extracellular. As shown in Fig. 5B, the F-actin molecules in the PV are short and rod-shaped.

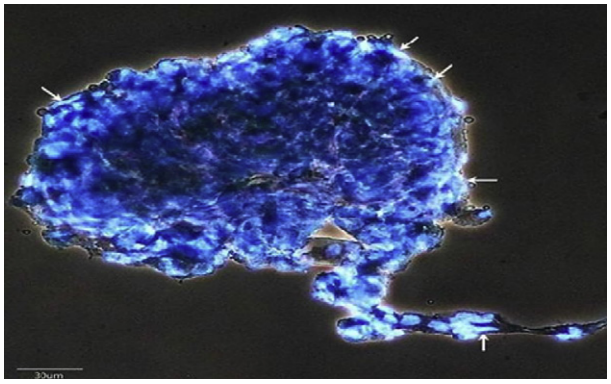

Figure 3 Merged microscopic image of the phase contrast and fluorescence images of primo vessels (PVs) and primo nodes (PNs) taken above the pia mater of the rat brain stained with 4′,6-diamidino-2-phenylindole (DAPI). The PN has a distinctive outermost boundary of rod-shaped nuclei, indicated by arrows. Inside the PN are many cellular structures stained with DAPI. A PV containing a rod-shaped nucleus (arrow) is connected with the PN. The scale bar is 30 μm.

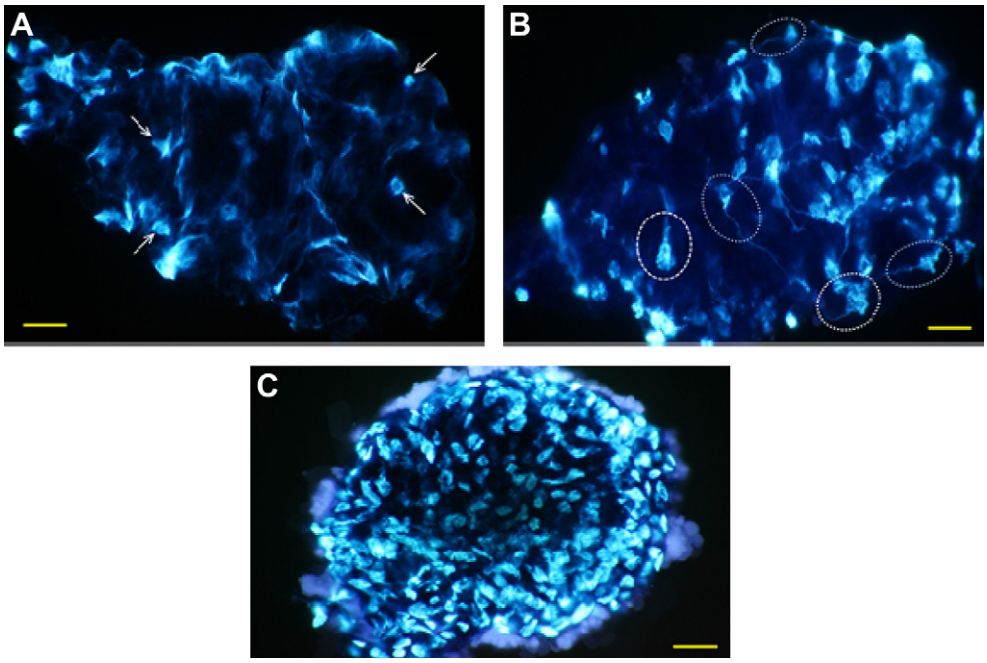

Figure 4 Three kinds of primo nodes (PNs) coexist above the pia mater of the brain and spine of rats. The three kinds of the PNs stained with 4′,6-diamidino-2-phenylindole (DAPI) are as follows: (A) The PN has extracellular DNA (eDNA) with rare nucleus-like ones (arrows). The eDNA images stained by DAPI look amorphous. (B) The PN has many nucleus-like ones (dotted circles) connected with threadlike structures, which are magnified in Fig. 5A. The eDNA images seem to be getting organized. (C) The PN has many normal nuclei that share the same image as in Fig. 3. Scale bars are (A) 25 μm, (B) 28 μm, and (C) 29 μm.

Fig. 6 shows a light microscopic sectional image of the cerebrum containing the pia mater stained by using toluidine blue. The outermost membrane of the pia mater consists of fibroblasts, indicated by arrows and dotted arrows. The PNs and the PVs are present above the pia membrane of the cerebrum. The PNs indicated by arrows A and B were examined by using transmission electron microscopy (TEM) as shown in Fig. 7. The first image of PN A is located above the fibroblast that forms the outermost membrane of the pia mater. Amorphous structures are observed in image a of PN A. As shown in image b of PN A, the fibroblast is surrounded by collagen fibers. The outermost membrane of PN A is also observed in image c, where sinuses are found. The fine fibers are found as a putative extracellular matrix. The first image of PN B shows membrane-surrounded, nucleus-like

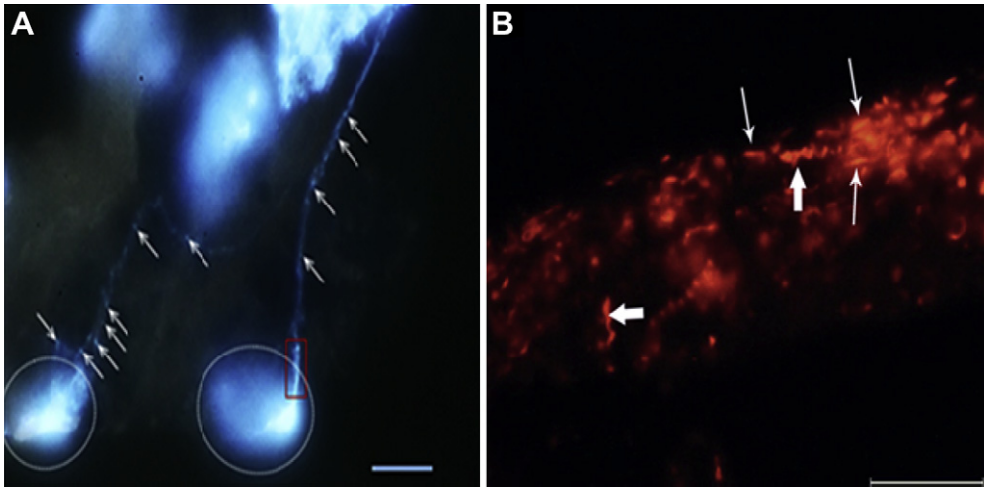

Figure 5 (A) Magnified image (×1000) shows extracellular DNA signals corresponding to the dotted circle in Fig. 4B. The granular forms (arrows) in extracellular DNA (eDNA) from a nucleus-like structure (dotted circle). The eDNA is threadlike and extends from nucleus-like structures in which distinctive eDNA is indicated by a red rectangle. (B) F-actin signals in a primo vessel (PV) stained by phalloidin (TEXAS RED, Molecular Probe). The PV consists of short rod-shaped F-actin molecules (arrows). Thick arrows indicate clusters of curved, short, rod-shaped F-actin molecules. The scale bars are (A) 13 μm and (B) 46 μm.

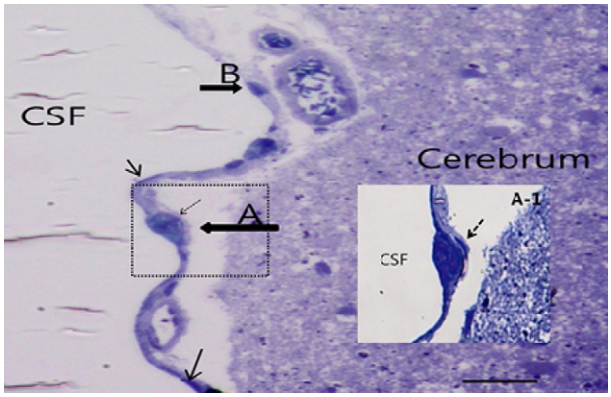

Figure 6 Light microscopic sectional image of the cerebrum containing the pia mater stained by using toluidine blue. The pia mater consists of fibroblasts (arrows and dotted arrows) above which primo nodes (PNs) are located as indicated by thick arrows A and B (subject no. 11 in Table 1). In the PN indicated by thick arrow A, the dotted arrow indicates a blurred image of a fibroblast, which is clearly captured (dotted arrow) in the next section of the same PN as in A-1. CSF = cerebrospinal fluid between the pia mater and the arachnoid mater of brain. Scale bar = 36 μm.

structures and a distinctive outermost membrane. Image b of PN B shows clustered granules, indicated by a dotted rectangle, which are magnified into image c. Distinctive granules and fine fibers are observed in image c of PN B.

4. Discussion

A question arises as to why so many neuroscientists have not noticed the network above the pia mater of the brain and spine. Considering that the PVS is transparent, thin (about 20 μm in diameter for a PV), and without blood capillaries, the fact that it has not been noticed until recently is understandable. However, since the development of the visualization method using TB, the existence of the network named the PVS (formerly Bonghan system) in subarachnoid spaces of the brains and spines of rats has been gradually recognized [2,3,10—12]. Unfortunately, however, the complete network, including distinctive PNs above the pia mater of the central nerve system, remains as a subject for further studies.

Another question also arises as to the reason Alcian blue preferentially visualizes the threadlike PV and corpuscular PN over surrounding tissues, such as blood capillaries and

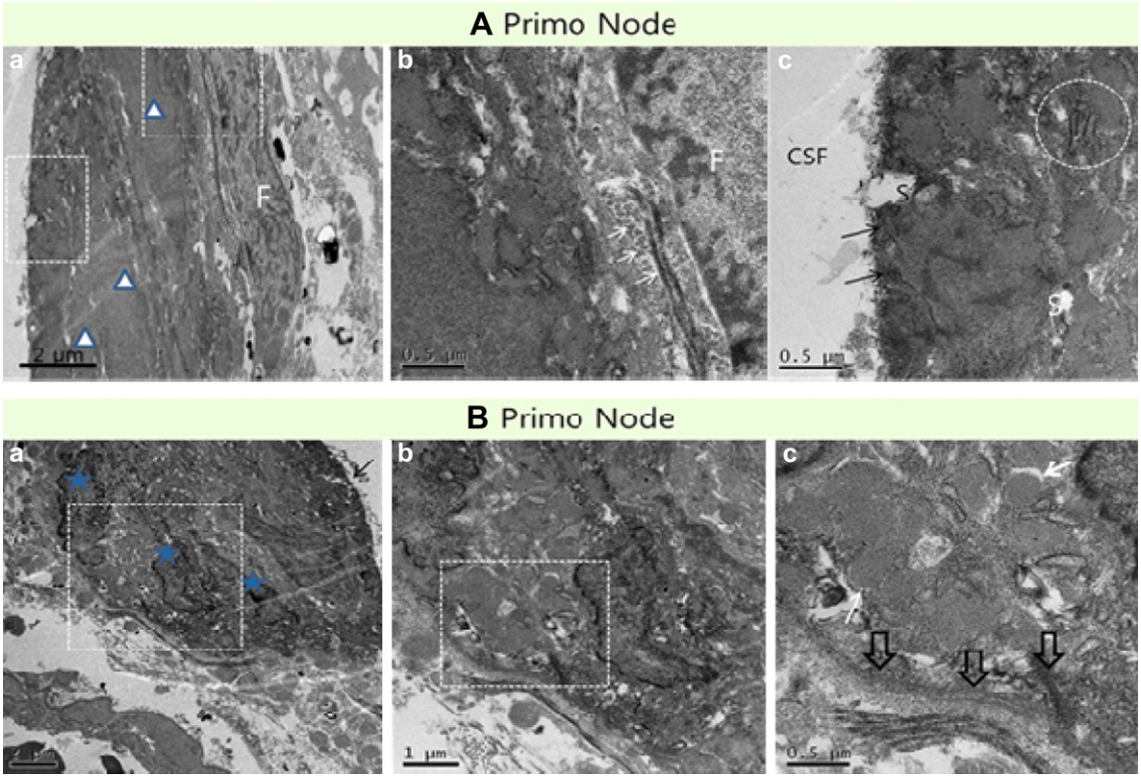

Figure 7 Transmission electron micrographs of primo nodes (PNs) A and B corresponding to the light microscopic image in Fig. 6. The first image of PN A (a) is located above a fibroblast (F) that forms the outermost membrane of the pia mater. Amorphous structures (three triangles) are observed in image a for PN A; dotted rectangles are magnified into images b and c, respectively. As shown in image b for PN A, fibroblast F is surrounded by collagen fibers (arrows). The outermost membrane of PN A is indicated by arrows in image c, where sinuses (S) are found. The fine fibers are also found as a putative extracellular matrix (dotted circle). Image a for PN B shows membrane-surrounded, nucleus-like structures (stars) and a distinctive outermost membrane (arrow). Image b of PN B shows clustered granules, indicated by a dotted rectangle, which are magnified into image c. Distinctive granules (white arrows) and fine fibers (thick arrows) are observed in image c of PN B.

the fascia covering the brain and spine. This question is more complicated than the previous question to clearly answer. We conjecture that PVs and PNs may have a great amount of carbohydrate such as hyaluronic acid, which has a strong affinity for Alcian blue. Our previous proteomic analysis on the PVS demonstrated that abundant proteins are closely related with carbohydrate metabolism in PNs and PVs [13]. However, the reason Alcian blue preferentially stains the network of the NPVS still represents a question in need of an answer.

From phase contrast and fluorescence microscopic examination, as shown in Fig. 3, we are sure that PVs and PNs are cellular structures consisting of rod-shaped nuclei. As summarized in Table 1, we collected PNs and PVs and examined them by using DAPI staining. We found that at least three types of PNs, in terms of DAPI-stained patterns, as shown in Fig. 4, and their DNA signals coexist above the pia mater of the brain and spine of a rat. Unfortunately, we do not understand why granular DNA and eDNA forms were observed in the PNs above the same pia mater of the brain and spine of rats. By definition, eDNA has been considered a kind of circulating cell-free DNA [14,15]. A report on eDNA in bacteria suggested that eDNA was not a remnant of lysed cells, but had a function to form a defined network-like structure [16]. Recently, clinical interest in the function of eDNA has been growing [17]. The eDNA is usually found in the blood circulatory system, implying its fibrinolysis function [18]. Beyond the growing interest in eDNA in modern biology, Bonghan Kim in the 1960s insisted that a great quantity of cell-free DNA (eDNA or circulating DNA in terms of modern terminology) flowed in the PVS [19]. He suggested with some data that cell-free DNA played pivotal role, implying PVS's regenerative function via the formation of new cells even in the central nervous system [1,19]. We discussed the regenerative function in detail in our previous work on the PVS in the rat brain [10,11]. However, at this stage, we conjecture that various-staged PNs, in terms of development related with DNA, may exist above the same pia mater. Moreover, F-actin molecules in the PVS present distinctive and interesting images. As shown in Fig. 5B, F-actin molecules are disassembled differently from the strait pattern in general cells. One significant report stated that the disassembled F-actin molecules were related with catecholamine releases in biological tissues [20]. In previous studies, we detected two kinds of catecholamine, adrenaline and noradrenaline, in the PNs and the PVs taken from the internal organs of rabbits [21]. Molecular and proteomics studies and analyses of this novel network of the NPVS in a future study may solve many of the above mysteries.

On the other hand, our finding on the novel network, PVs and PNs, above the pia mater of the brain derives from the new acupuncture meridian concept proposed by Bonghan Kim in the 1960s [1]. According to Bonghan Kim's idea on acupuncture meridians, these structures are connected with other PVs and PNs ubiquitously throughout a living body. Since 2002, Soh [22] investigated his idea with anatomical and histological methods, and results of this study imply that another circulatory system may exist. Bonghan Kim also suggested that PVs and PNs in the brain had a unique function to regenerate the damaged nerve system via DNA granules named "Sanals" [23], implying that a certain regenerating system existed in the brain and the spine. Given that there is no lymphatic system in the central nervous system that presents a defense against various forms of damage, Bonghan Kim's idea on the NPVS in the brain and spine must be considered if the NPVS is to be understood.

For further study, we would like to recommend that one should focus on three topics to understand the function of the novel network: (1) characterization of cells inside PNs, (2) analysis of the chemical components and extracellular DNA existing in PVs and PNs, and (3) visualization of the connection between the network above the pia mater and distant organs, including the skin where acupuncture points reside. In the future, we hope that the establishment of the physiological function of the nerve primo vascular system in the central nervous system will usher in a new paradigm to bridge between Western medicine and oriental traditional medicine.

Acknowledgments

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