



RESEARCH ARTICLE

The Flow Path of Alcian Blue From the Acupoint BL23 to the Surface of Abdominal Organs

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Abstract

Two hours after Alcian Blue (AB) dye was injected at the rat acupoint BL23, the abdominal cavity was examined and AB-stained threadlike structures were observed on the right abdominal cavity. Those threadlike structures were mainly distributed on the surfaces of the duodenum, colon and cecum. These threadlike structures were thin (about 50 μm) and moved freely, and were connected to corpuscles that were about 500 \times 200 μm wide and also stained with AB. On analyzing the histology of the threadlike structures, rod-shaped nuclei, bundles of collagen fibers, reticulofibers, and squamous-like epithelial cells were observed. Immune cells and some sinuses were inside the threadlike structures. These characteristics describe those of Bonghan ducts. The flow paths from the acupoint to internal organs can possibly be used as paths for drug delivery.

1. Introduction

In veterinary physiology, the only known circulatory systems are the blood and the lymphatic systems. Contrary to this knowledge, we found a threadlike duct that was neither a blood nor a lymph vessel that allowed flow from a specific area in the dorsal skin of a rat to the surfaces of the internal organs around the duodenum. A staining dye, Alcian Blue (AB), was injected into the BL23 acupoint. The dye appeared in a threadlike duct that was on, but did not adhere, to the stomach and the duodenum and that entered the large intestine.

An anatomical and morphological study with light and electron microscopes revealed that this threadlike structure was completely different from

a blood or a lymph vessel, and from a nerve; rather it was identified as a Bonghan duct (BHD), whose existence was first observed by Bonghan Kim [1] in the 1960s. He claimed that the BHD was a novel circulatory system connecting acupuncture meridians in the skin to internal organs. His claim, however, was not reproduced by any other group and was forgotten. Only recently, have several groups rediscovered BHDs in rabbits [2–4], rats [5], and mice [6], and its anatomical structure has been reported in detail [2,3,7,8]. In brief, BHDs are 50–100 μm thick semitransparent threadlike ducts on the surfaces of internal organs, such as the liver, stomach, small and large intestines and urinary bladder. They freely move and are sparsely and irregularly fixed to the peritonea. Oval or cucumber-like bodies, called

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Bonghan corpuscles (BHCs), are located irregularly along the BHD and both ends of a corpuscle are connected to BHDs.

Structural evidence for the circulatory function of the BHD was obtained using electron microscopy, which showed that the BHD had a plant-rootlet-like structure with multiple channels. Cryo-scanning electron microscopy disclosed traces of flowing liquid in these channels [7,9]. The speed of the liquid flow was directly measured by injecting AB dye into a BHC. The flow turned out to be very slow, 0.3 mm/sec, compared with the speed of blood or lymph flow [9]. The liquid contained neurotransmitter hormones, adrenaline and noradrenaline [10,11]. In addition, chromaffin cells that produce or store these hormones were present in the BHC [10]. A proteomics study of this liquid has been performed [12].

As to the physiological significance of the BHD, the most crucial point is to verify that it forms a circulatory system connecting the skin and internal organs. Until the present study, no researchers, including Bonghan Kim, have shown such flow from a specific area in the skin to specific organs via a BHD. For the first time, we demonstrate flow of liquid from the dorsal skin, through BHDs, to the omentum of the stomach and extending over the duodenum toward the large intestine of a rat.

This work is important not only as a new finding in physiology but also for its implications in medicine. For example, it can provide a new drug delivery path from the skin to a target intestinal organ, which may be applicable for cancer therapy. In contrast to the other forms of administration which transport drugs in the body through the circulatory systems, the BHD may carry the drug to the target organ exclusively and directly from the skin, thus reducing unwanted side effects. Since the BHC and BHD are known to contain catecholamine [10,11] they may be hitherto unknown hormone paths. Anatomically, physiologically and medically, this novel path of flow from the skin to internal organs reveals a challenging original research area.

2. Materials and Methods

2.1. Animal preparation

Twenty-nine female Sprague-Dawley rats were used in this study. They were 8 weeks old, and their body weights ranged from 150–200 g. The animals were housed in a temperature-controlled environment (23°C) with 60% relative humidity and a 12 hour light/dark cycle. They had *ad libitum* access to food and water, and the procedures involving the animals and their care were in full compliance with current international laws and policies (Guide for

the Care and Use of Laboratory Animals, National Academy Press, 1996).

2.2. Alcian Blue (AB) injection and surgical procedures under a stereomicroscope

Filtered 1% AB dye was prepared in this study to visualize the threadlike structures and to trace their paths. Also, the bilateral Bladder meridian (BL) 23 was selected as the injection point because of its accessibility and clinical significance. However, there was no recognized meridian in rats, so bilateral low-electric-resistance points between the 2nd and the 3rd lumbar spine were selected in this study based on the acupoint of a dog. In all animals, low-electrical-resistance points were displayed at locations 4–6 mm bilaterally off the dorsal midline. Those points were placed on an extension line between the 2nd and the 3rd vertebra. They were very similar to the existing BL23 points in humans and dogs.

Under general anesthesia (xylazine, 10 mg/kg, plus ketamine, 70 mg/kg, intramuscular injection), a bolus injection of 300 μ l of 1% AB was injected into the bilateral BL23 points subcutaneously or intramuscularly using a 31 G needle. Subcutaneous injection of AB was applied to 24 rats, and muscular injection was used in the other five rats.

Two hours after AB injection, the rats were euthanized using ether, and the subcutaneous areas of the whole back region and the abdomen were observed by using an incision of the dorsal midline and a laparotomy, respectively. This allowed the detection of AB-stained threadlike structures. These observations were performed under a stereomicroscope (SZX12; Olympus, Japan), and images were taken with a digital camera (Nikon, Japan).

2.3. DAPI staining and phase-contrast microscopy

The samples were fixed in a 4% paraformaldehyde solution for 1 hour, washed with phosphate buffered saline, and then stained with DAPI solution (SlowFade[®] Gold, Invitrogen, USA) for 1 minute in a dark place. After staining, the samples were covered with a coverslip and examined under a phase-contrast microscope (BX 51, Olympus, Japan) at an excitation wavelength of 350 nm (emission wavelength: 470 nm) to detect the DAPI fluorescence from the cell nuclei.

2.4. Transmission electron microscopy

For transmission electron microscope (TEM) examination, samples were fixed with 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at 4°C for 4 hours. The specimens were postfixed in 1% OsO₄

in a 0.2M sodium cacodylate buffer for 1 hour, stained in 0.5% uranyl acetate, dehydrated with ethanol and propylene oxide, and embedded in epoxy resin (Epon 812). Ultrathin sections were collected on 200 mesh copper grids, contrasted by 2% uranyl acetate and Reynolds' lead citrate. Samples were then examined with an energy filtering transmission electron microscope (Libra 120, Carl Zeiss, Germany).

3. Results

A typical threadlike BHD with two corpuscles was observed on the duodenum and appeared blue as the AB dye flowed through the duct (see Figure 1). The BHD was thin, semitransparent, and flexible, and it did not adhere to the surface of the duodenum and was thus free to move. Its diameter was 50 μm . Two cucumber-shaped BHCs, 500 $\mu\text{m} \times 200 \mu\text{m}$ and 400 $\mu\text{m} \times 200 \mu\text{m}$ are linked by BHDs at both their ends. Without the blue color of AB, due to their transparency, the BHDs would be very difficult to detect.

BHDs and BHCs were observed in ten rats. Five rats were from the 24 receiving subcutaneous injections, and the remaining five were from the rats receiving intramuscular injections. Thus, the intramuscular injection was highly effective, but the flow paths in the abdominal cavity showed no apparent difference between the two injection methods. The distribution of ten BHDs through which AB flowed is shown in Figure 2. The first five curves are for BHDs through which flow was obtained by subcutaneous injection, and the remaining curves are for

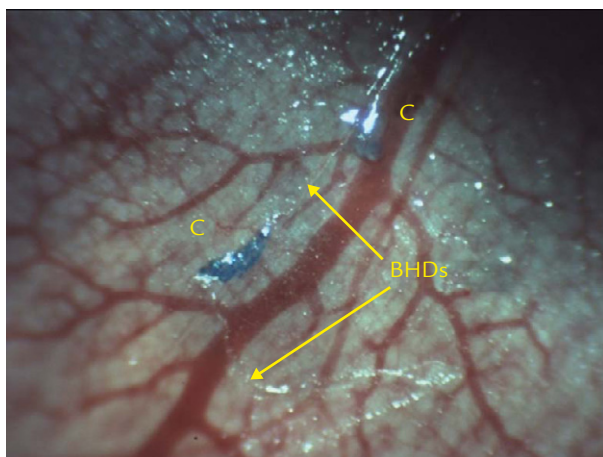


Figure 1 Overall characteristic features of Alcian Blue (AB)-stained threadlike Bonghan ducts (BHDs) and corpuscles. BHDs are thin, semitransparent, and freely movable. A corpuscle (C) is linked to BHDs on opposite sides (arrows). Most of the BHDs stained partially or wholly by the AB solution. The thicknesses of the BHDs were about 50 μm , and those of corpuscles were about 400–500 μm .

BHDs through which flow was obtained using intramuscular injections.

The BHDs were mostly observed on the right side of the abdominal cavity. They started from the liver, the great omentum of the stomach or the duodenum, and passed through the right abdomen to the small intestine and the large intestine. The thicknesses of the BHDs ranged from 50 to 100 μm , and the corpuscles were about 500 \times 200 μm in diameter.

BHDs and corpuscles were most frequently observed on the duodenum, cecum, and ascending and transverse colon. An example (number ① in Figure 2) showing the emerging and disappearing points of a BHD on the transverse colon is given in Figure 3. The BHD had large and small corpuscles (Mean \pm SD of diameter of BHC: 528.5 \pm 78.10 \times 226 \pm 43.57 μm), which were conspicuously blue due to the heavy flow of AB. The BHD did not adhere to the surface of the colon, so it moved freely. The BHD emerged from inside the fat tissue just before the large corpuscle and ended by entering into the colon after the small corpuscle. We were not able to trace the BHD either inside the fat tissue or beyond the colon membrane.

The BHD is sometimes anchored at the abdominal wall (number ② in Figure 2), as shown in Figure 4.

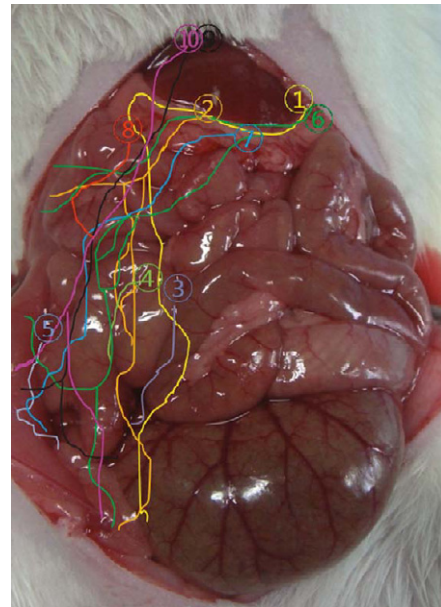


Figure 2 Distribution of Alcian Blue (AB)-stained threadlike Bonghan ducts (BHDs) and corpuscles after AB injection at Urinary Bladder 23 (BL23). The AB-stained BHDs and corpuscles are mostly observed on the right sides of abdominal organs, including the right sides of the small intestine, the large intestine, the abdominal wall, the liver, and the stomach (dotted line: overall pathway; solid lines: individual pathways). The BHDs start from the liver or the great omentum of the stomach or duodenum and pass through the right abdomen. BHDs and corpuscles are mostly observed on the duodenum, the cecum, and the ascending and transverse colon.

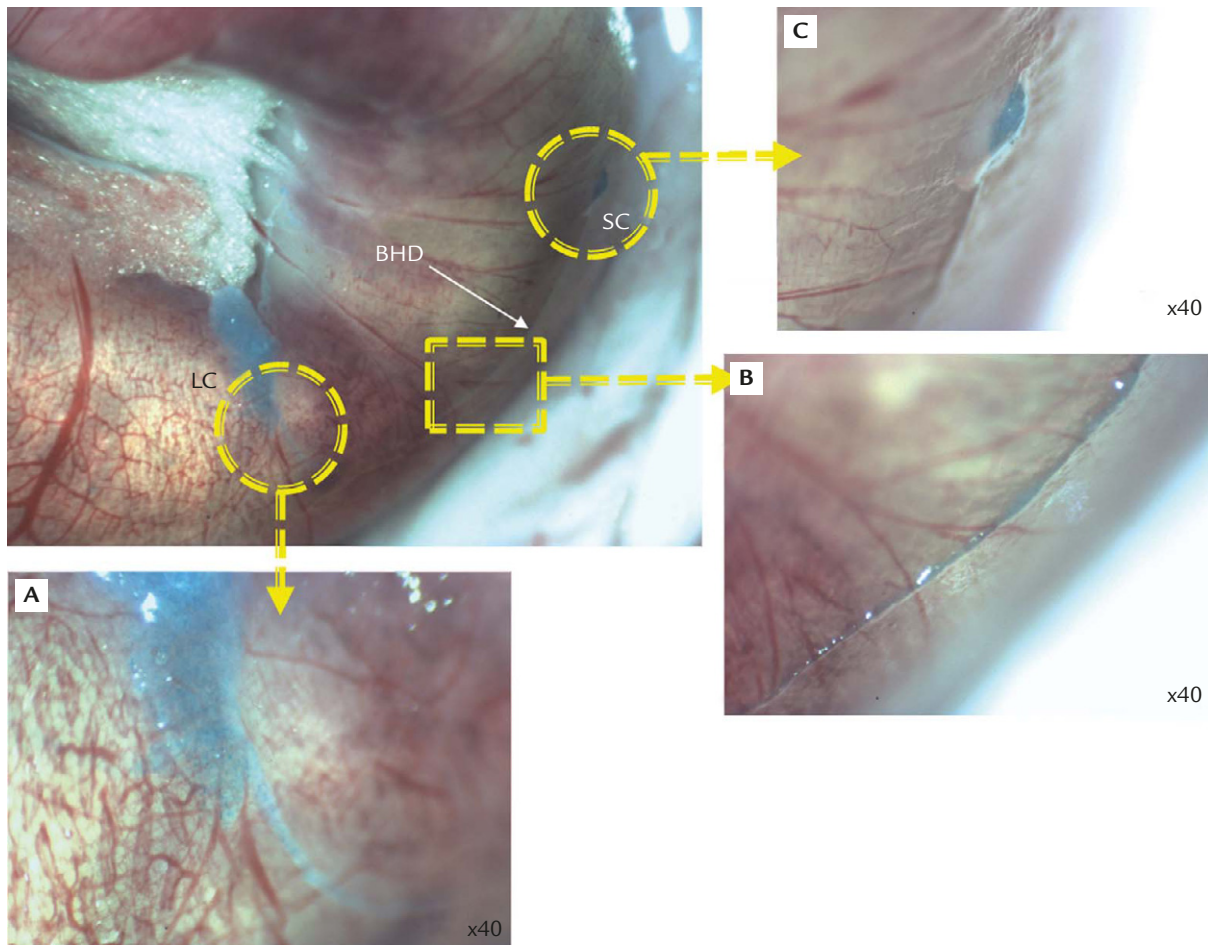


Figure 3 Stereomicroscope image of Alcian Blue (AB)-stained threadlike Bonghan ducts (BHDs) and corpuscles on the transverse colon. Large (LC) and small (SC) corpuscles are linked by threadlike BHDs, and all parts of the BHDs are heavily stained by AB. The corpuscles are adhered to the surface of the intestine connected to the colon (Figures 4A,C) while the threadlike BHD is not adhered to the surface and moves freely (Figure 4B).

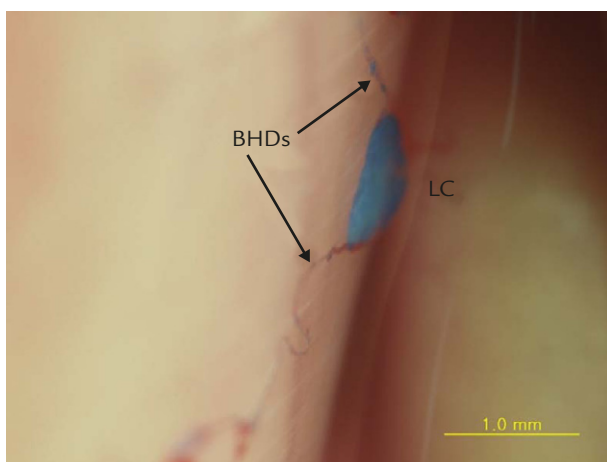


Figure 4 Stereomicroscope image of Alcian Blue (AB)-stained threadlike Bonghan ducts (BHDs) and a corpuscle on the surface of the right abdominal wall. The large corpuscle (LC) and the threadlike BHDs freely move and are heavily stained by AB. The size of the LC is about 1 mm, and the BHDs are about 30 μ m.

Neither the BHD nor its corpuscle adhered to the wall, and both moved freely. They became blue as AB passed through them. The BHD started from the pylorus region of the stomach and passed the duodenum, the right abdomen, the ascending colon, and the cecum. It was cut accidentally, so further tracing of the BHD was not possible because the remaining part of the duct coiled up and disappeared from view.

Like the three BHDs passing over the duodenum in Figure 5A (number ⑥ in Figure 2) and those on the colon in Figure 5B (number ② in Figure 2), the BHDs frequently branched to form a network. The latter BHD apparently showed no blue color at all in low magnification, but only with higher magnification. In Figure 5B, it seemed that there was no evidence of flow of AB through BHD. But under higher magnification (Figure 5C), AB spots were revealed in the BHD as evidence of flow of AB. The magnified view also revealed a bundle structure with channels, which is one of the characteristic features of the BHD.

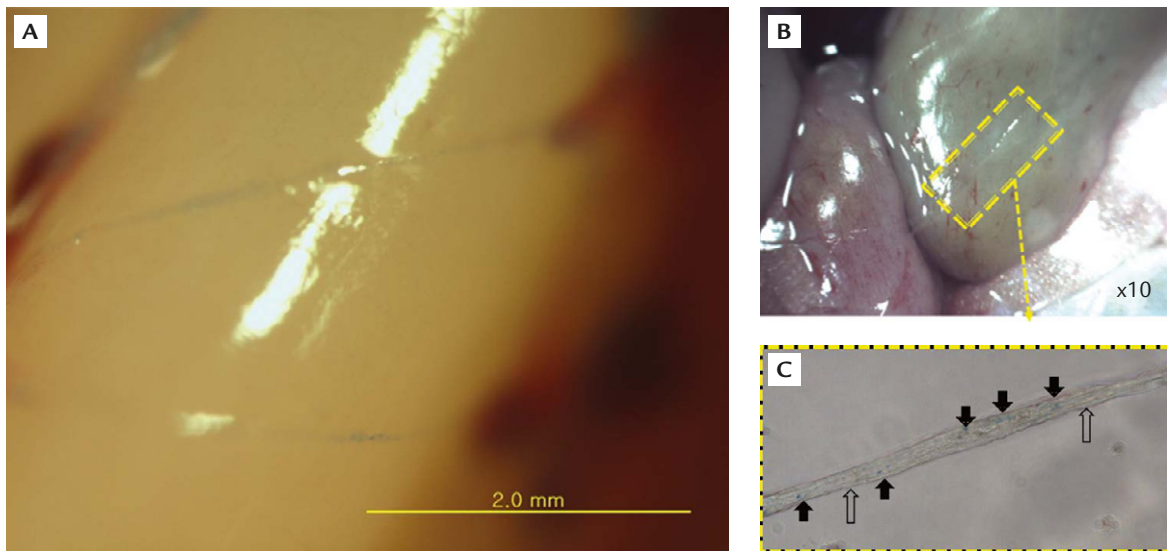


Figure 5 Network of Alcian Blue (AB)-stained threadlike Bonghan ducts (BHDs) on the intestines. (A) Three AB-stained BHDs pass over the duodenum and are connected to each other. (B) BHDs form a network on the colon. These appear to be transparent on lower magnification. (C) Under higher magnification (rectangular part of B), these structures have some AB spots (closed arrows) and a bundled structure containing some subducts (open arrows).

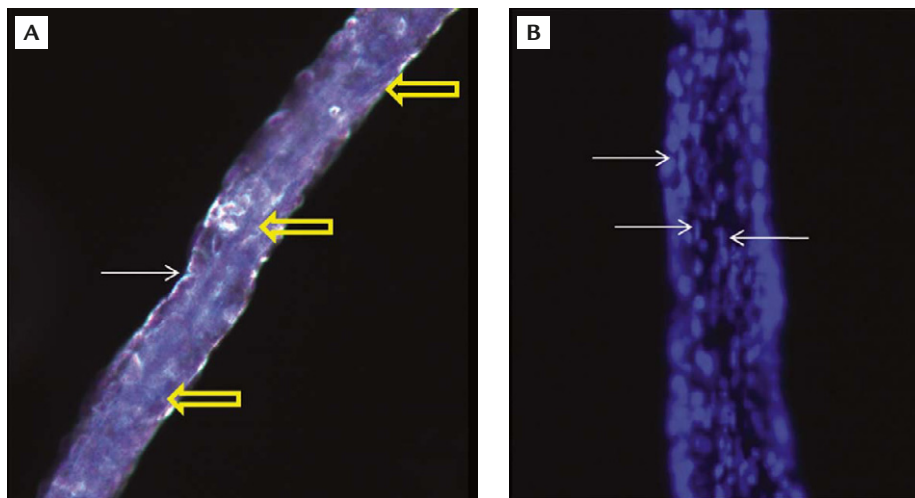


Figure 6 Phase-contrast microscope image and nuclear staining using DAPI of an Alcian Blue (AB)-stained threadlike Bonghan duct (BHD). (A) Under a phase-contrast microscope, the threadlike BHD clearly reveals three subducts (dotted black arrow) and evidence of AB flowing (opened arrows) in the direction of the subducts. (B) Rod-shaped nuclei are observed on the BHD (arrows).

The images in Figure 2–5 were taken *in situ* with a stereomicroscope. More detailed examination of the sampled BHDs with a phase-contrast microscope exposed a bundle structure composed of three channels, in which some marks of AB flow were present (Figure 6A). In addition, DAPI staining revealed rod-shaped nuclei longitudinally arranged in rows, as shown in Figure 6B, which is another characteristic hallmark of a BHD and has been reported in previous works [3,7,13–17].

The threadlike BHD sample in Figure 3B was analyzed by using TEM. The matrix of the BHD mainly

consisted of abundant collagen fibers, immune cells like macrophages, and flattened squamous-like epithelial cells, as shown in Figure 7A. Higher magnification of the rectangular region in Figure 7A disclosed subunits formed of collagen fibers and squamous-like epithelial cells, as shown in Figure 7B. There were macrophages between the subunits. The squamous-like epithelial cells formed the outer boundary of the BHD and had some granules and ribosomes inside its cytoplasm (Figure 7C). Between these squamous-like epithelial cells wrapping the collagen bundles, collapsed sinuses were located (Figure 7D). The sinuses

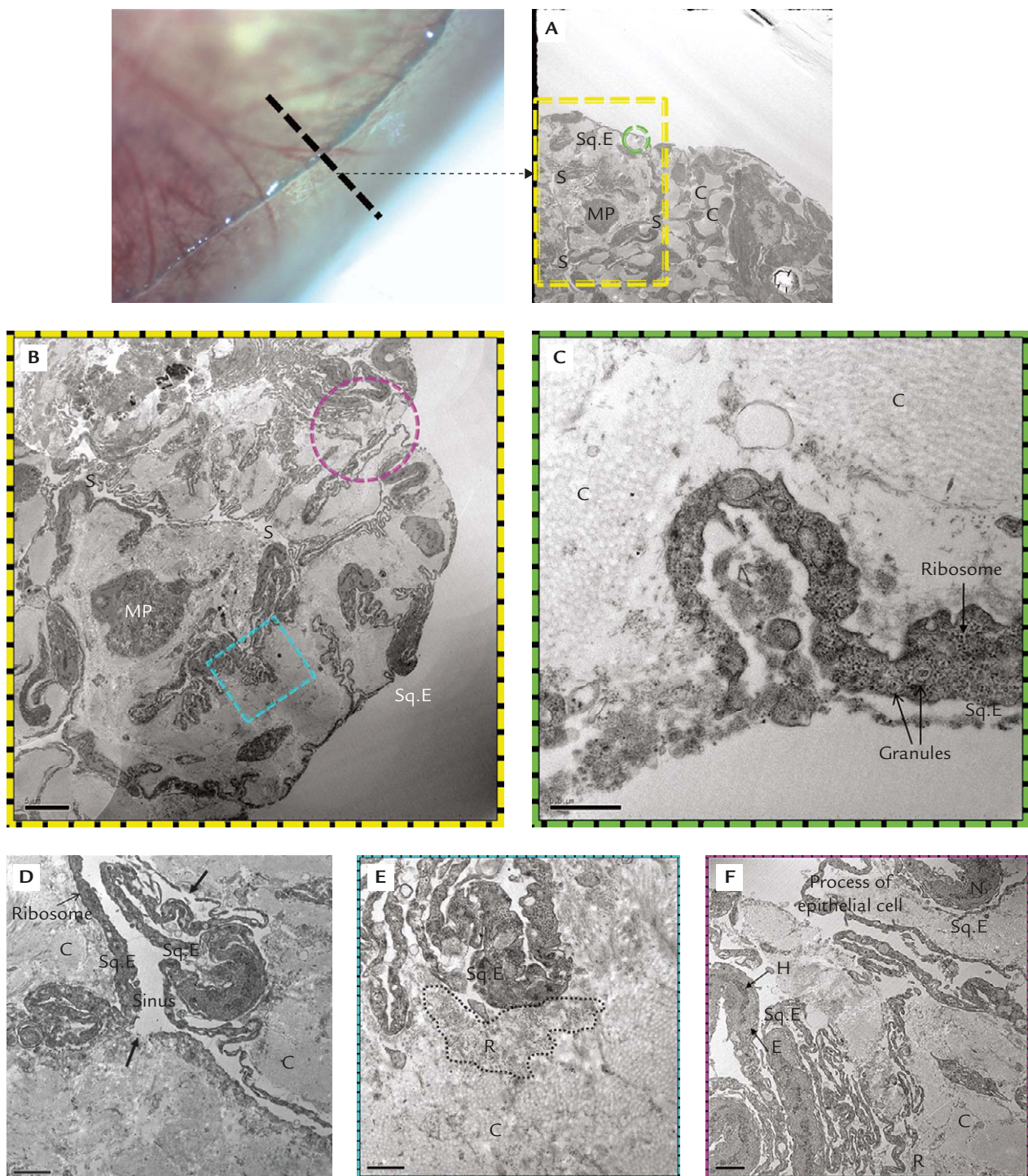


Figure 7 Transmission electron micrograph of a threadlike Bonghan duct (BHD). (A) The matrix of a threadlike BHD mainly consists of abundant bundles of collagen fibers (C), immune cells such as macrophages (MP) and flattened squamous like epithelial cells (Sq.E). (B) Higher magnification of rectangular area of A. Bundles of collagen fibers (C) and squamous-like epithelial cells (Sq.E) compose a subunit, and there are some collapsed sinuses and macrophages (MP) between individual subunits. (C) Higher magnification of the round area of A. A Sq.E, enveloped by collagen bundles (C), has some granules and many ribosome inside its cytoplasm. (D) The sinus between each subunit is surrounded by Sq.Es and their processes. There are some openings, about 2–3 μm , between the processes (arrows). (E, F) Higher magnification of the rectangular area E and the round area F of B. Reticulofibers (R, dotted line) are revealed between the Sq.E and the bundle of collagen fibers. The nucleus (N) of the Sq.E consist of heterochromatin (H) and euchromatin (E) in about a 1:3 (H:E) ratio.

were surrounded by squamous-like epithelial cells and their processes, and were 2–3 μm openings between the processes.

There were reticulofibers between the squamous-like epithelial cells and the bundle of collagen fibers, as disclosed by a high-magnification image of the rectangular area in Figure 7B (See Figure 7E). The nucleus of the squamous epithelial cells contained heterochromatin (H) and euchromatin (E) in about a 1:3 (H:E) ratio (Figure 7F). These electron microscopic images are consistent with our previous work [7,9,15], but detailed observations of the squamous-like epithelial cells of a BHD has not been reported before.

4. Discussion

For the structural identification of the threadlike duct in which AB flowed as the BHD, we considered its anatomical and morphological characteristics. First of all, it was not a blood vessel because it did not have red blood cells, and TEM image did not show any blood vessel wall structure, which also definitely excludes a lymphatic vessel or a nerve interpretation [3,7,9]. Its semitransparency, free movement without adherence to organs, thickness, and associated corpuscles are in agreement with the BHD description given by Bonghan Kim [1] and by other previous works [13,15,18]. The multiple channels and rod-shaped nuclei observed in the phase-contrast microscope image (Figure 6) are characteristic features of the BHD [19]. The extracellular matrix of collagens and the presence of immune cells (Figure 7) are also corroborative of a previous electron microscopic study of the BHD [7].

In addition, some sinuses, surrounded by squamous-like epithelial cells, were observed. We suppose that AB flows through these sinuses of the BHD, but we could not definitely prove this presumption. AB flow was not seen in the TEM image of the sinuses because AB did not remain during TEM processing. In the phase-contrast microscope image (Figure 6), AB was located inside subducts that seem to correspond to the sinuses.

As to the circulatory function of the threadlike duct, we only observed that the AB injected into the skin flowed through the duct to the wall of the large intestine. Whether the threadlike BHD forms a closed circulatory system, like the blood system, or an open one, like the lymphatic system, is unknown. It apparently has no pump like a heart, thus, it may maintain its flow in a way similar to lymph flow. The BHDs in Figure 2, obtained by combining the results of ten experiments, showed a general tendency to lie on the right hand side starting from the upper organs like the liver and the stomach and

terminating at the large intestine. However, the beginning and ending positions and terminals in the ten experiments were not the same. There were also variations in their development, with some having multiple branches, as shown in Figure 5.

The injection point was the acupoint BL23, the Urinary Bladder meridian. BL23 is the kidney association point, which is a very important acupoint in Traditional Chinese Medicine for treating and managing the kidney and urogenital function [20]. Therefore, we hypothesized that BHDs may be distributed over organs in the region of the urinary tract, such as the kidney or the urinary bladder. However, in this study, AB-stained threadlike ducts were located mainly on the surfaces of the duodenum, the right abdominal wall, the cecum and the colon. These were surprising and unexpected results, which indicated the possibility that BL23 could manage and control the right abdomen, especially the right part of the small and the large intestine. This disparity between the expected function of BL23 and the path of AB flow requires further study.

AB stains hyaluronan under high acidic conditions and was used for histological study of acupuncture meridians in humans because the acupoint has many mast cells that are stained well by AB [21–25]. It was also used even at pH 7.4 as a non-toxic staining agent for studying the preparation and evaluation of physiologic solutions of AB for *in vivo* use [26,27]. Given these observations, we used AB dye to trace the BHDs in this study, because the BHD is known to have an abundant supply of hyaluronic acid and mast cells [1,7,9]. However, AB was easily removed by histologic processing due to its water-soluble nature, and it disappeared during the washing process. This characteristic is a serious shortcoming in any histological study of the BHD in which AB was retained.

Due to the conflict between AB tracing and histological processes, we could not investigate the precise input position in the skin and trace the point at which the duct emerged in the abdomen or on the surface of the large intestine. Without tracing AB, it is not possible to detect the BHD in tissues of internal organs.

The method with AB has serious limitations when searching for the entire BHD path. This points to the need for a new staining agent that remains even after histological processes and that also flows well in the BHD. Nevertheless, it was essentially the first demonstration of the circulatory function of the BHD from acupoint BL23 in the skin to the large intestine of a rat. The medical significance of the BHD as a potential new drug delivery path seems to be especially suited for cancer therapy. This route of administration would produce fewer adverse effects in the kidney or liver, in contrast to intravascular injection. This possibility provides motivation for

further research to disclose the complete network of BHDs, including other acupuncture points and corresponding organs.

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