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RESEARCH ARTICLE

Visualizing the Network of Bonghan Ducts in the Omentum and Peritoneum by Using Trypan Blue

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Abstract
A visualizing agent, Trypan blue, was found to be preferentially effective for Bonghan ducts (BHDs) and corpuscles compared to blood vessels or adipose tissues. By using it, we observed a weblike network of BHDs which was in various membrane structures, such as the peritoneum, and omenta. This network of BHDs in the membrane structures was connected to the freely movable BHDs which did not adhere to the surfaces or wrapping membranes of internal organs. In addition, tracing BHDs in adipose tissues became possible because Trypan blue does not stain adipose tissue.

1. Introduction

The broad range of omental applications and functions has attracted much attention both from clinical and basic researchers [1]. For example, omental transposition yields an accelerated wound-healing process [2–7]. In tissue engineering, new organs have been developed by implantation of embryonic tissues into the omentum of the recipient [8,9]. The omentum provides the primary site for neutrophil exudation and a local site for peritoneal leukocyte proliferation and macrophage differentiation [10–12]. Also, a novel role for mast cells in omental angiogenesis was recently reported [1].

In this article, we report finding a novel anatomical structure on the omentum and the visceral peritoneum in the form of a web, which may arouse an enlightening new vista for understanding the omental functions in tissue engineering, peritoneal defense, adhesion formation, and tumor growth. Surprisingly, this novel web is related to acupuncture, one of the increasingly popular alternative medicines.

The acupuncture meridians, which are known to be the pathways of the Qi, are at the heart of traditional Chinese medicine. Establishment of their physical reality is crucial for scientific investigation of the 1,000-year-old practice of acupuncture. In the early 1960s, Bonghan Kim claimed to have discovered an anatomical structure corresponding to acupuncture meridians [13,14]. Despite the potential significance in both Western and Eastern medicines,

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his work has been ignored for many years due to the absence of verification from other researchers. Only one Japanese anatomist, Fujiwara, has ever managed to replicate his results [15].

With modern fluorescence and microscopy technologies, rediscovery of the Bonghan system has become possible, and this has led to a number of new research efforts. Scientists have explored the intravascular Bonghan ducts (BHDs) and Bonghan corpuscles (BHCs) in blood vessels [16–18] and lymphatic vessels [19–21], as well as organ-surface BHDs and BHCs [22–24]. Further progress was made when BHDs were found in the brain ventricles and the central canals in the spines of rabbits [25] and when putative acupuncture muscle channels were observed in the skin [26].

A series of investigations to elucidate the details of the anatomy and the morphology of a BHD have been performed using scanning probe microscopy [27], various electron microscopy techniques [28], X-ray microtomography [29], and immunohistochemical techniques [30]. Measurement of the flow speed of the Bonghan liquid in a BHD was performed by injecting Alcian blue; the speed of travel was found to be 0.3 ± 0.1 mm/sec [31]. Researchers also confirmed that a BHC has chromaffin cells that produce and store catecholamine, suggesting the medical significance of the BHD as a hormonal pathway [32].

In rediscovering the Bonghan system finding the right staining dye to visualize them is the most critical factor. In fact, Bonghan Kim stated that he used a blue dye to trace the whole circulatory system, but he kept the material and method secret [13,14]. This was the main reason no one was able to reproduce his results. We have tried various staining dyes and have found Janus Green B [19], Alcian blue [29], and Chrome hematoxylin [26] to be partially satisfactory; fluorescent nanoparticles [20,21,26] were also found to be useful. However, they were not specific enough to distinguish the BHD from other tissues, such as lymph vessels, connective tissues, or adipose tissues.

In the present work we report a new staining dye, that is, Trypan blue, which is by far the most efficient among the ones we have tried in detecting the BHD. It stains preferentially the BHD rather than blood or lymph vessels, nerves, adipose tissues, or other membranous structures wrapping internal organs. For the first time, we were able to find weblike networks of BHDs on the omentum and the visceral peritoneum of a rat. In previous works [22–24,28,31,32], only large BHDs were observed, and no network of fine BHDs was noticed, but it was visualized in the present report. In addition, we made a new progress to visualize the BHD in adipose tissue. BHDs often entered adipose tissues, which hinders tracing them any further.

2. Materials and Methods

2.1. Animal preparation

Rats (Wistar, both sexes, ~200 g) were obtained from Jung-Ang Laboratory Animal Company for this study. The animals were housed in a constant-temperature controlled environment (23°C) with 60% relative humidity under a 12-hour light/dark cycle. All rats had adlibitum access to food and water. The procedures involving the animals and their care were in accordance with international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996). The rats were anesthetized with urethane (1.5 g/kg) administered intraperitoneally, and all surgical procedures were performed under systemic anesthesia.

Under deep anesthesia, we cut the medial alba of the abdomen and observed BHDs above the internal organs of a rat after Trypan blue staining. For the tracing experiment with Trypan blue, we longitudinally incised the abdominal skin beside the medial alba of a rat.

2.2. Trypan blue staining

We used a 0.4% Trypan blue solution (Sigma, USA) for staining BHDs on the internal organs of a rat. After exposure of the internal organs of the rat, we drop several milliliters of Trypan blue on internal organs such as the small intestine, the large intestine, and the stomach. About 1-minute later, we washed the internal organs several times with phosphate-buffered saline, pH 7.4 (PBS). Under a stereomicroscope (STZ10, Olympus) with a CCD camera (DP70, Olympus), we observed BHDs stained by Trypan blue and took their images.

2.3. Tracing experiment with Trypan blue

We injected about 1 mL of 10% dimethyl sulfonic oxide (DMSO, Sigma, USA) and 0.4% Trypan blue solution (Sigma, USA) into the subcutaneous layer in the abdominal skin near the umbilicus of a rat. Twenty-four hours after the injection of Trypan blue, we longitudinally dissected the abdomen of the rat along the medial alba. We traced the flow of Trypan blue in the BHDs from the skin to the internal organs.

2.4. DAPI staining

In order to characterize the nuclei in a BHD, we stained the Bonghan specimens by using 4′,6-diamidino-2-phenylindole (DAPI). For quick and photo-stable staining, we used Prolong Gold antifade reagent with DAPI (Molecular Probes, USA). The specimens were examined with a phase-contrast

microscope (Axiophot, Zeiss, Germany) and a confocal laser scanning microscope (LSM510, Zeiss, Germany).

3. Results

Figure 1 compares the surfaces of the small intestine of a rat before and after staining with Trypan blue, which stained the BHDs preferentially compared to other tissues, such as blood vessels, adipose tissues, and organ surfaces. For example, a BHD on the adipose tissue around the small intestine was hardly noticeable before Trypan blue treatment, as shown in Figure 1A, but was clearly displayed after Trypan blue staining, as shown in Figure 1B. The milky white BHC also became blue colored by staining.

The weblike fine network of BHDs on the visceral peritoneum around the stomach near the spleen of a rat (Figure 2A) was revealed by Trypan blue visualization. It is remarkable that the BHD-web on the surface of the omentum was well stained while the blood vessels in the omental tissue were not colored at all. Scattered small knot-like BHCs were also observed at some of the crossing points of BHDs.

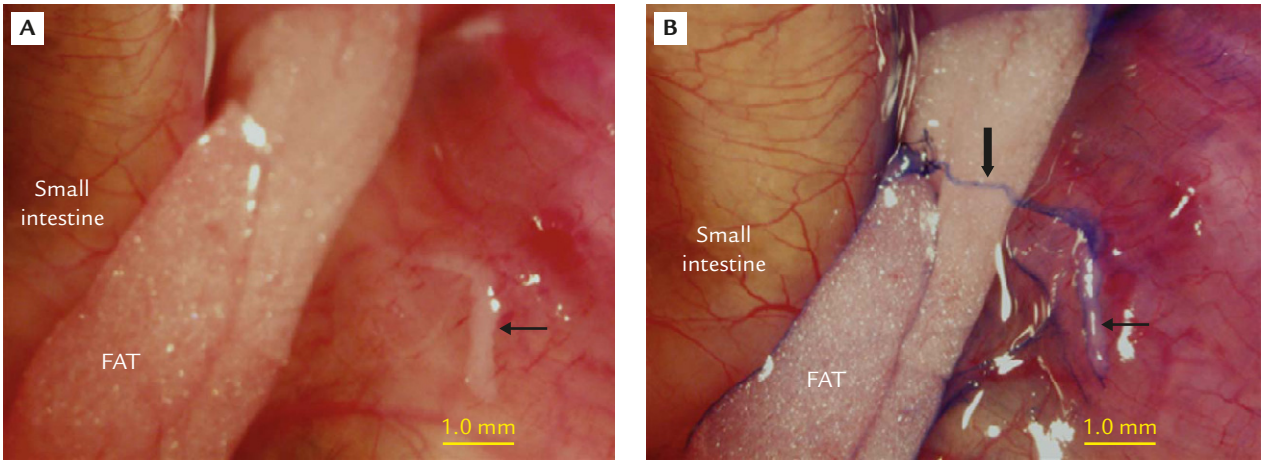

Figure 1 Comparison of a Bonghan duct (BHD) before and after Trypan blue visualization. (A) Adipose tissue around the small intestine. A Bonghan corpuscle (BHC, arrow) was detected, but its BHD was barely observable. (B) After staining with Trypan blue, the BHD (thick arrow) on the adipose tissue was clearly visualized. Blood vessels and adipose tissues were not stained.

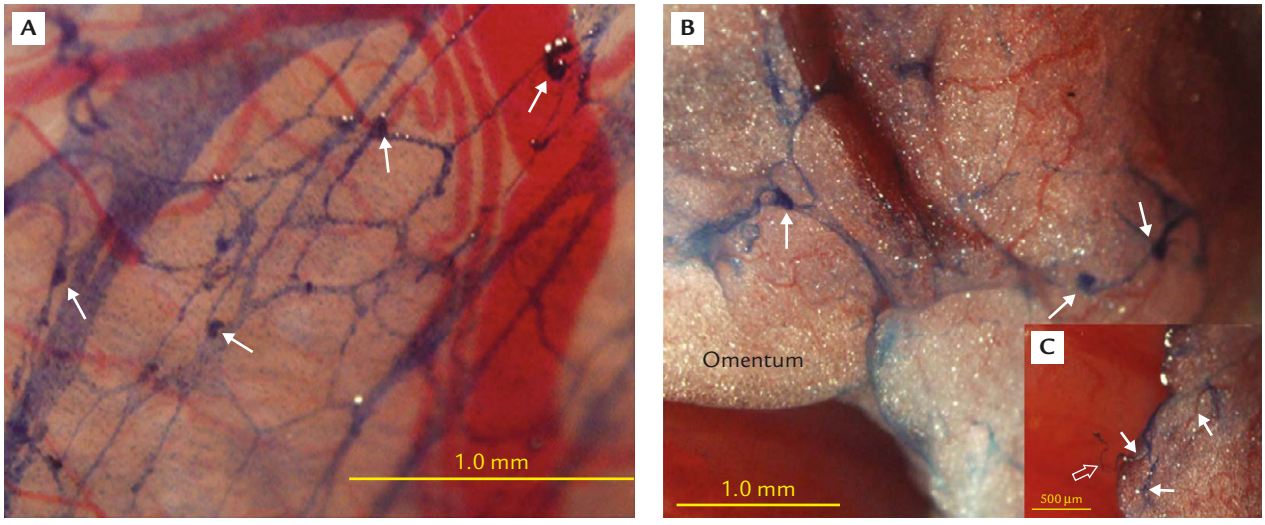

Figure 2 Weblike network of BHDs revealed by using Trypan blue. (A) The web of BHDs on the visceral peritoneum around the stomach near the spleen of a rat. There were several small BHCs at crossing points, as indicated by the arrows. The blood capillaries were not stained. (B) The network of BHDs on the omentum below the stomach and over the small intestine. Three small corpuscles at the crossing points of BHDs are indicated by the arrows. (C) In the inset another part of the same omentum as in (A) is shown. Here, the floating BHD (open arrow) was connected to the BHDs (arrows) in the omentum. This figure shows that BHDs on the omentum was a part of the larger network of freely movable BHDs on the surfaces of internal organs which were previously reported [24,28].

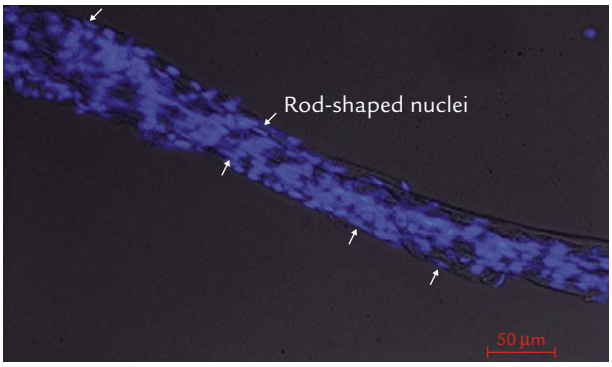

Figure 3 A phase-contrast image of a BHD merged with an image of nuclei stained by using DAPI (blue color). The figure shows the characteristic features of a BHD, rod-shaped nuclei, and a bundle structure, which provide simple criteria to identify a BHD from among similar looking structures. Some of the rod-shaped nuclei are indicated by arrows.

Another network of BHDs on the omentum around the stomach of a rat is shown in Figure 2B. The appearance of the web was not so prominent, but complicated net-structure was perceptible. Three BHCs were indicated by arrows. In the inset (Figure 2C) another part of the same omentum is presented. Here, a freely movable BHD (open arrow) was seen connected to the network of BHDs on the omentum.

The hallmark of a BHD is the presence of rod-shaped nuclei aligned longitudinally along the BHD, which was demonstrated by the nuclei stained with DAPI, as shown in Figure 3. This BHD was obtained from the surface of the small intestine of a rat after Trypan blue visualization. The phase-contrast image displays the bundle structure of the BHD, which is another characteristic feature of a BHD.

4. Discussion

The importance of visualizing agent for finding and identifying the BHD from among other various tissues cannot be over emphasized. Without a proper dye, a target tissue will probably not be noticed, not even with highly-magnifying microscopes. No one has yet found the secret blue dye that Bonghan Kim used when discovering the whole network of BHD [13,14]. We have tried various materials, such as methylen blue, methyl green, Janus green B, Alcian blue, hematoxylin, Chrome hematoxylin, and fluorescent nanoparticles, and all were partially useful. In this work, we found an efficient staining dye, Trypan blue, that preferentially stained the BHD rather than blood vessels and adipose tissues.

The dye Trypan blue is used in histology for staining connective tissues, such as collagen, muscle, and cornified epithelium [33]. Another common use is to discriminate dead cells from live ones. Trypan blue is also useful for vivistaining of vitereoretinal membranes in ophthalmic surgery [34].

In the current work, Trypan blue was used as a visualizing agent *in vivo* and *in situ*, not as a staining dye of tissue specimens for histological purposes. By using it, we were able to make the following significant contributions: (1) A weblike network of BHDs on the omentum and the visceral peritoneum was observed. (2) As shown in Figure 2C, the network of BHDs on the omentum was connected to the freely movable BHDs that did not adhere to internal organs, and were extensively studied in previous works [24,28,31,32]. (3) Trypan blue stained the BHD, but not adipose tissue, which enabled the tracing of BHDs even in adipose tissues.

The medical significance of the omentum in connection with tissue engineering and tumor growth [35,36] calls for special attention to be given to the BHD-web that is on the omentum because the Bonghan system is a regenerative system [14]. Further research aiming at investigating the connection between cancer and the BHD-web is extremely desirable. Finally, the presence of BHDs in adipose tissues should not be overlooked in the study of adipogenesis and other physiological processes in connection with obesity.

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