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In vivo visualization of Bonghan ducts inside blood vessels of mice by using an Alcian blue staining method

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An *in vivo* method using Alcian blue (AB) was developed for visualizing floating threadlike tissues inside blood vessels of mice. These novel structures called intravascular Bonghan ducts (IBHDs) are considered as extension of acupuncture meridians. For *in vivo* imaging of IBHDs, AB solution (*p*H 7.4) that stains mucopolysaccharides like hyaluronic acid was used. After injecting AB solution into the femoral vein of a mouse, the threadlike structures, stained deep blue, inside the inferior vena cava. The histological results, such as hematoxylin, eosin, and AB staining, show the compositions of the cells and the extracellular matrix in the IBHD. Further studies are needed to investigate their physiological functions, especially in relation with those of other circulatory systems.

Keywords Acupuncture meridian, Alcian blue, Bonghan duct, Hematoxylin and eosin, Hyaluronic acid

Novel threadlike structures inside the blood vessels of rats and rabbits that are called intravascular Bonghan ducts and corpuscles (IBHDs and IBHCs) have been reported[1-3]. The IBHD was first found by Bonghan Kim (1963) as part of an anatomical basis of the meridian system[4]. Kim reported that an IBHD was very thin (20 ~ 100 μm) and transparent. He also reported that a fluid containing hyaluronic acid among other biochemicals flowed through the IBHD system. Also the IBHD was deeply related with hematopoiesis; thus, in modern terms, it might be the source and the channel of hematopoietic stem cells. Kim's work was ignored until recently. One technical reason for this neglect was the difficulty in discerning the IBHD from ubiquitous fibrin strings in surgical situations. Both have about the same thickness, are transparent, and look similar under a light microscope.

A method that infused 0.1% (w/v) acridine orange (18.3 ml/kg) into a blood vessel was developed in order to discern the real duct and the fibrin coagulation by identifying the characteristic rod-shaped nuclei of the IBHD[2,3]. However, it was not possible to observe an IBHD in an *in vivo* condition by using this method. In this communication a new method to detect IBHDs in mice is reported. The new method uses Alcian blue (AB) injection to make *in vivo* visualization of the IBHD possible.

AB is one of the most widely used cationic dyes for the demonstration of mucopolysaccharides or glycosaminoglycans.[5-7] As an IBHD could be stained very well by AB, it was used as an *in vivo* tracer of IBHDs. An entire IBHD inside the inferior vena cava was visualized as a blue line due to AB staining. This implies that IBHDs contain many muco-polysaccharides, such as hyaluronic acid. Thus, the present work not only provides a new method for observing IBHDs but also confirms Bonghan Kim's claim on the presence of hyaluronic acid in Bonghan ducts[4].

Materials and Methods

Alcian blue (AB) solution — Cationic dye AB 8GX were obtained from Sigma (St Louis, MO, USA). AB solution (0.1 g AB, 10 U heparin in 10 ml phosphate buffered saline, *p*H 7.4) was used as an *in vivo* tracer of the IBHD.

Surgical preparation — Male mice (C57BL/6J, HSF-1, BALB/C, 20 to 30 g) were anesthetized with urethane (1.5 g/kg, Sigma, St Louis, MO, USA) administered on an intraperitoneal route. Their body temperature was kept at 37±0.1C by using a thermostatic heating pad (homemade). Institutional guidelines for animal care and use for research

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purposes were followed (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996).

In vivo imaging of an IBHD with AB staining— AB solution (200 μl/ 20 g) preheated at 37 °C was injected into a left femoral vein by using an insulin syringe with 30 gauge needle (ULTRA_FINEμ; BD, Franklin Lakes, NJ, USA). After injection, the abdominal sides of the mice were incised, and inferior vena cava was exposed. The adipose surrounding the inferior vena cava was separated and removed. Then, inside the inferior vena cava, an IBHD (mean diameter = 20 μm), stained deep blue, was visualized. Images of the IBHD under a stereomicroscope (SZX12; Olympus, Tokyo, Japan) were captured using a charge coupled device camera (DP70; Olympus, Tokyo, Japan).

Propidium-iodide staining — Samples of the IBHD were extracted in the inferior vena cava and were fixed in a fixative consisting of 0.5% paraformaldehyde (Sigma, St Louis, MO, USA) and 15% (v/v) saturated picric acid (Sigma, St Louis, MO, USA) in 0.1 *M* sodium phosphate buffer (*p*H 7.0) for 30 min, followed by several washes in phosphate-buffered saline (*p*H 7.0) in cover glass-bottom dish (confocal dish; SPL, Pcheon, Korea). The specimens were incubated in a propidium iodide (Molecular probes, Eugene, OR, USA) for 30 min and washed several changes with phosphate-buffered saline. Then, the samples were mounted with antifade reagent (SlowFade; Molecular probes, Eugene, OR, USA) and examined with a confocal laser scanning microscope (LSM 510; Zeiss, Goettingen Germany).

Hematoxylin, eosin, and AB staining — The samples of the IBHD with the inferior vena cava were fixed in 10% neutral buffered formalin and were frozen in an embedding medium (Tissue-Tek; Sakura Finetek, Torrance, CA, USA). Sections (20 μm thick) were cut with a cryotome (HM505E; Microm, Walldorf, Germany). The samples were washed with distilled water for 5 min, incubated in AB for 10 min and washed once again with distilled water for 5 minutes, followed by hematoxylin for 2 min. After several washings with tap water, the samples were soaked in 60% ethyl alcohol, incubated in alcoholic eosin, and dehydrated through a series of alcohols up to absolute alcohol. Next, the samples were mounted with 50% Canada balsam, examined with a light microscope (BX51/BX52 Olympus, Tokyo, Japan), and captured with a charge coupled device camera (Infinity 1; Lumenera, Ontario, Canada).

Results

Figure 1A is an illustration of the inferior vena cava (dotted arrows) and the femoral vein (arrowhead) of a mouse into which Alcian blue was injected. Figure 1B is a stereomicroscopic image of an IBHD inside the inferior vena cava. The IBHD was about 20 μm in diameter and was very transparent, so it was not visible without staining. In addition, it was easily broken during surgery. However, AB staining of the IBHD showed an intact IBHD under *in vivo* conditions without incision of the blood vessel.

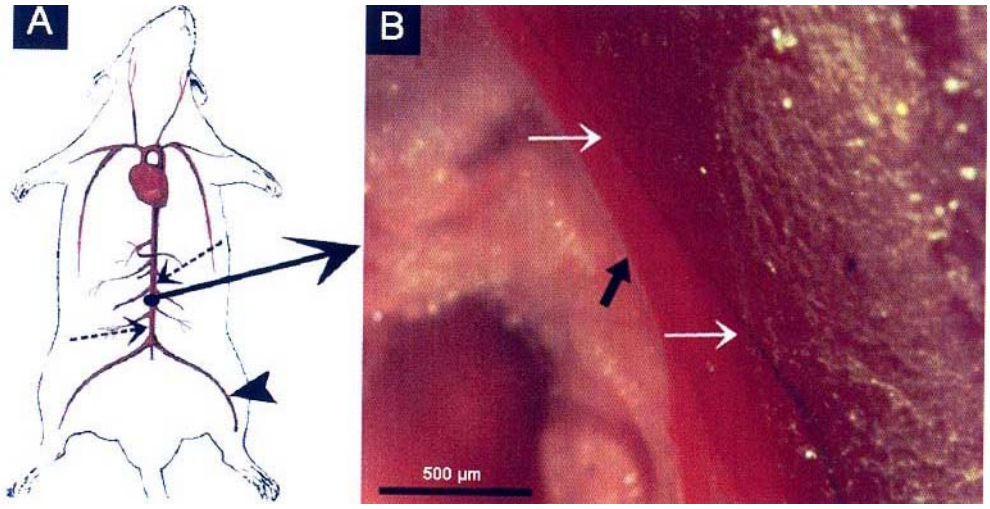

Fig. 1— (A) Schematic diagram of the inferior vena cava (dotted arrows) and the femoral vein (arrowhead) into which the Alcian blue was injected. (B) Stereomicroscopic image of a Bonghan duct (thin arrows) inside the inferior vena cava (thick arrow). The threadlike Bonghan duct was stained with Alcian blue *in vivo* and *in situ*.

Figures 2A and 2B show a confocal laser scanning microscopic image and differential interference contrast image of an IBHD from the inferior vena cava. The sample was taken from the inferior vena cava, put in a coverglass-bottom dish longitudinally, and stained with propidium iodide. The nuclei (arrowheads) of the IBHD were rod-shaped, about 10 μm long, and were distributed in a broken-line striped fashion.

The IBHD was connected with an IBHC whose size is about 50 ~ 150 μm in diameter. Figures 3A and 3B show cross-sectional images of the IBHC with the inferior vena cava after the cryo-section with a 20-μm thickness. The IBHC had many sinuses and various cells. IBHC in the inferior vena cava was stained deeply by AB compared with the vessel wall (Fig. 3A). AB preferentially stained the threadlike structure because the IBHD has more mucopolysaccharides than the blood vessel wall. In Fig. 3B, the staining colors of the blood vessel wall and the IBHC look different. The extracellular matrix of the IBHC was more basic than the blood vessel wall, which implies that the IBHC did not originate from the blood vessel wall.

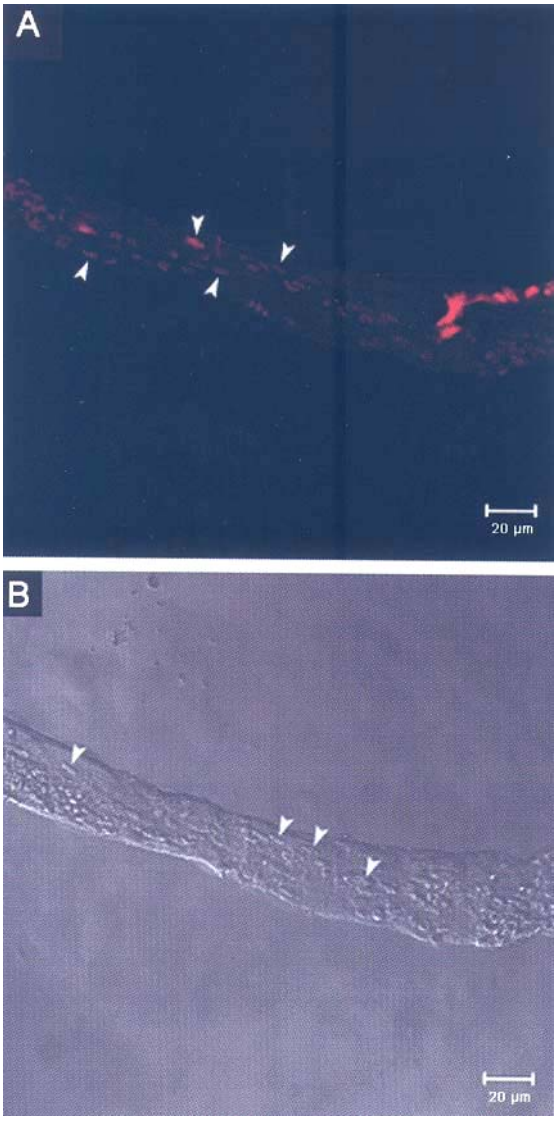

Fig. 2— (A) Confocal laser scanning microscopic image of the IBHD stained by propidium iodide. The rod-shaped nuclei (arrowheads) form a broken-line array. The diameter of the IBHD is about 20 μm. (B) Differential interference contrast image of the same sample. Arrowheads indicate rod-shaped nuclei.

Discussion

A new method was developed to visualize novel threadlike structures (intravascular Bonghan ducts) inside the blood vessel of mice by using Alcian blue injection into the femoral vein. Without incision of the blood vessel, we showed an *in vivo* image of a long IBHD inside the inferior vena cava was shown.

Propidium-iodide staining and hematoxylin, eosin, and AB staining results showed a set of histological features unique to IBHDs and IBHCs, which are different from those of blood vessels, nerves, and lymphatic vessels. The presence of rod-shaped nuclei, a critical feature that distinguishes IBHDs from similar-looking fibrin-coagulated strings was confirmed by propidium-iodide staining. In addition, many large sinuses were present in the cross section of the corpuscle. These sinuses were seen as long and narrow in the longitudinal image of the duct. Around the interior boundaries of the sinuses were the above-mentioned rod-shaped nuclei. Long and narrow lumens in the IBHD are considered to be the sub-ducts that form the bundle-like structure of the Bonghan duct. This bundle-like structure is a hallmark of the Bonghan duct and distinguishes it from a lymph vessel, which has only one lumen[8].

Kim claimed that the threadlike structures formed a circulatory network throughout the bodies of animal through which some liquid flowed[4]. The network was distributed inside blood and lymphatic vessels, on the surfaces of internal organs, and under the skin. Those under the skin were nothing but the classical acupuncture meridians, thus providing the anatomical basis of Traditional Chinese Medicine[4,9]. His theory was largely ignored, except by a Japanese anatomist, Fujiwara, who confirmed the existence of the intravascular and organ-surface threadlike structures[10]. Only recently were these works again verified by several groups. The threadlike structures inside blood vessels

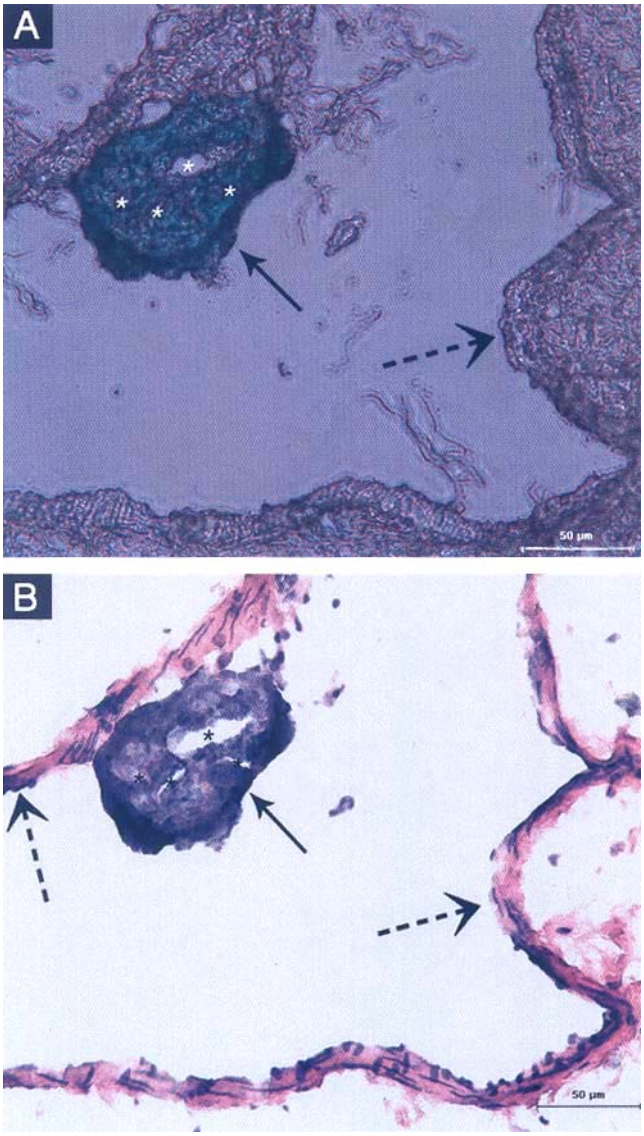

Fig. 3— (A) Phase contrast image of the cross-sectioned IBHC. Compared with the vessel wall, the IBHC in the inferior vena cava was stained deeply by AB. (B) Hematoxylin, eosin, and AB staining image of the same sample. The IBHC has many sinuses (asterisks), and its size is about 50 × 100 μm. The solid arrows shown the IBHC, and the dashed arrows show the inferior vena cava walls. ged

were observed in animals such as rats and rabbits[1-3]. Those on the surfaces of the internal organs of rabbits and rats were also observed and examined in detail[11,12]. The physiological role of the new circulatory system is considered to be deeply related to development processes and to the hematopoietic function[13].

In the present study, by using AB staining visual evidence of an IBHD inside the inferior vana cava that did not adhere to the vessel wall has been provided. The clear *in vivo* demonstration of the existence of this novel structure inside blood vessels gives substantial proof for Bonghan Kim's theory. Further studies are needed to develop specific imaging methods for IBHDs, such as sensitive visualization using nanoparticles and quantum dots[14]. Also, their functions, especially those related with therapeutic effects, as the physical basis for the acupuncture system need to be investigated.

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References

1 Jiang X, Kim H K, Shin H S, Lee B C, Choi C H, Soh K S, Cheun B S, Baik K Y, Soh K S, Method for observing intravascular Bonghan duct, *Kor J Oriental Preventive Med Soc*, 6 (2002) 162.

2 Lee B C, Baik K Y, Johng H M, Nam T J, Lee J, Sung B, Choi C, Park W H, Park E S, Park D H, Yoon Y S, Soh K S, Acridine Orange staining method to reveal characteristic features of intravascular threadlike structure, *Anat Rec B New Anat*, 278 (2004) 27.

3 Baik K Y, Lee J, Lee B C, Johng H M, Nam T J, Sung B, Cho S, Soh K S, Acupuncture meridian and intravascular Bonghan duct, *Key Engineering Materials*, 277-279 (2005) 125.

4 Kim B H, On the Kyungrak system, *J Acad Med Sci DPR Korea*, 90 (1963) 1.

5 Ghitescu L, Fixman A, Surface charge distribution on the endothelial cell of liver sinusoids, *J Cell Biol*, 99 (1984) 639.

6 Velleman S G, Yeager J D, Krider H, Carrino D A, Zimmerman S D, McCormick R J, The avian low score normal muscle weakness alters decorin expression and collagen crosslinking, *Connective Tissue Res*, 34 (1996) 33.

7 Budak Y, Demirci H, Akdogan M, Yavuz D, Erytrocyte membrane anionic charge in type 2 diabetic patients with retinopathy, *BMC Ophthalmology*, 4 (2004), 14.

8 Lee B C, Yoo J S, Baik K Y, Kim K W, Soh K S, Novel threadlike structures (Bonghan Ducts) inside lymphatic vessels of rabbits visualized with a Janus Green B staining method, *Anat Rec B New Anat*, 286 (2005) 1.

9 Langevin H M, Yandow J A, Relationship of acupuncture points and meridians to connective tissue planes, *Anat Rec B New Anat*, 269 (2002) 257.

10 Fujiwara S, Yu S B, 'Bonghan theory' morphological studies, Igaku no Ayumi, 60 (1967) 567.

11 Lee K J, Kim S, Jung T E, Jin D, Kim D H, Kim H W, Unique duct system and the corpuscle-like structures found on the surface of the liver, *J ISLIS*, 22 (2004) 460.

12 Shin H S, Johng H M, Lee B C, Cho S I, Soh K S, Baik K Y, Yoo J S, Soh K S, Feulgen reaction study of novel threadlike structures (Bonghan ducts) on the surfaces of mammalian organs, *Anat Rec B New Anat*, 284 (2005) 35.

13 KIM B H, Sanal Theory, *J Acad Med Sci DPR Korea*, 168 (1965) 39.

14 Gao X, Cui Y, Levenson R M, Chung L W K, Nie S, *In vivo* cancer targeting and imaging with semiconductor quantum dots, *Nature Biotech*, 22 (2004) 969.