The Origin of Endothelial Cells in Novel Structures, Bonghan Ducts and Bonghan Corpuscles Determined Using Immunofluorescence

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Abstract
Bonghan ducts (BHDs), and their associated Bonghan corpuscles (BHCs), which are novel threadlike structures, were recently observed in rats and rabbits by using various methods. As further support for the putative circulatory function of the novel threadlike structures (NTS), we investigated the presence and the origin of the endothelial cells within these structures. We immunostained the NTS with anti-CD146, an endothelial cell marker, and with anti-podoplanin, a lymphatic cell marker. Positive expression of CD146 in the BHDs was obtained, and the distribution of endothelial cells showed that the inner boundaries of the channels in the subducts branched from the BHDs and curled around, in a complicated manner, inside a BHCs. The negative expression of podoplanin implies that the endothelial cells in the BHDs are likely to be of vascular and not of lymphatic origin.

1. Introduction
Although there have been many attempts to elucidate the physical basis of acupuncture points [1–3], the identification of anatomical structures corresponding to acupoints and meridians has not yet been achieved. The only clear anatomical claim was made by Bonghan Kim in the early 1960s [4], and Fujiwara subsequently confirmed part of the Bonghan theory [5]. After being neglected for some time, new interest in Bonghan theory has been aroused by recent developments in methods to observe and identify Bonghan ducts (BHDs). BHDs are novel threadlike structures (NTS) in blood vessels [6], lymphatic vessels [7,8], and in the brain ventricles of rabbits and rats [9]. BHDs and Bonghan corpuscles (BHCs) have also been observed on the surfaces of various mammalian internal organs by three independent groups of researchers [10–12].

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The BHDs on the surfaces of internal organs are thin, semi-transparent, freely movable strands [12] and have intermittent thickened parts called BHCs. The ultra-structures of BHDs and BHCs were studied by using scanning transmission electron microscopes [13]. The BHD has a bundle structure that consists of many subducts, with each subduct having a diameter of about 10 μm. Through these subducts, liquid flows at a slow rate, measured at 0.3 ± 0.1 mm/sec [14].

According to Kim, the network of BHDs connects to form a circulatory system [4], an observation strongly supported by the flow of liquid. The presence of endothelial cells in the BHD would be another critical factor in identifying the BHD as part of a circulatory system, but no investigations have been conducted to identify and characterize these endothelial cells. In the present work, we found CD146 and podoplanin antibodies to be effective for this purpose. CD146, which is involved in the control of endothelial cohesion and permeability, is currently used as a marker for endothelial cell lineage [15–18]. CD146 is located at endothelial junctions but outside adherent junctions [16]. Podoplanin is a lymphatic endothelial cell marker that is highly expressed in proliferating lymphatic endothelial cells [19,20]. Podoplanin expression has been reported in the choroid plexus in rat brain and in the ciliary epithelium of the rat eye [21]. Schacht and colleagues reported podoplanin in salivary gland myoepithelial cells and testicular fibromyocytes, as well as in many other types of cells [20].

The NTS are thought to exist and are presumably found in the body (as shown by previous studies), however, no one has investigated whether the NTS originate from vascular or lymphatic vessels. Therefore, we used CD146 and podoplanin antibodies to determine whether the endothelial cells in the BHDs are of vascular or lymphatic origin.

2. Materials and Methods

2.1. Animal preparation and surgical procedures

New Zealand White female rabbits (1.5 kg) were used in this study. The animals were housed at an appropriate temperature (23°C), with a controlled humidity of 60%, a 12 hour light and 12 hour dark cycle, with free access to food and water. The procedures for handling and caring for the animals complied with the guidelines of current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). The rabbits were anesthetized with intraperitoneal administered urethane (1.5 g/kg/5 mL), and all surgical procedures were performed under general anesthesia. The abdominal wall was dissected under deep anesthesia. The hair of the abdominal region was removed before surgery by using clippers, and hemostasis was performed on the large skin vessels around the incision in order to avoid contaminating the abdominal and thoracic surfaces with blood.

The BHDs and the BHCs on the organ surfaces were identified using surgical instruments, such as iris scissors and microforceps, under a stereoscopic microscope (Olympus, Japan). The images of the threadlike structures were obtained with a digital camera (Olympus, Japan).

2.2. Histological findings

The threadlike structures were fixed in a 4% paraformaldehyde solution for 3 hours for histological procedures. The overall morphology of the BHDs was determined by examining 10 μm tissue sections cut using a cryotome (Leica CM1850, Germany) and stained with hematoxylin and eosin (HE; Sigma, Steinheim, Germany). Sequential HE stainings were performed with adjacent immunofluorescence slides. Frozen 10 μm sections of BHDs, including BHCs, were pretreated with phosphate-buffered saline (PBS) and blocked with 5% normal horse serum for 30 minutes. The abdominal aorta and the lymphatic vessels in the portal area of the rabbit were used as positive controls. Samples were incubated overnight with anti-CD146 (diluted 1:500, Chemicon, USA) and anti-podoplanin (diluted 1:500, Abcam, Cambridge, UK) antibodies at room temperature. The tissues were exposed to FITC-conjugated anti-mouse IgG at a 1:200 dilution (Jackson Immunoresearch Lab, PA, USA) for 2 hours at room temperature for anti-CD146 antibodies. Tissue used for podoplanin studies were exposed to Cy3-labelled goat anti-mouse IgG at a 1:200 dilution (Amersham Biosciences, UK) for 2 hours at room temperature. Incubation of tissue in mouse IgG, at the same concentration as that used for primary antibodies, created negative controls for immunofluorescence. The negative controls had no immunoreactivity in the identified structures. After the final wash, the samples were mounted using the VECTERSHIELD™ fluorescence system with 4’,6-diamidino-2-phenylindole (DAPI) mounting medium.

3. Results

3.1. Novel threadlike structure

NTSs had a thick corpuscle-like body called a BHC, as shown in the dotted circle in Figure 1A. The BHC is connected by BHDs at both ends, as indicated by
the two arrows in Figure 1B. As depicted in the inset, the corpuscle can be held and lifted with a pin set and displays the tensile force of the BHDs. The locations, sizes, shapes of the BHDs in rabbits and rats vary widely, depending on the individual subject [17], and the BHC in Figure 1A is larger than average. The BHDs were removed and placed in tubes with 4% paraformaldehyde for 4 hours.

3.2. HE staining

Figure 2 displays HE staining of a part of the NTS in Figure 1. Panel A includes the BHD connected to the left end of the BHC. Panels B−E correspond to rectangle A and are serial sections adjacent to the immunofluorescence sections, and F and G are magnified views of B. Apparently, the BHC has various cell types and fibers. The BHD entered the BHC and divided itself into many subducts [17] that twirl around in a complicated manner in the extracellular matrix of the BHC. Thus, the rod-shaped nuclei of the endothelial cells in the subducts are seen as being aligned (arrows) or scattered around.

3.3. Immunofluorescence for CD146

Abdominal aortas from rabbits were used as positive and negative controls. Figure 3A and B are negative controls without anti-CD146. Figure 3C and D are positive controls: The abdominal aorta samples which were incubated with anti-CD146 antibody showed positive staining, and resembled vascular endothelial cells in the internal elastic lamina. Anti-CD146 positive cells were observed in the BHC (Figures 3E, 3F). The positive cells formed variously shaped closed curves, as expected from the many subducts of various sizes and shapes twirling around in the BHC.

3.4. Immunofluorescence for podoplanin

Livers from the rabbits were used as controls. Portal lymphatic vessels incubated with anti-podoplanin antibody displayed positive staining (Figure 4A) while negative controls not incubated with anti-podoplanin showed no signal (Figure 4B). Some podoplanin positive cells were detected in the BHC (Figure 4C). Nuclei distribution was revealed by DAPI staining (Figure 4D), and the merged image (Figure 4E) of podoplanin positive cells and DAPI stained nuclei showed five positive cells. A magnified portion of the image is shown in the inset.

4. Discussion

NTS with BHDs and BHCs are distinctly different from nerve, blood vessels or capillaries, and lymph vessels. Studies of their anatomical and morphological features were performed using light microscopy [17] and various electron microscopes [8]. The flowing speed of the liquid through the BHD was measured [14], and the neurotransmitter hormone, adrenaline, was observed in the BHD and in the BHC [22]. These studies provide supporting evidence for the circulatory function of the NTS, which, in turn, suggests that the presence of endothelial cells forming channels in the BHD and BHC is an important criterion for the postulated circulatory function.

Conventional HE staining could not reveal the endothelial cells in the NTS because it is very difficult to discern them from among the various types of cells, as shown in Figure 2. An ultra-structural study with electron microscopes [13] yielded results consistent with the current HE data, showing fibers and various cell types, including macrophages, eosinophils,
Figure 2  The serial HE staining of Bonghan structures adjacent the immunofluorescence slides. (A) Bonghan duct (BHD) and its connected corpuscle (BHC). (B–E) Serial sections of the BHC. (F, G) are magnified views of B (bar=100 μm). The threadlike BHD enters the BHC and divides into many branches to form a very complicated ball of subducts in a pool of other cells and extracellular matrices. The rod-shaped nuclei (arrows) in G indicate alignment of the endothelial cells of a subduct. Such an alignment is rarely seen because the subducts do not arrange themselves in order, but rather twirl around in a complicated manner. * = inlet of BHC.
mast cells, monocytes and fibroblasts. However, in previous studies, no definite identification or characterization of endothelial cells could be done with HE staining or electron microscopic observations [13]. Thus, an immunofluorescence investigation was needed for this purpose.

In the present study, we applied anti-CD146 antibody to obtain positive expression in the BHC (Figure 3). These results provide supporting evidence for the presence of endothelial cells in the NTS. We obtained a negative result in the podoplanin staining study (Figure 4). The few podoplanin-positive cells were somewhat large, round, and ovoid in shape, unlike the CD146-positive cells. These may be flattened endothelial-like reticular cells [23].

Podoplanin reactivity yielded unclear images from

Figure 3 CD146-positive endothelial cells on Bonghan ducts. (A−D) Rabbit aorta was chosen as a vascular endothelial control. A and B are negative controls. C and its magnified view (D) show positive responses to the anti-CD146 antibody. Fluorescence signal is primarily on the internal elastic lamina (white arrow). E and F: CD146-positive responses on the Bonghan ducts. The positively stained cells formed many closed curves of various shapes and sizes as expected from the structure of the BHC, where the main ducts subdivided and branched into various sized subducts which twirled around (bar=100 μm).
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many experiments, as the reactivity was somewhat weak and the number of positive cells was low. Thus, we stained with podoplanin and DAPI at the same time and merged the two images to indicate that the podoplanin positive cells were real.

The distribution of CD146-positive cells is consistent with the morphological features of the BHD with multiple subducts. For example, in Figure 3, the CD146-positive cells formed scattered groups of several closed curves, which could be interpreted as the inner boundaries of subducts forming a bundle. The bunches of endothelial curves are sections of the BHD that twirled around inside the BHC. The BHD with many subducts could be sectioned at various angles, sometimes very obliquely. Thus, the diameters of the closed loops formed by the CD146-positive cells varied. The diameters of the two loops in the rectangle (Figure 3F) are about 10–20 μm, which is in agreement with Bonghan Kim’s original data [4] and with other previous data on sinuses in electron microscopic images [13].

In conclusion, we found anti-CD146 positive expression and no podoplanin staining in the BHC, thus obtaining supporting evidence that the origin of endothelial cells is likely to be vascular rather than lymphatic. In addition, we observed that inside the BHC, the BHD had twirling bundles of subducts lined with endothelial cells.
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