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BRIEF REPORT

DiI Staining of Fine Branches of Bonghan Ducts on Surface of Rat Abdominal Organs

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Abstract
Novel thread-like structures and corpuscles, designated Bonghan ducts (BHDs) and corpuscles (BHCs), are known to form a system of networked channels. Here, we tested the effectiveness of a fluorescent carbocyanine dye, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), in staining BHDs and BHCs. DiI solution was infused into a BHC on the surface of a rat abdominal organ at a steady rate and the resulting labeling of neighboring BHCs connected via BHDs was examined, as identified by the red fluorescence of DiI. BHDs diameters tapered away from BHCs and formed tree-like branches with fine arborizations embedded in the membranous tissues at their terminal parts. In the proximal parts, DiI fluorescence appeared as continuous lines within BHDs, but a large portion of BHDs remained unstained. In the distal parts of BHDs, discontinuous elongated DiI microparticles were identified along the sinuses within BHDs. The results showed that inner spaces within the BHDs allowed DiI to flow and that BHDs have tree-like branches and terminal arborizations. In conclusion, DiI can be used in visualizing BHDs fine structures.

1. Introduction

Bonghan ducts (BHDs) and corpuscles (BHCs) were initially discovered in the 1960s by Bonghan Kim [1], who proposed that they formed a novel circulatory network distinct from the nervous, blood, and lymphatic systems. Bonghan systems were recently rediscovered in various tissues, including on abdominal organ surfaces [2–5], within blood vessels [6–8] and lymphatic vessels [9–11], and in the central nervous system [12]. To detect these novel structures, several labeling methods have been applied, including acridine orange [6,7], alcian blue [5,7,8,10], hematoxylin [12], Janus green B [9], and the Feulgen reaction [2]. According to Kim [1,13], BHDs and BHCs have tree-like branches and form terminal arborizations at cellular target contacts. The dyes tested so far have been useful in identifying BHDs and BHCs, but they have not allowed detection of fine structures, such as small

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branches and terminal plexi. Here, we used DiI a fluorescent carbocyanine dye that stains fine processes of neurons, to visualize the detailed structure of BHDs [14].

2. Materials and Methods

Rats (Wistar/ST males, ~200 g, Jung-Ang Laboratory Animal Co., Seoul, Korea) were housed at 23°C and 60% relative humidity under a 12/12 hour light/dark cycle with *ad libitum* food and water. Animals were handled in accordance with the guidelines of the Laboratory Animal Care Advisory Committee of Seoul National University. Under urethane anesthesia (1.5 g/kg), the medial alba of the abdomen was dissected and searched for BHCs on the internal organs using a stereomicroscope (SZX 12, Olympus, Tokyo, Japan). Following identification of BHCs *in situ* and *in vivo*, the largest corpuscle was selected for injection with 1,1′-dioctadecyl-3, 3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). DiI solution (10 μM) was infused into the BHC with a glass capillary at a rate of 0.02 μL/minute for 12 hours using an injection system (KD 310 NanoPump; KD Scientific, Holliston, MA, USA; Figure 1A). Twelve hours after injection, the DiI-stained BHDs were traced by viewing with a fluorescence microscope (MVX 10, Olympus, Tokyo, Japan) and images of traced BHDs were obtained using a CCD camera incorporated with the microscope and under dim white light from a halogen lamp (Figures 1 and 2). Dim light was required to locate BHD processes emanating from the BHC (Figure 1A). The distribution of DiI within a BHD was identified from images of DiI-traced BHDs fixed with 10% neutral buffered formalin under a confocal laser scanning microscope (LSM 510, Carl-Zeiss, Jena, Germany). Optically-sectioned images were obtained at every 2.4 μm (Figure 3). Staining of DNA particles and nuclei in the BHD was accomplished using acridine orange (0.01% w/v in saline), producing green fluorescence.

3. Results

A representative sample of a BHC connected to BHDs (marked ‘a’ and ‘b’) located on the surface of abdominal organs is illustrated in Figure 1. A BHC (~2 × 3 mm) was detected on the left surface of the abdominal organs between the bladder and large intestine. About 1 hour after DiI injection into the BHC, strong red fluorescence was observed in the central core and proximal processes (Figure 1B) and visible branch thickness appeared to decrease with distance from the BHC. Interestingly, the proximal processes (open triangles; Figure 1B) were only partially stained with DiI, with a large proportion remaining unstained. A branching point and two sub-branches of the BHD were observed about 5 mm away from the BHC (arrows; Figure 1B). At 12 hours after dye injection, two other organ surface BHCs were detected stained with DiI: one corpuscle located on the abdominal cavity right surface (circle; Figure 1A), approximately 20 mm from the initially-injected BHC and another labeled BHC identified in a deep dorsal area, close to the spinal cord, between folds of the small intestine. Figure 2

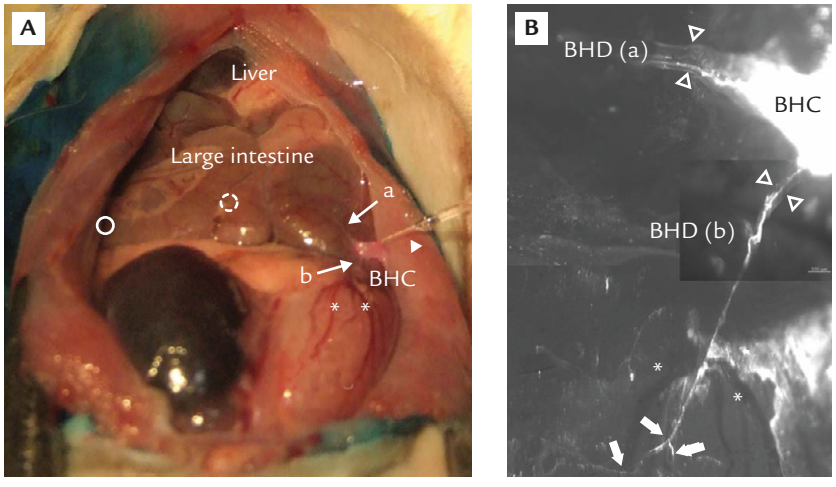

Figure 1 Labeling of rat organ surface Bonghan ducts (BHDs) with DiI. (A) Photomicrograph of abdominal surface showing organ surface Bonghan corpuscles (BHC) and needle (arrowhead) used for DiI injection. The two BHDs (a,b) emanating from BHC (two circles) on organ surface (solid line) and deep abdominal region (dotted line) 12 hours after DiI injection. (B) Fluorescence images of organ surface BHC and attached BHDs (a,b) as shown in A, branches of the BHD (b) (white arrowheads) and blood vessels (asterisks) are shown. Bright signals (white arrows) in lower right part not attributable to DiI fluorescence but to reflection of white light required to locate target object.

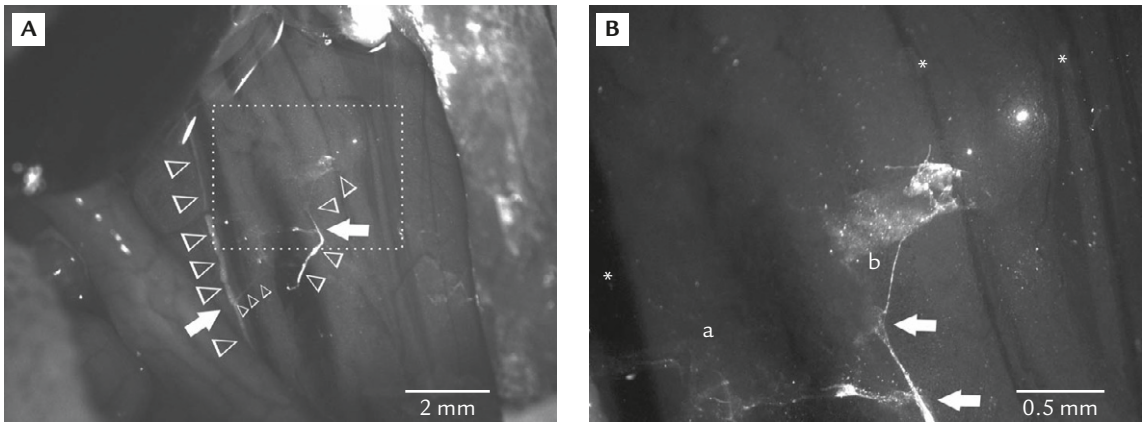

Figure 2 A DiI-labeled organ surface BHD in rat deep abdomen. (A) Labeled BHD shown in Figure 1A at 12 hours after DiI injection, branches of BHD (arrowheads) ran parallel to blood vessel (dark lines) and crossed by smaller branches of blood vessels. (B) Enlarged view of boxed region in A showing network-like structures of BHD terminals embedded in membranous tissues (a,b) with nearby fat tissues, branching points (arrows) and blood vessels (asterisks). Images obtained in partially darkened conditions.

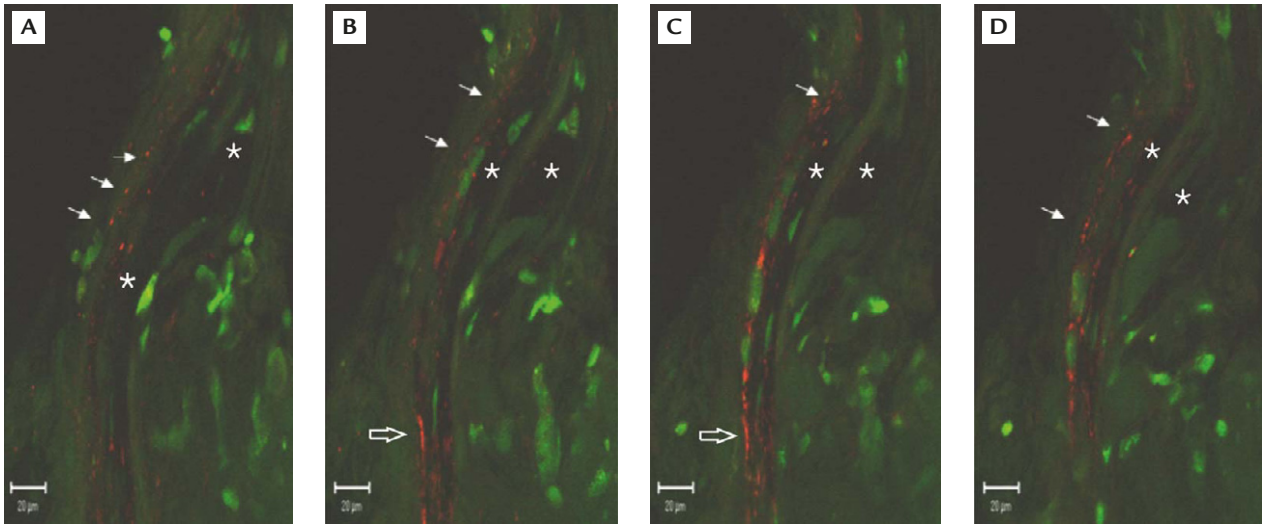

Figure 3 Confocal imaging of a BHD containing DiI (red fluorescence; arrows) on rat abdominal organ surface. Optical sections (2.4 μm) from A to D reveal short (solid arrows) and long (open arrows) DiI particles around the BHD sinuses; DNA particles, (green fluorescence) in BHD were stained with acridine orange; sinuses in BHD [3] appeared as dark areas (asterisks) in each panel. The scale bar is 20 μm.

illustrates the fine structures of BHDs originating from the third BHC described in Figure 1. Upon fluorescence illumination with weak normal light, BHDs appeared as bright lines (open triangles; Figure 2) and blood vessels as dark lines of various thicknesses. Two primary BHDs emitted secondary and tertiary branches at the branching points (arrows; Figure 2A). In general, the process thicknesses decreased with increasing levels of branching. Two BHD networks appearing as diffuse web-like structures embedded in the membranous tissues are shown in Figure 2B. One BHD network (marked 'a'; Figure 2B), located next to a blood vessel (dark area), whereas the other (marked 'b') was connected to adipose tissue.

DiI is known to stain neuronal processes by lateral diffusion rather than fast axonal transport [14]. We examined the DiI particle distribution within the BHD using a confocal microscope to further elucidate the labeling mechanism (Figure 3). DiI and DNA particles (stained with acridine orange) appeared as red and green fluorescence, respectively, whereas the sinuses of BHD, reported by Lee et al [3], appeared as dark regions (asterisk; Figure 3). DiI particles were dense in the lumen of the sinuses and in surrounding tissues. DiI particle sizes were 1–3 μm and 2–20 μm in width and length, respectively. Particles were not evenly distributed in BHD tissues and mostly limited to a sinus running parallel with the BHD long axis. A large proportion

of the BHDs did not contain DiI, which was consistent with data from Figure 1B, in which DiI fluorescence was found in a limited area of the proximal processes.

4. Discussion

The most salient finding of the present study was the successful visualization of BHD fine structures using the fluorescent carbocyanine dye, DiI. BHDs originating from dyed BHCs on the surface of abdominal organs tapered with distance, formed two or more smaller sub-branches, and generated web-like terminal networks. Our results are consistent with Kim et al (Picture 29) [13] on the fine structure of BHDs. Further studies are required to clarify the anatomical details of BHD-target organ contacts. In addition, the finding here that BHCs with connecting BHDs could be visualized by DiI injection into BHCs supports previous findings that BHD networks can circulate substances through the ducts [1,5].

The majority of dyes tested to date for BHD and BHC detection stain DNA in the nucleus of BHD cells and DNA particles inside the duct [2,6,7,9]. Alcian blue binds to acidic substances, such as hyaluronic acid [15] which is rich in the liquid in BHDs [1], and is extensively used to visualize BHDs in blood vessels [7,8], lymphatic vessels [10], and on organ surfaces [5]. None of these dyes clearly reveal the fine network structures of BHDs, but DiI is different because it is highly lipid-soluble and able to stain cell membranes [16] and has been extensively used as both a retrograde and an anterograde tracing agent in nerve tissue [14,16]. The tracing property of DiI is known as "lateral diffusion" [14,17]. DiI labeling of fine networks and terminal arborizations of BHDs in this study were likely due to the mode of action of DiI and not the lateral diffusion in the proximal region of BHD, because the distribution of DiI fluorescence was not continuous and restrained within the sinuses or in the spaces surrounding the sinuses. Further study is needed to understand the detailed mechanisms of the DiI labeling of BHDs. In view of the minimal cytotoxicity of DiI and long-term dye stability restrained to within sinuses or surrounding spaces in animals [18], DiI appears to be a promising dye for analysis of the morphology and functions of BHDs and BHCs over prolonged periods *in vivo*.

In conclusion, we have shown that BHDs form multiple sub-branches leading to a web-like fine network on abdominal organ surfaces and that these ducts may circulate substances through their sinus interiors. The results indicate that DiI can be effectively used to visualize the fine structure of Bonghan systems in both normal and disease states.

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