FEATURE ARTICLE

Feulgen Reaction Study of Novel Threadlike Structures (Bonghan Ducts) on the Surfaces of Mammalian Organs

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Threadlike structures on the surfaces of internal organs, which are thought to be part of the Bonghan duct system, were first reported about 40 years ago, but have been largely ignored since then. Recently, they were rediscovered, and in this study we discuss the Feulgen reaction that specifically stains DNA in order to identify these structures on the surface of rabbit livers as part of the Bonghan system. The distribution, shapes, and sizes of their nuclei are found to be similar to those of intravascular threadlike structures. The endothelial nuclei are rod-shaped, 10–20 μm long, and aligned in a broken-line striped fashion. The threadlike structure consists of a bundle of several subducts, which is a characteristic feature of Bonghan ducts and distinguishes them morphologically from lymphatic vessels. In addition, the Feulgen reaction clearly demonstrates that the subducts pass through a corpuscle, which is usually irregular or oval-shaped and is connected to two or several threadlike structures that form a web on the surfaces of organs. Furthermore, spherical granules of about 1 μm in diameter are detected in the subducts. These granules were well stained by using the Feulgen reaction, which implies that they contain DNA. According to previous reports, a granule is a type of microcell and plays an essential role in the physiology and therapeutic effect of the Bonghan system and acupuncture. This role has yet to be elucidated. *Anat Rec (Part B: New Anat) 284B:35–40, 2005.* **© 2005 Wiley-Liss, Inc.**

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INTRODUCTION

A radical challenge to modern anatomy is to explain recent claims that hitherto unnoticed novel threadlike structures exist on the surfaces of the internal organs. These claims are based on recent reports of three groups (Cho et al., 2004; Lee BC et al., 2004a, 2004b; Lee KJ et al., 2004) and on the long forgotten work of Bonghan Kim (1963) and Fujiwara's follow-up (Fujiwara and Yu, 1967). In brief, 50–100 μm thick semitransparent threadlike structures have been found on the surfaces of internal organs, such as the stomach, liver, large and small intestines, and bladder, of rabbits and rats. These structures do not adhere to the surface, but move freely and are sparsely and irregularly fixed to the peritonea. These novel threadlike structures have been sought as part of the network of Bonghan ducts for which an intravascular component had been previously reported (Jiang et al., 2002; Lee BC et al., 2004a).

Bonghan Kim (1963) sought the anatomical basis of acupuncture meridians in humans and animals and found a new circulatory system that was completely different from the vascular, nervous, and lymphatic systems. The meridians formed an anatomically distinctive system of threadlike ducts that spread under the skin. In addition, by tracing the ducts with a staining dye, he discovered that the ducts continued to spread onto the surfaces of internal organs and that they existed even inside blood vessels. He also found that a liquid flowed through the Bonghan duct system, and that the liquid played a physiological role akin to modern cell therapy by totipotent adult stem cells. The flow of this liquid was correlated with the therapeutic effects resulting from acupuncture treatments of damaged internal organs.

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Until recently, Bonghan Kim's discovery could not be reproduced, mainly because the formula of the staining dye, which was essential for identifying the Bonghan ducts, was kept secret. Without this secret formula, however, the existence of intravascular Bonghan ducts inside the blood vessels of rabbits and rats was recently confirmed (Jiang et al., 2002), and the acridine orange fluorescence method (Lee BC et al., 2004a) was found to distinguish clearly the threadlike structures from fibrins. Failure to make this distinction had been a major obstacle to the detection of Bonghan ducts in numerous previous experiments. Another step toward rediscovering the Bonghan system was the observation of threadlike structures on the internal organs of rabbits and rats (Cho et al., 2004; Lee BC et al., 2004b; Lee KJ et al., 2004).

The purpose of this article is to report on Feulgen reaction studies of novel threadlike structures taken from the surfaces of rabbit livers. To investigate whether the threadlike structures are indeed organ surface Bonghan ducts (OSBHDs), Feulgen reaction studies were particularly important to use because they specifically stain DNA (Chieco and Derenzini, 1999). This identification procedure is necessary for OSBHDs because other similar-looking threadlike structures exist, especially lymph vessels, which can be mistaken for the OSBHDs. In the case of the intravascular threadlike structures, such an identification procedure would not be required because, according to our current knowledge of anatomy and physiology, no structure other than the intravascular threadlike Bonghan ducts reported by Kim (1963) could explain that observation (Guyton and Hall, 1996; Smith and Shenk, 2001).

In previous reports (Cho et al., 2004; Lee BC et al., 2004b; Lee KJ et al., 2004), OSBHDs were observed and simple morphological descriptions were given, but no effort was made to identify the structures unequivocally. Here, for the first time, Feulgen reaction studies were performed to establish the novel threadlike structure as OSBHDs. Since Feulgen staining had been used by Kim (1963, 1965) to study the nuclear distribution of Bonghan ducts, our achieving the same results would be strong support for the identification of our observed structures as OSBHDs. The distribution, shapes, and sizes of the nuclei were in agreement with Kim's description. In addition, our results are consistent with those of previous reports on intravascular threadlike structures (Lee BC et al., 2004a).

As we present in this article, the crucial difference between the organ surface Bohghan ducts we report here and lymph vessels is that the OSBHDs are found in a bundle structure, whereas lymph vessels are known to be single tubes. In addition, according to the Bonghan theory, we expected to observe 1–2 μm-sized round granules containing DNA flowing through the OSBHDs (Kim, 1965; Baik et al., 2004). Indeed, we detected such granules in the threadlike structures and the existence of DNA in them was demonstrated by using the DNA-specific staining of the Feulgen reaction. Hence, we clearly identified the novel threadlike structures as Bonghan ducts.

ANIMAL PREPARATION AND METHODS FOR IDENTIFYING BONGHAN DUCTS

The animal preparation and surgical procedures were performed as follows. Ten female New Zealand white rabbits of 9 weeks were obtained from Hanlym Lab Animal for use in this study. All of the animals had ad libitum access to food and water. The housing and experimental conditions were the same as those given in another work (Lee BC et al., 2004a). The procedure was in full compliance with current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996).

The rabbits were anesthetized with urethane (1.5 g/kg) administered intraperitoneally, and all surgical procedures were performed under general anesthesia. The large vessels in the skin of the abdomen and the thorax were held by hemostats for hemostasis so that blood flow over the organ surfaces was minimized.

The search for threadlike organ surface Bonghan ducts on the liver was carried out under stereomicroscope (Olympus SZX12), and if the search failed, Mayer's hematoxylin (0.4% in PBS, pH 7.4) was spread on the liver surface to stain the OSBHDs and then washed with PBS (pH 7.4). The in situ OSBHDs were recorded by using a CCD camera (Olympus DP70). The tracings of the OSBHDs were done using micromanipulators that were equipped with needles or micropipettes.

Samples taken from the surface of the rabbit's liver were stained by using the Feulgen reaction. The samples were fixed in neutral buffered formalin for 1 day. They were then hydrolyzed in 5 M HCl at room temperature (20–25°C) for 60 min and stained in Schiff's reagent for about 60 min at room temperature, followed by three 5-min washes in 0.5% potassium metabisulphite solution and two 10-min rinses in distilled water. Finally, the samples were dehydrated by using ethanol solutions at concentrations of 50%, 70%, 80%, and 99% for 1 min in turn. Immediately following Feulgen staining, we used sharp needles and microknives under a stereomicroscope to remove the connective tissues binding the threadlike structures. Differential interference contrast images (Axiovert S100, Carl Zeiss, Germany) and fluorescence microscope images (LSM 510, Carl Zeiss) were obtained after Feulgen staining of the nuclei of the samples from which the connective tissues had been removed.

VISUALIZING BONGHAN DUCTS

A threadlike structure on the surface of a rabbit liver is shown in Figure 1. This is an example of OSBHDs found on the surface of internal organs. The threadlike structures had well-developed branches and were joined to a corpuscle. For demonstration, the entire structure on the surface of liver was lifted with a microforcep. Usually, a corpuscle is connected to two threadlike structures (Fig. 2), so the branching is simpler than in this figure.

A piece of a threadlike structure similar to the structure shown in Figure 1 was stained using the Feulgen reaction, and the surrounding connective tissues were removed using sharp needles and microknives. Figure 3A shows three substructures of the threadlike tissue. Two of the three

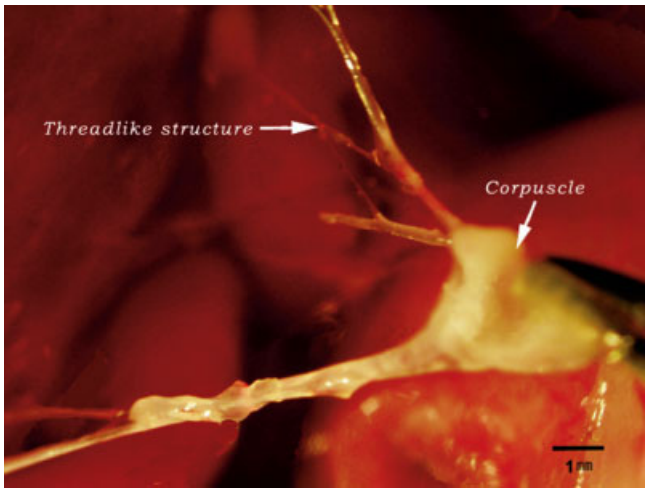

Figure 1. Stereomicroscope image of threadlike structures joining a corpuscle. The branching of the threadlike tissue was particularly well developed, and the corpuscle was connected to four threads. Usually corpuscles are connected by two threads, but some are multiply connected as shown here. The corpuscle and the threadlike structures were held above the liver surface for ease of exhibition.

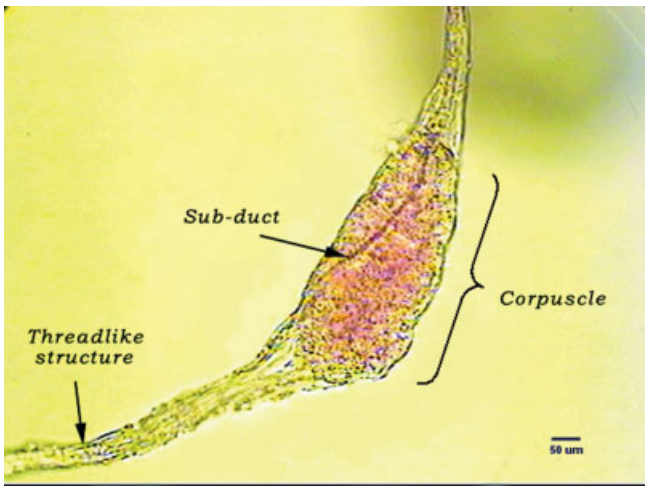

Figure 2. A typical corpuscle connected to a threadlike structure is shown. One subduct of the threadlike structure is clearly shown to pass through the corpuscle. The corpuscle was heavily stained using the Feulgen reaction because it contained abundant granules (see Fig. 4). Corpuscles are sometimes connected to many threadlike structures as shown in Figure 1.

were broken while removing the connective tissues, so the peeled-off connective tissues dangled below the unbroken threadlike structure. The thickness of the threadlike structure was about 100 μm, and that of each substructure was about 30 μm. This picture was taken using an inverted microscope (Axiovert S100, Carl Zeiss) in the differential interference contrast mode.

A more highly magnified view taken using an inverted microscope (IX71, Olympus) of the rectangular part of Figure 3A is shown in Figure 3B. A bundle structure with four subducts (1, 2, 3, and 4 in the middle right of figure) is clearly seen. Notice that the fourth subduct (labeled 4) is bent at about the midpoint. The thickness of each subduct is about 6.5 μm. Apparently, the unbroken part of the bundle structure has four subducts, but this number may be misleading because the picture is only a two-dimensional projection.

Figure 3C is a confocal laser scanning microscope image of the unbroken part of the threadlike structure in Figure 3A. Feulgen reaction stains DNA specifically, so the figure shows the distribution and the shapes of the nuclei, which appear to be red because the fluorescence wavelength is 625 nm. One should note the rod-shaped nuclei distributed on the endothelial layers of the subducts; this characteristic is one of the hallmarks of Bonghan ducts. The nuclei are distributed along several broken lines in a striped fashion. The features of the nuclei of the threadlike structure are in good agreement with Bonghan Kim's original description (Kim, 1963) and our description in a previous report on the intravascular Bonghan ducts (Lee BC et al., 2004a).

We observed corpuscular structures that were sparsely located along the threadlike structures. Figure 2 shows a corpuscle that had been weakly stained using the Feulgen reaction and whose diameter and length were about 0.3 and 0.7 mm, respectively. The corpuscle was more heavily stained compared with the threadlike structure. This suggests that there is a much higher nuclear content inside the corpuscle than in the threadlike structure. Figure 2 clearly shows a subduct that passes through the corpuscle. Only one subduct is shown because the others are at different depths of focus. If one looks at the corpuscle very carefully, one can see the dim image of another subduct that passes through the lower middle portion of the corpuscle.

We found that small granules containing DNA flowed in the subducts. Figure 4 shows small spherical red balls (arrows) in the subducts. Their diameters are about 1–2 μm, and they are well stained by using the Feulgen reaction. Two subducts can be seen in Figure 4 and DNA-containing granules can be observed, as expected from the work of Kim (1965), which claimed that the subducts were flow channels for DNA-containing granules.

DISCUSSION

Bonghan Ducts Are a Distinct Though Elusive Network

The novel threadlike structures on the surfaces of internal organs were not detected and received no attention in numerous surgeries carried out in the past because several obstacles prevented their easy observation. First, they are semitransparent and thin, thus hardly visible to the naked eye or

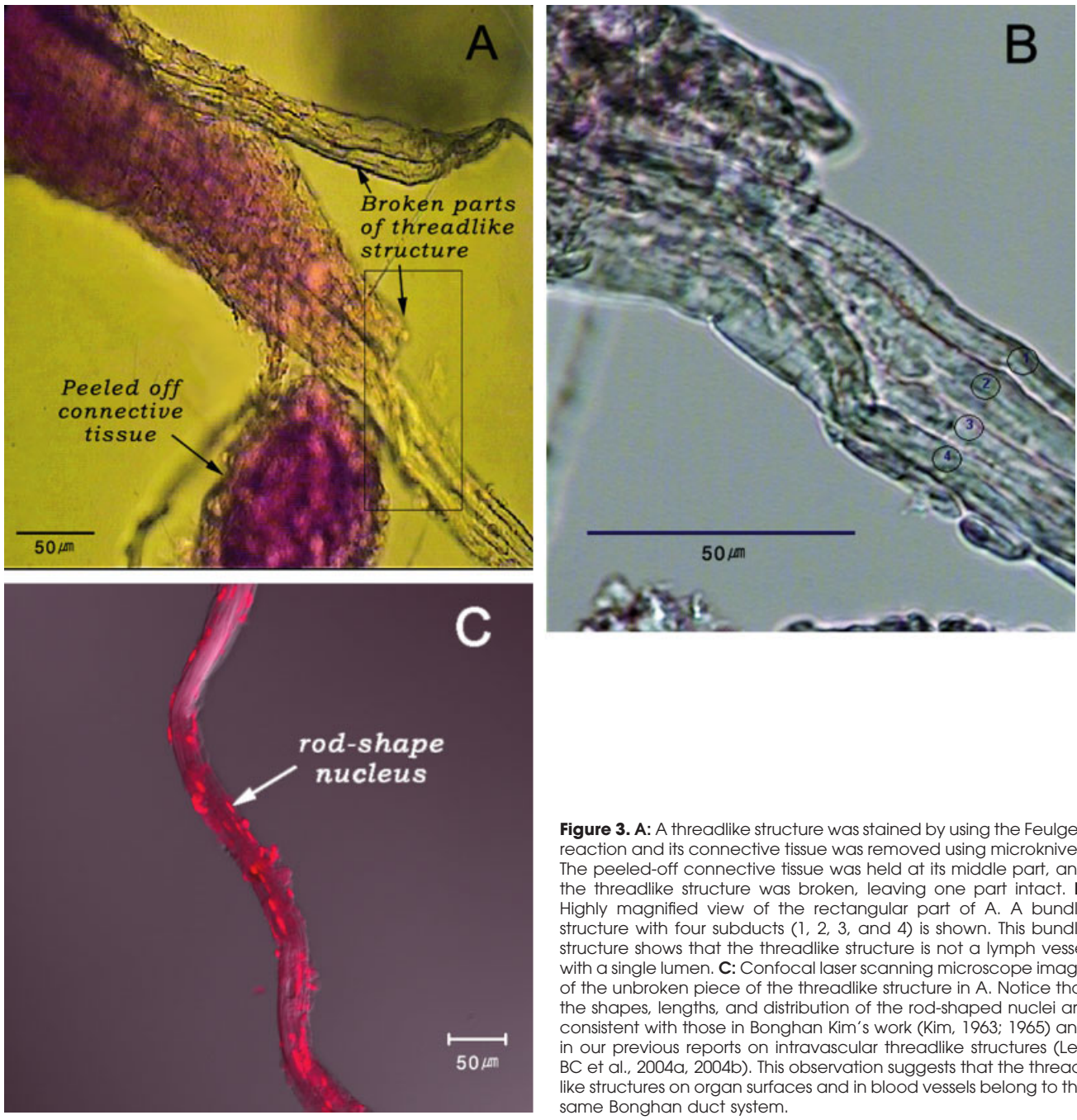

Figure 3. A: A threadlike structure was stained by using the Feulgen reaction and its connective tissue was removed using microknives. The peeled-off connective tissue was held at its middle part, and the threadlike structure was broken, leaving one part intact. **B:** Highly magnified view of the rectangular part of A. A bundle structure with four subducts (1, 2, 3, and 4) is shown. This bundle structure shows that the threadlike structure is not a lymph vessel with a single lumen. **C:** Confocal laser scanning microscope image of the unbroken piece of the threadlike structure in A. Notice that the shapes, lengths, and distribution of the rod-shaped nuclei are consistent with those in Bonghan Kim's work (Kim, 1963; 1965) and in our previous reports on intravascular threadlike structures (Lee BC et al., 2004a, 2004b). This observation suggests that the threadlike structures on organ surfaces and in blood vessels belong to the same Bonghan duct system.

low-magnification surgical microscope. A good stereomicroscope is required to observe some parts of their structure.

Second, bleeding during any surgery would need to be tightly controlled because fibrin in the blood has a strong affinity for the threadlike structures and enshrouds them once bleeding has occurred. Coagulated fibrin and the novel structure look alike and are indistinguishable unless a special staining technique is used (Lee BC et al., 2004a). Thus, in most surgeries, these threadlike structures would have been considered to be coagulated fibrins.

Third, similar-looking tissues from torn-off peritonea or capsules of internal organs might exist, and these could be distinguished from the novel structures only through careful morphological examination of such features as branchings, very long threads extending from one organ to more distant ones, the existence of corpuscles, and the flow of granules.

Fourth, even if the novel structures were to be noticed inadvertently, no motivation would exist to study them because they are not recognized in current theories of anatomy. They would be simply ignored as insignificant structures in the complexity of the abdomen.

The last but most significant obstacle is the difficulty in discerning the novel tissue from lymphatic vessels. In fact, this possibility of confusion with the lymph system is the usual

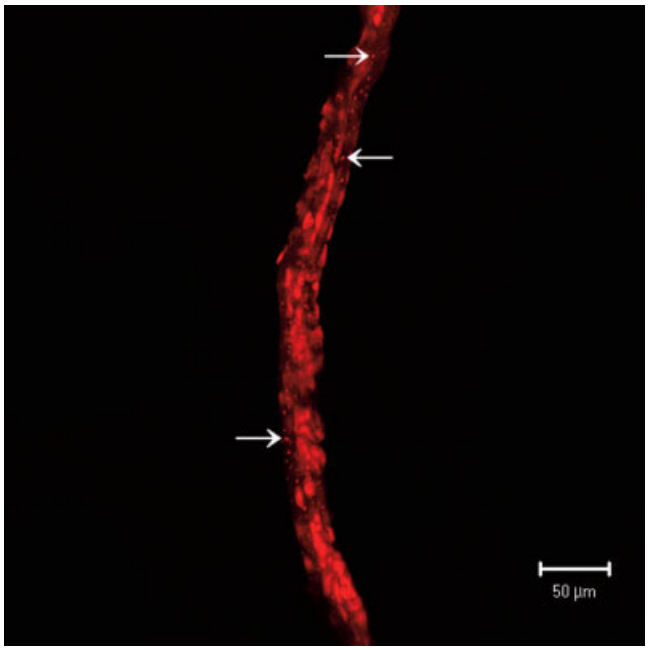

Figure 4. Round 1–2 μm diameter granules were observed in the lumens of the subducts. The granules were well stained using Feulgen reaction, revealing the DNA content. Bonghan granules may be naturally generated microcells that flow in acupuncture meridians (Bonghan ducts); thus, they may play an essential physiological function in the Bonghan system.

objection raised by medical or veterinarian experts to the existence of these structures (Kellner, 1966; Kroger, 1973). Indeed, both objects are semitransparent, have similar sizes, and carry liquid. One purpose of the present article is to present features that clearly distinguish the novel structure from a lymphatic vessel. First of all, the threadlike tissues do not adhere to the surfaces or capsules of internal organs and freely move, so they can be lifted by forceps as shown in Figure 1. In contrast, lymph vessels are fixed to the surfaces or the peritonea of organs. Second, the novel structures do not join at lymph nodes. Third, the most critical difference is that the novel threadlike tissues have structures with bundles of subducts as shown in Figures 2 and 3A and B. Lymph vessels and capillary blood vessels have only one lumen, i.e., they are single tubular structures. The striped broken lines of nuclei shown in Figure 3C represent the bundle structure. In the confocal scanning microscope images, the striped lines appear mostly at about the middle of the thread, which suggests that the thread is not a single tube. In a single tube structure, nuclei appear only at two boundaries in the midsection images. Last, the flow of 1 μm-sized granules in the subducts, as shown in Figure 4, is unique to the Bonghan threadlike structures, whereas lymph vessels carry 5 μm or larger-sized lymphocytes.

One incidental point concerning the difficulty in detecting the threadlike structures is their indefiniteness with respect to length, thickness, location, and weblike distribution. While their parameters are similar in a given case, no definitive description is possible, as the characteristic features vary from subject to subject. For some subject animals, the system is well developed and easy to find, while for others it is extremely difficult to detect even a single piece of thread. Our conjecture is that these threadlike systems have an indefinite structure and that physiological conditions, maybe even biorhythmic changes, determine the way in which they develop. The observational skill of the surgeon or the researcher is an essential factor when attempting to identify these threadlike structures on organs.

Bonghan Ducts and Acupuncture Meridians

We emphasize that the novel threadlike structures found on internal organs are essential subsystems of the third circulatory system, the intravascular and the subcutaneous acupuncture meridians being other major subsystems. Until the present time, intravascular and organ surface threadlike structures have been observed by several groups (Fujiwara and Yu, 1967; Jiang et al., 2002; Cho et al., 2004; Lee BC et al., 2004a, 2004b; Lee KJ et al., 2004), but the complete network, including its subcutaneous aspects, has yet to be isolated.

At this point, we should remind the reader that there are many other hypotheses for the physical basis of acupuncture meridians. Many researchers in the field tend to believe that the function of acupuncture can be understood via neurophysiological theories (Pearson, 1987; Mann, 1998). A reasonable alternate hypothesis is that acupuncture points and meridians are parts of a network formed by interstitial connective tissue (Langevin and Yandow, 2002). The liquid-crystal collagen fibers that make up the bulk of connective tissue may conduct sound or electricity (Ho and Knight, 1998). Until now, however, no anatomical or histological structures corresponding to acupoints have been found (Zhang et al., 1982; Heine, 1988; Shang, 1989).

Even though the hitherto unknown major anatomical structure for acupoints is not yet proved, its physiological and medical implications may still be very interesting. Liquid flowing through the Bonghan duct includes 1–2 μm-sized DNA-containing granules (Bonghan granules), as shown in Figure 4, and Bonghan granules are like microcells that are naturally generated in the tissues of various organs and then flow through the network of Bonghan ducts. Microcells contain one or a few chromosomes surrounded by a thin rim of cytoplasm and a cell membrane (Prescott and Kirkpatrik, 1972; Buikis et al., 1999), and they have been widely used for cell fusion studies, especially for microcell-mediated chromosome transfer (Hunt, 1996), which has led to the study of cell senescence genes and in turn has provided a tool for investigating carcinogenesis (Rar and Pereira-Smith, 2000) and Down syndrome (Kabota et al., 2002).

Bonghan granules and microcells

are similar in their sizes, their round or oval shapes, and their intensively stained nuclei. In addition, both have a thin outer membrane and one chromosome amount of DNA inside (Kim, 1965; Buikis et al., 1999). However, microcells are produced in vitro by using a chemical substance like colcemid or in pathological conditions, such as in tumor tissues, whereas Bonghan granules are produced in normal physiology and function in the Bonghan system, which includes acupuncture meridians.

According to Kim (1965), Bonghan granules are involved in cell regeneration in damaged tissues. Thus, a scientific understanding of the therapeutic effect of acupuncture treatments, in line with modern biomedical concepts, will emerge by establishing an anatomical basis for acupuncture meridians.

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