원 저

형광염색을 이용한 혈관내봉한관의 관찰

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Fluorescent Method for Observing Intravascular Bonghan Duct

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국문초록

미분간섭현미경법과 아크리딘-오렌지(acridine-orange) 형광 염색을 이용하여 쥐 혈관 내의 실 모양 구조물을 관찰하였다. 공초점현미경법과 헤마톡실린-에오신(hematoxylin-eosin) 염색을 통해 피브린, 모세혈관, 소정맥, 소동맥 혹은 림프관의 핵 분포와 뚜렷이 구별되는 혈관 내 실 모양 구조물의 핵 분포 패턴을 얻어낼 수 있었다. 이 실 모양 구조물의 생리적 기능을 침술과 연관하여 논의하였다. 특히, 이 실 모양 관을 통한 약물의 흐름이 약침의 기전에 해당할 것이라는 가설을 제기하였다.

Abstract

Observation of intra-vascular threadlike structures in the blood vessels of rats is reported with the images by differential interference contrast microscope, and fluorescence inverted microscope of the acridine-orange stained samples. The confocal microscope image and the hematoxylin-eosin staining revealed the distinctive pattern of nuclei distribution that clearly discerned the threadlike structure from fibrin, capillary, small venule, arteriole, or lymph vessel. Physiological function of the intra-vascular thread in connection with acupuncture is discussed. Especially, this threadlike duct can be a circulation path for herb-liquid flow, which may provide the scientific mechanism for therapeutic effect of herbal acupuncture.

Key words : Bonghan duct, Intra-vascular thread, Confocal microscope, Acridine-orange, Herbal acupuncture

Ⅰ. INTRODUCTION

It is widely accepted in current anatomy that there is no threadlike structure afloat running along inside blood vessels. Thus it was received with skepticism when Bonghan Kim claimed in early 1960's that his group found intra-vascular duct (IVD) structures which do not adhere to the vessel walls[1], and no one has confirmed his results because the method to detect such an existence was not disclosed. Bonghan Kim mentioned only that he used blue-colored dye to trace the IVD without giving the material and process of staining.

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Very recently some of us were able to develop a new method to observe the intra-vascular threadlike structure from caudal vena cava, abdominal aorta, hepato portal vein etc. of rats and rabbits[2,3,5,6,7]. In this method dextrose solution was injected into the femoral vein to dilute the blood until the blood vessel becomes transparent, and strings of fibrin with coagulated blood were observed in the caudal veins and arteries. Pieces of the broken IVDs were obtained from the strings of fibrin using processes to separate them from the fibrin either by an electrical method[3,4] or by urokinase solution. Histological studies by hematoxylin and eosin, Malloy's triple, and Verhoff's elastic stain revealed unique distribution of nuclei, thin collagen fibers intermingled with connective tissues, and abundance of elastic fibers, respectively[5,6].

The above injection method of dextrose solution has a serious short coming: The IVD is not discriminate from fibrin string. In this paper we report an improved method overcoming this deficiency for obtaining the intra-vascular threadlike structure from the blood vessels of rats. The key technique we contrived is to inject acridine-orange at femoral veins to circulate along the blood vessels and to stain nuclei in the intra-vascular threads. When observed with fluorescence microscope, the thread was distinguished by its peculiar pattern of nuclei distribution that forms a longitudinal striped pattern. We present the fluorescence images along with differential interference contrast (DIC) images of the intra-vascular thread, and compare with confocal microscope images, and hematoxylin and eosin studies which clearly show that the new structure was distinctively different from small capillaries, fibrins or lymph vessels.

The intra-vascular thread is semi-transparent, white, and elastic. It is easily broken, shrinks and twines itself round once it is cut. It collapses to be flattened by a cover glass. The diameter is about 20~100㎛.

One naturally wonders why such a microscopic scale structure has not been observed in numerous surgical operations and hematological studies. The first reason is the invisibility due to transparency of the IVD. It can not be seen either in situ or in vitro when it stays in the blood vessels which are filled with blood. When a piece of blood vessel is cut, put on slide, and examined with ordinarily light microscopes or stereoscopic microscope the threadlike structure is hardly noticeable even with conscious efforts. With a phase contrast or differential interference contrast microscope there appears longitudinally striped patterned strips of tissues, but it is hard to discern from fibrin strings coagulated with blood clots. These are enough reasons to miss its existence in many surgical situations. In addition to them the IVD is too elusive to catch it by cutting pieces of blood vessels and searching inside for the IVDs. It shrinks quickly once it is broken where, due to its weakness, it is usually broken into many small pieces. The broken pieces would be enshrouded with blood coagulated fibrins, and no one would have tried hard enough to remove the coagulated clots to expose the thin structure. Using the acridine-orange staining and fluorescence stereoscopic microscope we were able to identify the intra-vascular thread lying inside a piece of the blood vessel which was taken after cutting both ends of caudal vena cava. This process yielded, however, broken pieces of the IVD with unpredictable yield. We have not yet understood the mechanism that the IVDs evade our capture strategy. It would be most desirable to devise a method to observe the IVDs in situ.

In the next section we present the method and procedure to make the invisible threads perceptible. The images of differential interference contrast microscope, fluorescence inverted microscope, confocal microscope, and hematoxylin and eosin staining study will be presented in the result section. The physiological functions, the full network of the Bonghan ducts, and further research directions will be given in the discussion section.

II. MATERIAL AND METHODS

1. Animals and surgical procedures

Male Sprague-Dawley (SD) rats obtained from the Breeding and Research Center of Seoul National University were used in this study. The animals were housed in a constant temperature-controlled environment (23℃) with 60% relative humidity. All animals were fixed at 12 hr light-

dark cycle, and had ad libitum access to food and water. Procedures involving animals and their care conformed with the institutional guidelines, which are in full compliance with current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996).

A rat is anesthetized with urethane (1.5 g/kg) administered intraperitoneally. All surgical procedures were performed under general anesthesia. In deep anesthesia, the rat was infused with 0.1% (w/v) acridine orange (Sigma) (18.3 ml/kg) into the femoral vein for 5 min. The infusion of 120% (w/v) dextrose solution (20.2 ml/kg) was done at another femoral vein. The jugular vein was cut to exsanguinate for 10 min. The frontal side of the rat was incised, and stomach and intestines were removed such that vascular systems were exposed for easy operation.

2. Search with fluorescence stereomicroscope

In search for the intra-vascular threads, caudal vena cava or abdominal aorta were incised to open. Under the fluorescence stereoscopic microscope (Stemi SV11, Carl Zeiss, Germany), the search for the threadlike structure was done with small surgical instruments such as iris scissors and microforceps for the manipulation. We searched for threadlike structures from inside of the vessels.

3. Sample collection and microscope images

Once the intra-vascular threads were found they were observed and recorded with the inverted microscope (Axiovert S100, Carl Zeiss, Germany). We noted the differential interference contrast (DIC) images to see the characteristic semi-transparent striped pattern, and then compared with the fluorescence images of the acridine orange stained spindle-shaped nuclei whose distribution was easily discernible from that of other tissues.

The thread specimens were further examined using confocal microscopes (LSM510, Carl Zeiss, Germany) to study the distribution of nuclei inside the thread as we were able to take sectional views of 2㎛ thickens

Light microscope procedure for hematoxylin and eosin stained sample.

Some of the collected intra-vascular threads were fixed in 10% neutral buffered formalin and then cryoprotected by transferring to 30% sucrose in 0.1 M phosphate buffer. All tissues were then frozen in OCT embedding medium (Tissue-Tek, Sakura). The sections of 20㎛ thickness were cut with a freezing microtome and mounted on the gelatin-coated slides and stained with hematoxylin and eosin. The sections were observed and photographed under the light microscope (BX40, Olympus, Japan).

III. RESULTS AND DISCUSSION

In the caudal vena cava and abdominal aorta filled with the mixture fluid of blood and saturated dextrose solution, the intra-vascular threads were observed under stereoscopic microscope and taken by microforcep to be sampled on a slide. The sample was usually taken with blood coagulated fibrins which occasionally include the intra-vascular threads. One of the shortcomings of the present technique is the irregular yield of the desired threadlike structure included in the fibrin-string.

The fibrin-string on the slide was scrutinized under fluorescence microscope in order to search for the intra-vascular thread which was clearly and distinctively recognized by its yellowish-green fluorescence of the acridine-orange stained nuclei. The characteristic distribution of the spindle-shaped nuclei was seen as a striped thread.

The intra-vascular threadlike structure is semi-transparent and almost invisible when it is inside blood vessel. Its differential interference contrast (DIC) image (Fig. 1(a)) is barely discernible, but not clearly distinguished from fibrins. The fluorescence image of the same thread is shown in Figure 1(b) where one can clearly perceive its peculiar pattern of nuclei distribution. As mentioned by Bonghan Kim[1], the nuclei are spindle-shaped, and they form broken lines of striped pattern.

As shown in Fig 2 the confocal microscope images of the same thread sample provide clearer impression of the pattern.

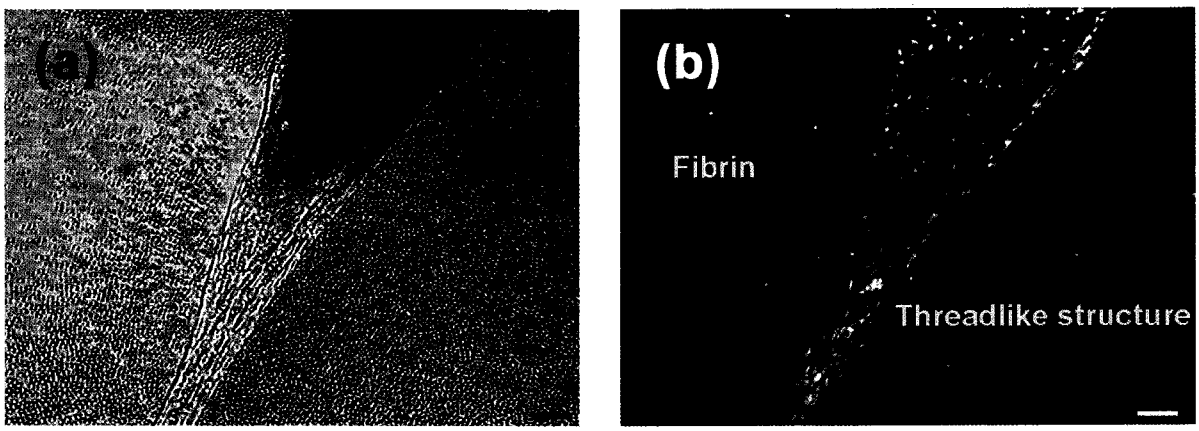

Fig. 1 Images of an intra-vascular thread obtained from the caudal vena cava of a rat. (a) Differential interference contrast image. The scattered spheres are red blood cells. The right hand-side boundary is the threadlike structure, and the triangular shape above it is fibrin. (b) Fluorescence inverted microscopic image of the acridine-orange stained sample shown in (a). The white dots in the triangle region are white blood cells. The rod shaped nuclei are located in stripe fashion on the right hand-side boundary (bar 50 ㎛).

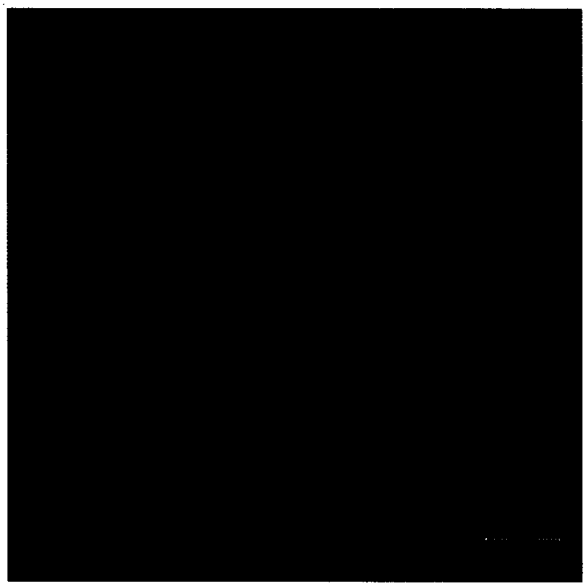

Fig. 2 Confocal microscope image of the threadlike structure. The bright objects are the fluorescent images of spindle-shape nuclei (bar 20 ㎛).

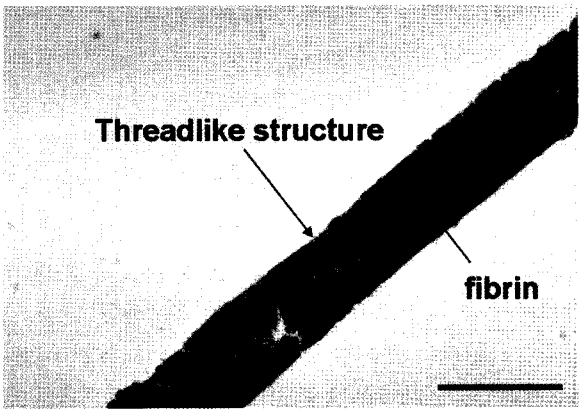

Fig. 3 Hematoxylin and eosin stained section image of the threadlike structure embedded in fibrin. The same sample shown in Fig. 1 is used (bar 50 ㎛).

The diameter of the intra-vascular threads in the caudal vena cava and abdominal aorta is about 20~100 ㎛.

As show in Fig 3 the hematoxylin and eosin stained thread shows most of the threadlike structure containing many spindle-shaped nuclei (about 3.6-5.4 ㎛ in length) was covered with lots of fibrins.

As we had already mentioned the intra-vascular structure has escaped detection in ordinary surgical situations due to its invisibility and elusiveness. According to Bonghan Kim[1] the intra-vascular Bonghan duct is only a part of a new circulatory system where extra-vascular ducts that are distributed on the surface of various organs, and superficial duct under skin are other parts. The superficial ducts are the anatomical basis of the acupuncture meridians.

Although we have not been able to trace the whole network of Bonghan ducts, we encountered occasionally samples of

extra-vascular ducts whose systematic studies require more efforts and techniques. The intra-vascular threadlike structure may exist in every vertebrate including human, which should be confirmed in future. We checked a few cases with rabbits and found the intra- and extra-vascular threads. Systematic studies on the Bonghan ducts of rabbits and other animals are clearly desirable.

Physiological functions of the intra-vascular threadlike structures might be the scientific basis for the mysterious therapeutic effects of acupuncture treatment as they are part of a new circulatory network including acupuncture meridians[1]. Some liquid (Bonghan Liquid) flows along the network of Bonghan ducts which plays important roles in development, and cell-therapy[1]. Thus the intra-vascular threadlike structure might provide the underlying anato-physiological basis of the Traditional Chinese Medicine as well as opening of a new area in biology and medicine. Especially, the existence of intravascular Bonghan ducts will provide a proper explanation to how herbal acupuncture works. That is to say, the Bonghan system can deliver drugs from an acupuncture point to a specific organ. This model might be a more persuasive assert for the mechanism of herbal acupuncture than that based on the acupoint-brain-viscera model.

At present we saw only the invisible nature of the intra-vascular threadlike structure, but have not understood the elusiveness: why it is broken to small size and hard to capture inside the pieces of blood vessels. We do not understand the mechanism of dextrose solution, but have used to help the fibrin to capture the elusive intra-vascular threads. Through many experiments we found that dextrose helps to increase the yield of the desired sample, but have not been able to figure out its working principle[5,7] The most desirable technique would be to observe them in situ, and trace the network inside and outside of all vessels and organs, which we hope to develop in future.

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