Development of an Experimental Apparatus for Investigating Lymphatic Pumping Activity of Murine Mesentery In Vivo

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Abstract: The present study has been attempted to establish a modified intravital microscope system for investigating murine lymphatic pumping activity in vivo and evaluate whether or not there is rhythmic pumping activity of murine mesenteric lymphatic vessels in vivo. We designed and constructed a custom organ chamber with a semicircular channel (8 mm in radius, 5 mm in width, 3 mm in depth), being suitable for the superfusing of murine mesentery in vivo. A marked lymphatic pumping activity was observed in the mesenteries of DDY mice. The maximal and minimal diameter and frequency in the pumping activity were 60.9±1.0 μm, 53.7±1.8 μm and 12.8 min⁻¹ (n=5), respectively. Both NE (norepinephrine, 10⁻⁸–10⁻⁶ M) and TEA (tetraethylammonium, 1–10 mM) caused dose-dependent constriction of the mesenteric lymphatic vessels in the mice. These findings suggest that a modified intravital microscope system with a specially designed and constructed edge-monitoring device enables us to investigate in vivo lymphatic circulation in murine mesenteries. [Japanese Journal of Physiology, 50, 25–31, 2000]

Key words: modified mesenteric chamber for mice, lymphatic pumping activity, murine mesentery, in vivo experiments, norepinephrine, TEA.

The lymphatic system plays an important role in regulating the transport of excess extracellular fluid and macromolecular substances, albumin and immunoglobulin, in tissues and organs. The lymphatic system also plays a key role in the absorption of fluid and fat from the lumen of the small intestine. The central lacteals begin in the intestinal villi. These lacteals converge to form submucosal lymphatic-collecting vessels which emerge from the mesenteric border as mesenteric lymphatics.

The transport of lymph depends on passive and active driving forces as well as on the rate of lymph production in organs and tissues [1]. The active driving mechanism may play a significant role in the centripetal propulsion of lymph, which is due to the intrinsic contractility of the lymphatic vessels [1–3]. There are marked species differences in existence of the intrinsic contractions and corresponding rhythmic pumping activity in vivo; sheep, cow, cat, guinea-pig and rat mesenteric lymphatic vessels demonstrate the contraction in vitro and the pumping activity in vivo [1, 2, 4–9]. No report, however, shows the rhythmic contraction in vitro and the pumping activity in murine mesenteric lymphatics in vivo.

Recently, development of molecular bioengineering enables us to utilize transgenic and knock-out mice, which have been used to understand the mechanisms of cardiovascular disease as well as physiological functions [10–14]. The mouse is one of the best species to establish gene-mutant animals. A specialized vital microscope system, however, has not been developed to study the mesenteric micro- and lymph-circulation in transgenic and wild mice.

Therefore, the present study was undertaken to es-

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establish an intravital microscope system for investigating mesenteric lymphatic circulation and then evaluate whether or not rhythmic pumping activity exists, similar to rat, in murine mesenteric lymphatic vessels in vivo.

MATERIALS AND METHODS

Animals. Male DDY mice (5 to 6 weeks old, 28.8±1.0 g, n=5) were used for the present studies. The mice were housed in an environmentally controlled vivarium and fed a standard pellet diet and water ad libitum. Access to food but not water was discontinued 18–20 h before experimental use. All experimental protocols were approved by the Animal Ethics Committee, Shinshu University School of Medicine, in accordance with the principles and guidelines of the Council of the American Physiological Society.

Surgical procedure. The mice were anesthetized by intraperitoneal injection of a mixture of 2% α-chloralose and 10% urethane (0.7 ml/100 g b.w.). Additional anesthetic was supplemented (0.1 ml, i.m.) when the mice produced body movement, hyperventilation or increment of arterial blood pressure. Each mouse was tracheotomized and polyethylene tubing (SP102, Natsume, Japan) was inserted to ensure spontaneous respiration with room air. To measure the arterial blood pressure, the right carotid artery was cannulated by an elongated polyethylene tube (SP45, Natsume) connected to a pressure transducer (PD104, Toyoda, Japan). Changes in arterial blood pressure were recorded on a direct-writing recorder (Recti-Horitz 8K, Sanei-Sokki, Japan). The left femoral vein was also cannulated for intravenous infusion of 0.9% physiological saline (0.1 ml/h) with a syringe pump (STC-525, Terumo, Japan).

Figure 1 shows an experimental layout of the modified intravital microscope system (A) and a custom-made mesenteric chamber suitable for superfusion of murine mesentery in vivo (B, C and D). The mice were placed on a heated plate to keep the rectum temperature at 37–38°C throughout the experiments (Fig. 1A). After a midline incision of the abdomen was made, the mesentery adjacent to a segment of small intestine was exteriorized on the baseplate of the mesentery chamber (Fig. 1B, C and D). The exteriorized small intestine was positioned in a semicircular channel (8 mm in radius, 5 mm in width, 3 mm in depth, Fig. 1C) on the baseplate of the chamber, and the mesentery was spread over an observation window in the chamber (Fig. 1B). The space between the intestine and semicircular channel was filled with physiological saline-soaked cotton strips. Care was taken not to twist or occlude the mesenteric lymphatic vessels as well as blood vessels. The exposed small intestine was covered with a layer of cotton and high vacuum silicone grease (Dow Corning, Japan). A plastic plate (intestine cover) was placed over the semi-circular channel in a manner that produced a seal between the intestine and the mesentery but did not obstruct blood and lymph flow. The mesenteric chamber was superfused with 37°C Krebs-bicarbonate solution (in mM): NaCl 120, KCl 5.9, NaHCO₃ 25.0, NaH₂PO₄ 1.2, CaCl₂ 2.5, MgCl₂ 1.2, and glucose 5.5, which was aerated with a gas mixture of 5% CO₂–95% N₂ to keep a pH of 7.4. The rate of superfusion was kept at 6 ml/min during the experiments.

Analysis of murine lymphatic pumping activity. The custom-made murine mesenteric cham-

Fig. 1. An experimental layout of the modified intravital microscope system (A) and a custom-made mesenteric chamber suitable for superfusion of murine mesentery in vivo (B, C and D). B, C and D show top, front and sectional views of the chamber, respectively. Heated water (37°C) was circulated at the bottom of the baseplate.
The lymph was transferred to the stage of an intravital microscope (BH-2, Olympus, Japan) (Fig. 1A). To measure the lymphatic pumping activity in murine mesentery, we selected a lymphatic vessel clearly identified from the adipose and surrounding tissues. The mesenteric lymphatics chosen for the observation were located 2 to 5 mm from the intestinal wall. Experiments were terminated if the mean arterial blood pressure fell below 80 mmHg. The image of lymphatic vessel was obtained through a 35 water-immersion objective lens with a numerical aperture of 0.1 (Nikon, Japan), a photoeyepiece lens (×5), and a monochrome CCD camera (KP-M1, Hitachi, Japan), and then the image was recorded on a video cassette recorder (BR-S800, Victor, Japan).

**Description of the edge-monitoring device for measuring intraluminal diameter of the lymphatic vessel.** In this study, we designed a new edge-monitoring device to measure changes in the intraluminal diameter of lymphatic vessels. The image of a lymphatic vessel obtained using the CCD camera was displayed on a monochrome TV monitor (PVM-1454Q, Sony, Japan) via the device. The intraluminal diameter of the lymphatic vessel was calculated by an edge-detection method [15,16].

Figure 2 shows a block diagram of the electrical circuit in the edge-monitoring device. The video signals obtained by the CCD camera were used as an input signal of the device (Fig. 2, video input). The device also had two edge-detection circuits which consisted of an analog comparator (Fig. 2, COMP. 1 and 2) and an edge detector (Fig. 2, ED. 1 and 2). The changes in the diameter of the lymphatic vessels were measured automatically using the electrical circuit. Figure 3A shows a representative picture on the TV monitor equipped with two superimposed windows. Figure 3B also demonstrates a schematic diagram of electrical processing to measure the intraluminal diameter of the lymphatic vessel. Firstly, two electrical windows were adjusted manually on both sides of the lymphatic wall displayed on the TV monitor. The changes in the optical density on a horizontal line at
the top of the windows (Fig. 3A, arrow) were used to detect both edges of the image of the lymphatic vessel. To keep correct detection, the longitudinal axis of the lymphatic vessel was adjusted perpendicularly against the horizontal line by rotating the CCD camera. The video signals (Fig. 3B, W1 and W2) at the horizontal line were compared independently with the corresponding threshold voltages using the analog comparators. The outputs of comparator 1 and 2 (Fig. 3B, COMP. 1 and 2) were used to decide, electrically, both edges of the lymphatic wall. The total number counted by edge detectors (Fig. 3B, Nd) was converted into an output of analog voltage through a D/A converter and then recorded as the intraluminal diameter of the lymphatic vessel on a direct-writing recorder. Figure 4 demonstrates a calibration curve which shows a linear relationship between the output voltage of the device (V) and scale of an objective micrometer (µm). The relationship is that the scale (µm) equals 100 times V (V).

Measurement of lymphatic pumping parameters. After equilibration for 20 min, the maximum diameter (D_max, µm), minimum diameter (D_min, µm) and contraction frequency (min⁻¹) were measured for 10 min as control parameters of lymphatic pumping activity in the mesenteric lymphatic vessel of DDY mice. After another equilibration for 20 min, norepinephrine (NE, 10⁻⁸ to 10⁻⁶ M) or tetraethylammonium (TEA, 1, 5 and 10 mM) was added to the superfusing solution bathing all mesenteries of the mice for 5 min. The parameters of lymphatic pumping activity in vivo were also measured in the same manner as those in each control. The other parameters of the lymphatic pumping activity, stroke volume index and ejection fraction, were calculated as follows: stroke volume index = π(D_max/2)² − π(D_min/2)², ejection fraction = {π(D_max/2)² − π(D_min/2)²}/π(D_max/2)² [4].

Drugs. All salts (Wako, Japan), NE hydrochloride and TEA bromide (Sigma, St. Louis, USA) were used in the present study. Concentrations of the drugs were expressed as a final concentration in the mesenteric chamber. All salts and drugs were prepared on the day of the experiment.

Data analysis. Experimental values in the text, figures and tables are expressed as mean ± standard error of the mean. The n indicates the number of experimental animals examined in the present study.

RESULTS

The mean arterial pressure and heart rate of the DDY mice were 103 ± 3 mmHg and 444 ± 17 beats/min, respectively.

Lymphatic pumping activity in the mesenteric lymphatic vessels of DDY mice

Figure 5 shows representative tracings of changes in the arterial blood pressure (A) and the diameter of the mesenteric lymphatic vessel (B) in a DDY mouse.
respectively. The averages of the $D_{\text{max}}$, $D_{\text{min}}$ and frequency in the mice were 60.9±1.0 μm, 53.7±1.8 μm and 12.8 min$^{-1}$ ($n=5$), respectively. The stroke volume index and ejection fraction of the lymphatic pumping activity were calculated to be 641±113 μm$^2$ ($n=5$) and 0.22±0.04 ($n=5$), respectively.

**Effects of NE and TEA on changes in the diameters of mesenteric lymphatic vessels in DDY mice**

Figure 6 demonstrates representative tracings of the effects of $10^{-6}$M NE (A) and 5 mM TEA (B) on the diameters of mesenteric lymphatic vessels in DDY mice. Both $10^{-6}$M NE and 5 mM TEA caused a marked constriction of the lymphatic vessels superimposed with oscillatory changes of diameter in the mice. Thus, in response to the $10^{-6}$M NE, the diameter of the lymph vessels in the mice was reduced from 53 to 38 μm (Fig. 6A). The NE-induced maximal responses of the lymph vessels are summarized in Fig. 7A. TEA (5 mM) also caused a shortening of the diameter, from 51 to 39 μm, in the lymph vessels of the mice (Fig. 6B). Such data are summarized in Fig. 7B.

**DISCUSSION**

The major findings of the present study are: (1) a modified intravital microscope system enables us to study lymphatic circulation of murine mesenteries in vivo; (2) mesenteric lymphatic vessels in DDY mice exhibit rhythmic pumping activity in vivo; and (3) NE and TEA caused a significant constriction of the lymphatic vessels in the murine mesenteries.

**A modified intravital microscope system for murine mesentery.** To investigate the lymphatic pumping activity of murine mesenteries in vivo, major modifications of a classical intravital microscope system are summarized as follows: (1) improvement in the size and shape of the superfusion chamber for fitting murine mesenteries, and (2) construction of a cheaper and more concise edge-monitoring device, compared to the edge detection software systems on the market, which enables us to continuously measure changes in the diameter of the lymphatic vessels. The size and shape of the mesenteric chamber were constructed to provide the highest probability of success and to be suitable for the investigation of murine lymphatic pumping activity in vivo. Additionally, we have taken care in deciding the height of the chamber between the heated plate and the observation window, which has played a crucial role in preventing leakage of the superfused solution into the abdomen cavity.

A concise edge-monitoring device was also designed in the present study to measure continuous changes in the diameter of murine mesenteric lymphatic vessels in vivo using the technique with a binary image [17]. The techniques for recording of vascular diameters that have been widely used are as follows: image-splitting method using a mechanical splitter [18] or an electrical splitter [15, 16, 19], and a computerized image-processing method utilized with changes in the intensity of video signals [20–22]. Both of the methods were, however, difficult for recording real-time changes in the diameter in vivo. These methods also required complicated electrical and mechanical circuits. In addition, there is the other problem of deciding the real border between intraluminal space and the wall of the blood vessel. The blood flow in microcirculation consists of red blood cell columns in the intraluminal space, which causes a complex intensity profile of video signals corresponding to the real border between the space and wall.
Thus, it is difficult to detect the intraluminal edges of video signals clearly and automatically. The lymphatic vessels have, however, no blood cell columns, and then the edges of the lymphatic walls were able to easily detect the video signals as shown in Fig. 3.

On the other hand, there are a number of edge-detection software systems on the market which seem to do a very good job of following vessel diameters. Considering the situation, we designed and constructed a cheaper and more concise edge-monitoring device for continuously recording the diameter of murine mesenteric lymphatic vessels. The characteristics of the device are summarized as follows. (1) The electrical circuits were made very concisely with low cost. (2) Operation of the device is very easy because both the binary and original images are simultaneously displayed on the TV monitor. (3) The device is also able to detect real-time changes in the diameter of vascular vessels because the frequency characteristic of the device is kept at 60 Hz.

Existence of rhythmic pumping activity of mesenteric lymphatics in DDY mice. Several in vivo studies demonstrate that spontaneous oscillatory changes in the diameters of mesenteric lymphatic vessels are present in cats [7], guinea-pigs [4, 5, 23, 24] and rats [4, 25, 26]. There is, however, no information regarding the existence of rhythmic pumping activity in murine mesenteric lymphatic vessels in vivo. The present study is the first to demonstrate that a marked rhythmic pumping activity exists in vivo in the mesenteric lymphatic vessels of DDY mice. There are two studies that investigated lymphatic circulation in vivo in murine tails [27, 28]. Such rhythmic beatings in the murine tails, however, have not been confirmed. The maximum and minimum diameters of the lymphatic vessels corresponding to the rhythmic beatings were 60.9±1.0 and 53.7±1.8 μm, respectively. The frequency in the murine mesenteric pumping activity was 12.8±0.7 min⁻¹, being higher compared with other rodents [5, 25, 26]. In contrast, the ejection fraction (0.22±0.04) and stroke volume index (641±113 μm²) in the murine mesenteric lymphatic vessels were smaller than those obtained with rats (0.65±0.00 and 3,700±480 μm²) [26].

NE and TEA-induced constrictor responses of the lymphatic vessels in DDY mice. Both NE and TEA caused a dose-dependent constriction of the mesenteric lymphatic vessels in the mice (Fig. 6). These drugs also produced spontaneous oscillations of the diameters, which were superimposed on the drug-induced shortening of the diameters of the lymphatic vessels in the mice (Fig. 6).

It is well-known that humoral and neural factors affect the mechanical activity of the smooth muscles in collecting mesenteric lymphatics [29]. The rhythmic spontaneous activity of the mesenteric lymphatics in rats and cows depends on an extracellular Ca²⁺ and a voltage-dependent L-type Ca²⁺ channel [9, 30]. Recently, some investigators have also reported that K⁺ channels regulate the membrane potential of lymphatic smooth muscles in sheep and guinea-pig mesenteric lymphatics [23, 31]. We also demonstrated that ATP-sensitive K⁺ channels are involved in the regulation of spontaneous activity in lymphatic smooth muscles in isolated rat mesenteric lymphatic vessels [32]. Considering these findings for the lymphatic smooth muscles, TEA, a non-selective K⁺ channel blocker, seemed to reduce all kinds of K⁺ channels of the lymphatic smooth muscles, totally depolarize the membrane potential, activate the depolarization-mediated Ca²⁺ permeability and Ca²⁺-release from the plasma membrane, and then result in a marked constriction of the lymphatic smooth muscles. The hypothesis may be, in part, related to the evidence that ouabain-induced inhibition of the electrogenic sodium pump of the lymphatic smooth muscles in bovine mesenteric lymphatic vessels has produced a marked vasoconstriction [33].

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