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STRUCTURAL RELATIONSHIP BETWEEN MICROLYMPHATIC AND MICROVASCULAR BLOOD VESSELS IN THE RABBIT VENTRICULAR MYOCARDIUM

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ABSTRACT

We investigated the distribution and relationship between draining lymphatic vessels, lymphatic capillaries, and microvascular blood vessels in rabbit ventricular tissue. The left and right ventricular tissue from 15 healthy adult rabbits was obtained, processed, and sectioned for analysis. 5'-nucleotidase-alkaline phosphatase (5'-Nase-Alpase) double staining was first used to identify lymphatic and blood vessels. Dual fluorescent immunohistochemical technique was then utilized with lymphatic endothelial cell marker podoplanin and blood vascular marker PAL-E. In addition, five ventricular samples were examined for ultrastructure using transmission electron microscopy (TEM). Draining lymphatic vessels and both lymphatic and blood capillaries were observed in the ventricular tissue. The lumens of draining lymphatic vessels were larger and irregular while the lymphatic capillaries were small in diameter and abundant. All lymphatic vessels were located among blood capillaries in the myocardium and aligned with the longitudinal axis of myocardial cells. The immunofluorescence double staining demonstrated that draining lymphatic vessels, lymphatic capillaries, and microvascular blood vessels were adjacent to each other and the cardiac myocyte with a ratio of lymphatic to microvascular blood vessels of approximately 1:1. This study suggests that lymphatic and blood capillaries exist in abundance and in nearly identical numbers in the ventricular myocardium and that they interweave with each other to comprise a complicated vessel network.

Keywords: lymphatic vessels, microlymphatics, microvascular blood vessels, ventricular myocardium, rabbit heart, immunohistochemistry, lymphatic markers

The cardiac lymphatic vasculature comprises both capillaries and draining lymphatic vessels. These vessels are sometimes difficult to distinguish from each other and also from the capillaries derived from microvascular blood vessels using histology. Recently, techniques for molecular biology, enzyme histochemistry, immunohistochemistry, and lymphatic cast scanning electron microscopy have provided more specific and sensitive methods for analysis. 5'-nucleotidase-alkaline phosphatase (5'-Nase-Alpase) double staining is considered an accurate method to distinguish blood capillary vessels from lymphatic capillaries. Zhao et al observed lymphatic and blood vessels from multiple organs in the rabbit, guinea pig, and rat with this method (1,2). The discovery of specific markers for lymphatic (e.g., podoplanin, LYVE-1, and VEGFR-3) and blood [e.g., VEGFR-2 and CD31 (PECAM-1)] vessels has also provided

new and sensitive methods to distinguish lymphatics from blood vessels (3-5). Despite a variety of lymphatic vessels in animals, organs, and tumor tissues reported using these methods (5), there is very little reported on the lymphatic and blood vessels of the myocardium and even less concerning their arrangement, relationship, and ratio (6-8). Therefore, we undertook this study to examine the distribution and proportion of the myocardial microlymphatic and microvascular blood vessels using enzyme histochemistry, immunohistochemistry, immunofluorescence double staining, and transmission electron microscopy in the rabbit myocardium.

MATERIALS AND METHODS

Sample Preparation

Fifteen adult New Zealand white rabbits, weighing 2.5~3.5kg, were obtained from the experimental animal center of Xinxiang medical college. The experimental protocol was approved by the ethics committee of Xinxiang Medical University. Rabbits were anaesthetized with intravenous sodium pentobarbital (1.5ml/kg), an incision was made along the left sternal border, 2 to 3 rib cartilages were removed, and the pericardium was cut off to expose the ascending aorta. An infusion needle was placed about 1-1.5cm from the aortic root and fixed with a silk suture to prevent the needle from entering the aortic valve and into the left ventricle. 500ml of 4% paraformaldehyde was infused in 5-10 min. The left and right ventricles were divided into two parts. One part was kept in 4% paraformaldehyde for 24h, cleaned with running water, dehydrated, processed, embedded, and cut at 5 μm. The second part was embedded in OCT and cut at 5 or 30 μm (for alkaline phosphatase detection). Sections were mounted on glass slides coated with APES, pre-fixed in cold acetone for 30s, dried, and stored at -20°C.

5'-Nase-Alpase Double Staining

The Alpase reaction for blood vessels was followed by 5'-Nase staining for lymphatics. The slides were incubated for 60 min at 37°C in reaction solution I [0.2Mol/L Tris-BR dilute acid buffer 20ml, adenosine -5'-phosphate (5-AMP, Sigma) 20mg, 2.5% magnesium sulfate 5ml, sugar 3g, distilled water 22ml, 2% lead nitrate 3ml, final pH7.2], followed by incubation for 1-2 min at room temperature in 1% ammonium sulfide, and incubation for 60-90 min at room temperature in reaction solution II [naphthol AS-BI phosphate 20mg, DMF 0.5ml, solid blue BB 40mg]. Control slides were incubated with reaction solution I without 5'-AMP and reaction solution II without naphthol AS-BI sodium phosphate.

Immunohistochemistry

For immunohistochemistry, slides were dewaxed, rehydrated, incubated with 0.3% H_2O_2 for 10 min to eliminate endogenous peroxidase activity, and washed for 3 x 5 min with PBS. Slides were boiled in citrate buffer for antigen retrieval, cooled to room temperature, and washed for 3 x 5 min with PBS. Following blocking with 5% BSA for 10 min, slides were incubated with primary antibody (podoplanin, Sigma) in a humid chamber at 4°C. Negative controls were treated with PBS and washed for 3 x 5 min with PBS. Secondary antibody IgG (Beijing Zhongshan) was applied in a humid chamber at 37°C for 30 min, washed 3 x 5 min in PBS, incubated with SABC complex for 30 min at 37°C, and washed in PBS again. Peroxidase activity was detected with the substrate diaminobenzioline (DAB).

Dual Immunofluorescence

Slides were dewaxed, rehydrated, and underwent antigen retrieval as above. They were quickly fixed for 10 min, washed 3 x 5 min with TBS, and incubated with 5% BSA

for 30 min at 37°C for blocking. Diluted primary antibody mixture [goat anti-rabbit podoplanin (KPL) and mouse anti- rabbit PAL-E (Sigma)] was applied and incubated overnight at 4°C in a humid chamber. Control slides were incubated with PBS. A diluted mixture of secondary antibodies [anti-goat Cy3 (Pik-Day Institute of Biotechnology) and anti-mouse FITC (Ai Bikang Biological Products)] was applied and incubated for 1 hr at 37°C in a dark humidified chamber. Slides were then washed 6 x 5 min with TBS, immediately dehydrated with a hairdryer, and sealed using a coverslip with anti-quenching agent. Photographs were taken by fluorescence microscopy and laser confocal microscope after 30 min. Because PAL-E identifies arterioles, venules, and capillaries, we combined these for analysis under the term microvascular blood vessels. Similarly, draining lymphatic vessels and capillaries were collectively combined and called lymphatic vessels to facilitate description and analysis of the dual immunofluorescence.

Transmission Electron Microscopy

Specimens were post-fixed for 30 min at room temperature in 1% O_SO_4, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Semi-thin sections (1.0 µm thick) were stained with 1% toluidine blue for light microscopy. In total, 30 blocks were prepared for ultrathin sections (80-100 nm thick) and were stained with uranyl acetate and lead citrate for examination under transmission electron microscopy.

Image Analysis and Statistical Processing

Two sections were selected from left and right ventricles of 5 hearts and 3 fields were observed from each section for a total of 60 fields of view. Fluorescent points were counted in cross section and the fluorescent lines were counted in longitudinal sections. All values were expressed as mean plus or minus standard deviation (X±SD), and both the relationship and percentage of lymphatic and microvascular blood vessels were calculated.

RESULTS

5'-Nase-Alpase Double Staining for Lymphatic and Blood Vessels

Cardiac draining lymphatic and blood vessels (including microvascular lymphatic vessels and blood capillaries) were seen on 5'-Nase-Alpase double staining. The lymphatic vessels were stained brown and blood vessels were blue-black. Significantly fewer draining lymphatic vessels were located among the blood capillaries, and lymphatic capillaries were infrequently detected. The lumen of draining lymphatic vessels was larger and irregular with a thin lymphatic wall while blood capillaries were smaller with smaller lumens and were arranged in crisscross pattern which connected into a network (*Fig. 1*). The negative control sections did not identify either lymphatic or blood vessels (data not shown).

Immunohistochemical Expression of Podoplanin

Podoplanin positive sites, highlighted with a brown color, were observed in the endothelial cells of lymphatic vessels and capillaries. Larger lymphatic vessels were significantly less numerous than smaller lymphatic capillaries. All lymphatic vessels and blood capillaries were arranged irregularly and aligned with the longitudinal axis of myocardial cells (*Fig. 2*).

Dual Immunofluorescent Expression of Podoplanin and PAL-E

The endothelium of draining lymphatic vessels and lymphatic capillaries was highlighted red under the multi-function fluorescence microscope. Microvascular blood

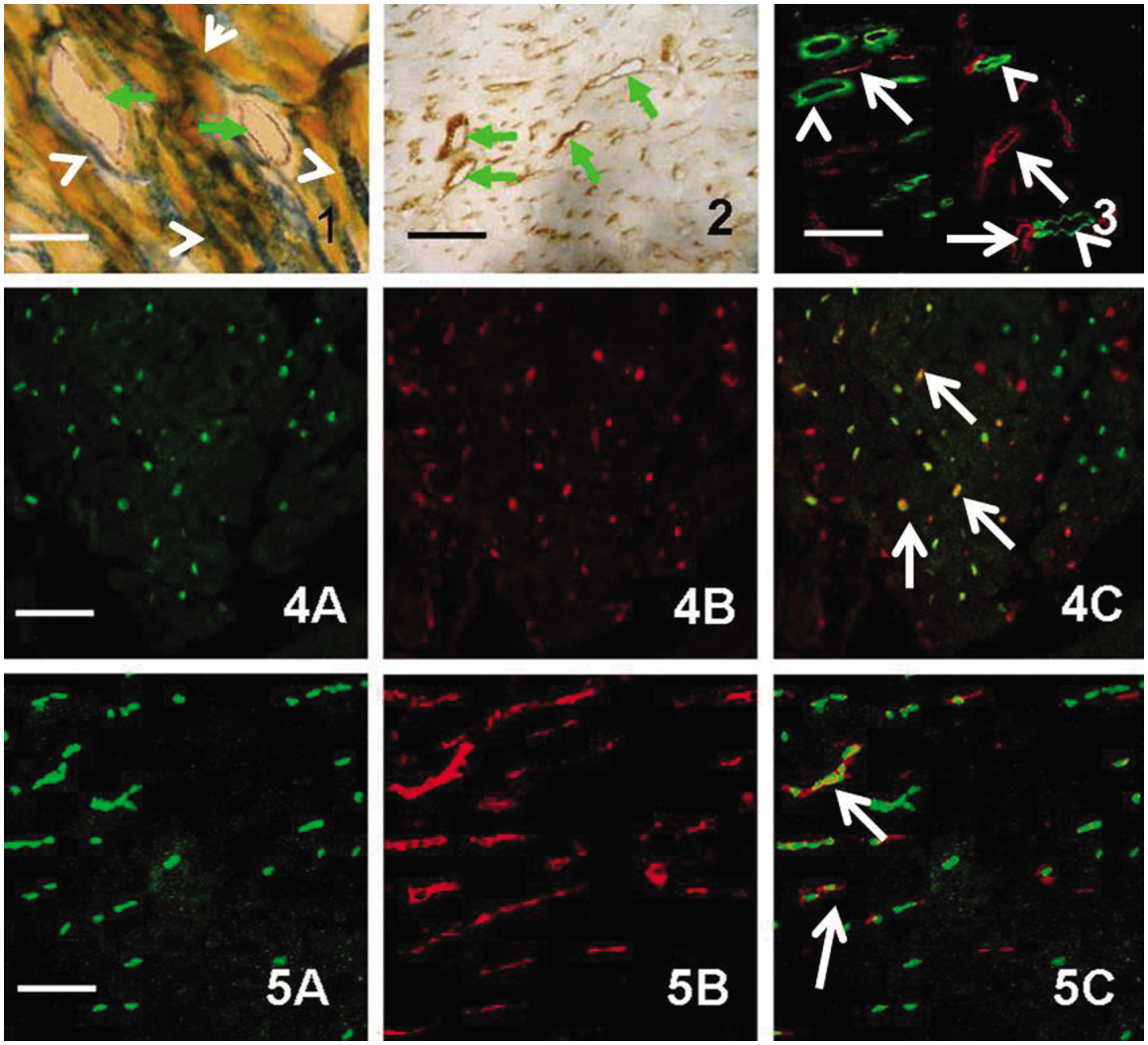

Fig.1. 5'-Nase-Alpase double staining for lymphatic vessels and blood capillaries. The arrows indicate draining lymphatic vessels and the blue-black vessels (arrowheads highlight some) are blood capillaries. x400

Fig. 2. Immunohistochemical staining for lymphatic vessels using podoplanin. The arrows indicate lymphatic vessels seen in this transverse section. x400

Fig. 3. Immunofluorescence double staining for lymphatic vessels and microvascular blood vessels. The red fluorescence (arrows) highlights draining lymphatic vessels and lymphatic capillaries using podoplanin, and the green fluorescence (arrowheads) highlights the blood microvascular vessels using PAL-E. x400

Fig. 4. Immunofluorescence double staining for transverse sections of lymphatic vessels and microvascular blood vessels under confocal laser scanning microscopy. 4A: microvascular blood vessels with PAL-E; 4B: lymphatic vessels with podoplanin; 4C: merged image (arrow indicates merged color). x200

Fig. 5. Immunofluorescence double staining for longitudinal sections of lymphatic vessels and microvascular blood vessels under confocal laser scanning microscopy. 4A: microvascular blood vessels with PAL-E; 4B lymphatic vessels with podoplanin; 4C: merged image (arrow indicates merged color). x200

TABLE 1
Ratio of Lymphatic to Microvascular Blood Vessels in the Left and Right Ventricle (Mean±SD)

	lymphatic vessels		blood vessels		lymphatic/capillaries	
	CS	LS	CS	LS	CS	LS
LV	1910±2.98	1135±2.89	1440±2.36	1350±3.21	1.33: 1	1: 1.01
RV	1919±2.08	1128±2.11	1429±1.98	1342±2.01	1.34: 1	1: 1.12
Total	3829±1.11	2263±1.08	2869±1.98	2692±1.65	1.34: 1	1: 1.12

LV= left ventricle, RV= right ventricle, CS= cross section, LS= longitudinal section

(including arterioles, venules, and capillaries) endothelium displayed green fluorescence. A few lymphatic and microvasculature blood vessels were aligned in parallel, but most demonstrated no relationship (*Fig. 3*). The number of draining lymphatic vessels, lymphatic capillaries, and blood microvessels seen was increased using laser confocal microscopy compared to the multi-functional fluorescence microscope. Lymphatic and blood capillaries displayed green and red dots in cross section (*Figs. 4A-C*) and were parallel to each other in longitudinal sections. Some lymphatic vessels were bifurcated and the lumen diameter was usually larger than that of blood microvessels (*Figs. 5A,B*). The merged image (*Fig. 5C*) demonstrated a very occasional overlap of the different vessel types. Count results from the 60 fields of view are shown in *Table 1*. The ratio of lymphatic vessels to blood microvascular vessels was approximately 1:1 in both left and right ventricular tissues and there was no significant difference between them ($p>0.05$). The proportional relationships of cross section and longitudinal section were also very close with no significant difference. Negative control sections did not produce any red or green fluorescence (data not shown).

Ultrastructure of the Cardiac Lymphatics

Generally, 4 vessels were identified around each cardiac myocyte using TEM with half of them identified as lymphatic capillaries and the other half as blood capillaries. Red blood cells were always observed in blood capillaries and not detected in lymphatic capillaries, which were filled with matrix (*Fig. 6*). The lymphatic capillaries were composed of a layer of endothelial cells, the lumen was irregular, and the wall was very thin *(Figs. 7-9*). The periphery of the capillary was enclosed with very thin connective tissue, and there rarely was a continuous basement membrane. Many processes (composed of plasma membrane) extended toward the lumen from the capillary endothelial cells (*Figs. 7-8*). Finger-like processes were also observed on the abluminal surface (superficies externa), and these connected the lymphatic capillary and connective tissue (*Figs. 7-8*).

DISCUSSION

Although studies have been reported previously concerning cardiac lymphatics, due to limitations of research methods, these primarily have been at the level of gross anatomy. Shimada et al (9) observed the distribution of lymphatics on the subepicardial layer by scanning electron microscope and found a well-developed lymphatic system including the lymphatic trunk and lymphatic vessels. But research on lymphatic morphology and distribution in the myocardium is limited, and specifically the proportion and

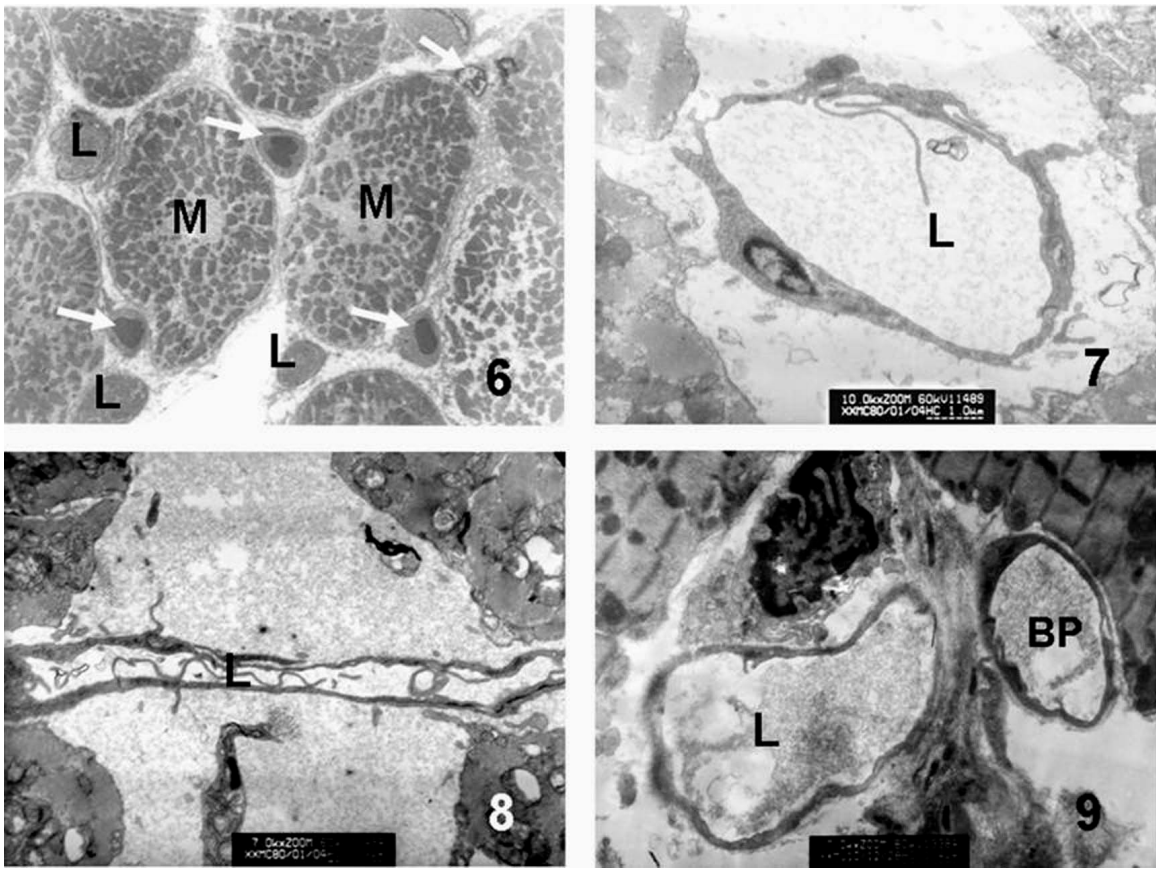

Fig. 6. TEM transverse section displaying lymphatic (L) and blood capillaries (õ) in relationship to the myocardial cells (M). x3000

Fig. 7. TEM transverse section of one lymphatic capillary (L) highlighting a long process in the lumen. x10000

Fig. 8. TEM longitudinal section of one lymphatic capillary (L) demonstrating many processes into the lumen and a few short processes seen on the abluminal side into the matrix. x7000

Fig. 9. TEM transverse section of a draining lymphatic (L) and blood (BP) capillary. x7000

relationship between lymphatic and blood capillaries is still largely unknown.

Since 5'-Nase is mainly located on the lymphatic endothelium and has weak or no activity on the blood vascular endothelium, this method is useful for distinguishing blood and lymphatic capillaries (10). In our study, 5'-Nase-Alpase double staining displayed draining lymphatic vessels and blood capillaries and clearly identified the relationship between them. It was clear that draining lymphatic vessels were less numerous than microvascular blood vessels in the myocardium. Using this method with the characteristics of large, irregular lumen and fewer in number, we were only able to identify draining lymphatic vessels and not lymphatic capillaries. Therefore, we concluded that 5'-Nase-Alpase double staining method was not ideal to examine the total lymphatic system in deep myocardium, and therefore, the overall distribution

between myocardial draining lymphatic vessels, lymphatic capillaries and blood vessels was not clear. In order to identify this relationship, we chose to use the dual-color immunofluorescent technique.

The morphology of myocardial draining lymphatic vessels, lymphatic capillaries, and blood microvasculature is very similar. Therefore, the use of specific markers is needed. Specific markers for lymphatics include: Prox-1 (11), podoplanin, LYVE-1 (12), VEGFR-3 (13), macrophage mannose receptor (14), and CCL21 (15). These markers have advantages and disadvantages in different tissues and different developmental stages, and reports about them have been conflicting (5). Podoplanin is a recently discovered lymphatic marker (2,16) that was first found as a glycoprotein in the cell membrane of glomerular foot processes. Breiteneder-Gelef et al reported that podoplanin only identifies small lymphatic capillaries and not larger vessels such as the thoracic duct. It is also present in the cell membrane of lymphatic sinus walls in lymph nodes and in lymphatic endothelial cell gaps (3). Up until now, it has not been reported that podoplanin is specific in myocardial tissue. Immunohistochemistry and immunofluorescence results from this study, however, showed that myocardial draining lymphatic vessels and lymphatic capillaries stain positive, suggesting that podoplanin is a reliable marker of lymphatic vessels in the myocardium.

There may be a very specific marker to highlight both blood microvascular and lymphatic vessels simultaneously. Although progress in this field has been made recently, the perfect marker for use in pathological and physiological conditions for various species, tissues, and vessels has not been found. LYVE-1, VEGFR-3, and Prox1 are very common markers for identifying lymphatic endothelia, but expression can also be seen in blood vascular endothelium in some tissues. Other markers like CD31 and VEGFR-2 can be expressed on both blood vessels and lymphatic endothelium. It is clearly very important to correctly select the specific markers for blood or lymphatic vessels (17). Markers for blood capillaries that work in the myocardium include VEGFR-1 (17) and PAL-E (18). PAL-E, an antigen associated with small vesicle endocytosis, can stain blood capillaries and venules but is negative or weakly positive in the lymphatic endothelium (3,4,19). Therefore, since PAL-E can be used to display the blood capillaries and venules, and podoplanin can display the lymphatics, we used these for our studies. A complicating factor we found in these experiments was that the number of lymphatic and blood capillaries identified was less using the fluorescence microscope compared to the laser confocal microscope due to reduced sensitivity and choice of equipment is important to consider.

Our studies have shown that a rich lymphatic system is distributed in myocardium with fewer draining lymphatic vessels. The results also demonstrated that these lymphatic vessels form with the blood microvascular system a complicated vascular web. Some blood vessels accompany lymphatic vessels while others are found singly among myocardial cells. Most lymphatic and blood vessels aligned along the long axis of myocardial cells with only a few connected in a transverse arrangement. We also found that the ratio of lymphatic to microvascular blood vessels is approximately 1:1, and this finding is supported by other reports where it is generally accepted that blood capillaries are arranged in lattices (20) and that the ratio is 1:1. The TEM results also support this observation with a 1:1 ratio also found in the vessels surrounding the myocytes. These results need further investigation and confirmation particularly in other species.

Recently, the concept of a "cardiovascular unit" (CVU) has been proposed by Breiteneder-Gelef et al, and this unit includes myocardial cells, capillaries, and fibroblasts (21) and their mutual interaction with each other for optimal function (22). Even if this

concept holds up in the future, it needs to include the lymphatic capillaries to be truly encompassing. The myocardial lymphatic and blood microvascular systems are interdependent systems constituting a very complex network, together forming the basis of the myocardial microcirculation. If myocardial draining lymphatics or lymphatic capillaries are damaged, the myocardium could be extensively impaired (i.e., conduction disorders, myocardial contraction weakness, coronary artery damage), and therefore, more research needs to be conducted on the lymphatic biology of the myocardium (23).

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