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Anatomy

The Morphology of Lymphatic Endothelial Microchannels in the Renal Cortex of the Mouse

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Abstract **Original Research Article**

Background: The knowledge of the intrarenal lymphatic vasculature, especially the lymphatic endothelial microchannel, still remains unclear, that restricts the development of modern scientific research to support clinical management. ***Methods:*** Twelve kidneys harvested from 6 white mice were used for the investigation. Four kidneys were histologically sliced for hematoxylin-eosin, immunohistochemistry and immunofluorescence staining examinations. Four of them were sliced horizontally and coronally into 1mm thick tissues after ventricular perfusion was performed by a solution of 4% paraformaldehyde mixed with Indian ink, and then were inspected by a confocal laser scanning microscope after immunofluorescence staining followed by tissue clearing. Four were sliced and examined by scanning electron microscope. ***Results:*** The lymphatic vasculature has been detected in the renal tissue of the mouse. The arrangement of renal lymphatic vessels of the mouse was irregular in the cortex. In the 3-dimensional confocal image, many lymphatic endothelial microchannels were visible in the wall of segmental vessels. The ultra-structural feature of the lymphatic endothelial microchannel was noticed in the SEM image. ***Conclusion:*** The intrarenal lymphatic vascularture in the renal cortex of the mouse has been presented and the lymphatic endothelial microchannel has been described in detail. The results will provide basic knowledge of animal experiment for further scientific study and clinical applications.

Keywords: Mouse; kidney; renal lymphatic vessel; lymphatic endothelial microchannel; tissue clearing technique.

INTRODUCTION

The renal lymphatic vessel is a part of the vascular system in the kidney, it plays an important role in collecting and returning excess interstitial fluid to the circulation system under both the physiological and pathological processes (Russell *et al*., 2019). Although the lymphatic distribution, vasculature and morphological characterization of the kidney have been investigated in normal and pathological conditions of the human and various animals in recent decades (Albertine *et al*., 1979; Ishikawa *et al*., 2006; Seeger *et al*., 2012; Tanabe *et al*., 2012; Yazdani *et al*., 2014; Breslin *et al*., 2018; Russell *et al*., 2019), some issues have not been resolved. For instance, how does the lymph enter the lymphatic vasculature from the interstitium of the kidney, and how does the micro-pathway of the lymph look like anatomically? In fact, the "genuine" lymphatic endothelial micro-pathway of the kidney has not been authoritatively confirmed anatomically, though some studies have shown that the lymphatic pathway can been found between their endothelial cells in some manner (Leak, 1971; Albertine *et al*., 1980; Yang *et al*., 1981; Mendoza *et al*., 2003; Baluk *et al*., 2007; Galie *et al*., 2009). Therefore, it not only hinders the progress of biotechnology research, but also affects the development of precision medicine, especially the 3-dimensional (3D) renal biomanufacturing (Wragg *et al*., 2019).

In recent years, a combination method of immunofluorescence (IF) staining and tissue clearing has been applied for detecting the neurovascular structures in cubic tissues (Ertürk *et al*., 2012; Merz *et al*., 2019; Ueda *et al*., 2020), which could make the structures appear comprehensively under both physiological and pathological conditions of the animal models. Therefore, we assume that if the same method is used for detecting the lymphatic structure in the thick tissue of the kidney, the equivalent result might be obtained.

In this study, the lymphatic vasculature is detected in the thick renal tissue of the mouse after using techniques of IF staining, tissue clearing and scanning electron microscope (SEM). Details of the distribution, structure and ultrastructure - especially the renal lymphatic endothelial microchannel, are revealed.

MATERIALS AND METHODS

Experiments in this study were approved by the Animal Ethics Committee of Xuzhou Medical University (L20210226466, Jiangsu, China) and performed under the guideline for Care and Use of Laboratory Animals of Xuzhou Medical University.

Animal preparation and renal tissues acquisition

A total of 6 adult male white mice (weighing between 28 to 36 g) were obtained from Laboratory Animal Resources, Chinese Academy of Sciences, Shanghai, China. Each mouse received an intraperitoneal injection with 10% chloral hydrate (0.35ml/100gm) (CAS# 302-17-0, BBI Life Sciences, Shanghai, China) for anesthesia before kidneys were removed. Four kidneys were harvested from 2 mice, which were sliced horizontally into 2 mm thick pieces of the tissue and were fixed in 4% paraformaldehyde (PFA) solution for histological section and hematoxylin-eosin (HE), immunohistochemistry (IHC) and IF staining. Two pairs of kidneys were gained from 2 mice after ventricular perfusion was performed with a mixture of 4% PFA 200 ml and Indian ink 1 ml (HR#204, Shanghai Hero group Stationery Sales Co., Ltd, Shanghai, China), and then were fixed in 4% PFA solution overnight at 4°C for thick tissue IF staining and transparency processing. Four kidneys were removed from 2 mice. From each of them, 3 pieces of 1 x 1 x 2 mm tissue block were harvested and immediately fixed in a solution of 4% PFA mixed with 2% glutaraldehyde (GA) for SEM examination.

HE, IHC and IF Staining

Paraffin blocks were made from 2mm thick of renal tissues by standard histological protocols. Three sets of 5 μm histological sections were obtained from each block to be mounted on glass slides. One set was conventionally performed for HE staining. Each remaining set was used for IHC and IF staining respectively. Slices were passed through xylol, alcohol solutions and distilled water. After heat-mediated antigen restoration with citrate buffer (pH 6) was performed, samples were blocked with 1% Bovine Serum Albumin (BSA ST023-50g, Beyotime Biotechnology P/L, Shanghai, China) for 30 min, and then incubated with primary antibody (LYVE-1: ab33682 1:500 dilution; Abcam) overnight at 4°C. Samples were then washed 3 times with PBS before being incubated with secondary antibodies (For IHC: Goat Anti-Rabbit IgG H&L ‹HRP› ab205718 1:5000; For IF: Goat Anti-Rabbit IgG H&L ‹Alexa Fluor® 488› ab150077 1:5000; Abcam) at room temperature in the dark for 50min. Subsequently, samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI: ab104139, Abcam) at room temperature for 10min after being washed 3 times with PBS. Finally, slides were mounted with Antifade Mounting Medium (P0128: Beyotime Biotechnology P/L, Shanghai, China) and were scanned by an automatic digital scanner (Pannoramic MIDI 3Dhistech, Hungary) for image analysis.

Thick renal tissue IF staining and transparency processing

Two pairs of kidneys, after ventricular perfusion with a mixture of 4% PFA and Indian ink, were sliced horizontally and coronally into 1 mm thick tissues that were then fixed in 4% PFA at 4°C for 4 hours. Subsequently tissues were washed 3 times with PBS, each tissue was placed in a 12-well plates respectively and blocked with 10% Goat Serum (C0265, Beyotime Biotechnology P/L, Shanghai, China) at 4°C overnight. The following day, blocking solution was removed and primary antibody (LYVE-1: PA5-19620 1:500, Thermo Fisher) was added for incubation on rotary shaker at 4°C overnight. After washing 3 times with PBS, tissues were incubated in secondary antibody (Goat Anti-Rabbit IgG H&L, Alexa Fluor® 488, A-11008, 1:500, Thermo Fisher) in the dark on rotary shaker at 4°C overnight. Tissue was washed 3 times with PBS again, tissues were placed successively in 50%, 70% and 80% Tetrahydrofuran (THF) solution in the dark on rotary shaker at 4°C for 30 min each. Then they were placed in 100% THF in the dark on rotary shaker at 4°C for 30 min by 3 times. Afterwards they were placed in dichloromethane in the dark on rotary shaker at 4°C for 20 min and in dibenzyl ether for 15 min. Then they were observed under a Confocal Laser Scanning Microscope (CLSM: TCS SP8, Leica Microsystems Trading Co. Ltd, Shanghai China) with software of Leica Application Suite X (LAS X, Leica Microsystems Trading Co. Ltd, Shanghai China).

SEM scanning

Small block of renal tissues (1 x 1 x 2 mm) were harvested and immediately fixed in a solution of 4% PFA mixed with 2% glutaraldehyde (GA) for scanning electron microscope (TEM: HT7700, Hitachi High-Technologies Co. Ltd, Japan) examination by standard protocols.

RESULTS

Distribution, morphological and histological structures, ultra-structure of the lymphatic vessels in the renal cortex of the mouse have been revealed as follows.

In the thick section of the mouse kidney

Abundant segmental lymphatic capillaries were seen in the thick section of the renal cortex of the mouse after IF staining and transparent processing was performed (Fig 1). The arrangement of avalvular lymphatic capillaries was irregular in the cortex (Fig

1B). The average diameter of the vessel was about 30 µm.

In the 3-dimensional confocal image, many lymphatic endothelial microchannels of the mouse kidney were visible in the wall of segmental vessels (Fig 2A). On the plan of the cross section, vessels were presented in different shapes – round, oval and irregular and constituted by endothelial cells where the nuclei could be seen (Fig 2B).

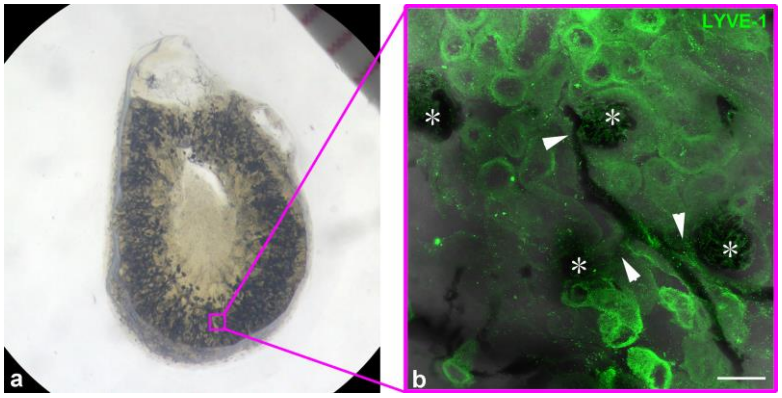

Figure 1: Distribution of lymphatic vessels in the kidney of the mouse. (A) A thick section of the mouse kidney after IF staining and tissue transparent processing are performed. (B) A magnified image from the purple boxed area in (A) showing the irregular lymphatic vessels arrangement in the cortex. *= Glomerulus; White bar = 50 µm

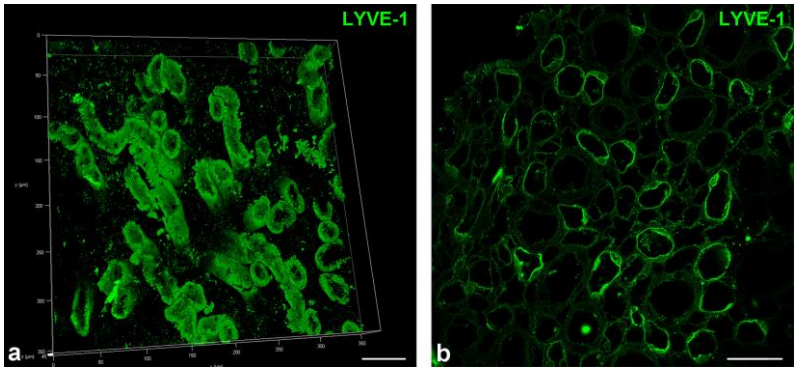

Figure 2: Mophology of lymphatic vessels in the thick renal tissue of the mouse. (A) 3D confocal image shows endothelial microchannels in the wall of lymphatic vessels. (B) The section of the lymphatic endothelial structure. White bars = 50 µm

In the thin (histological) section of the mouse kidney

Lymphatic vessels were hardly recognized in the HE staining section of the mouse kidney, but they could be detected by IHC and IF staining of LYVE-1. Vessels were expressed in different shapes in 2-dimensional images (Fig 3).

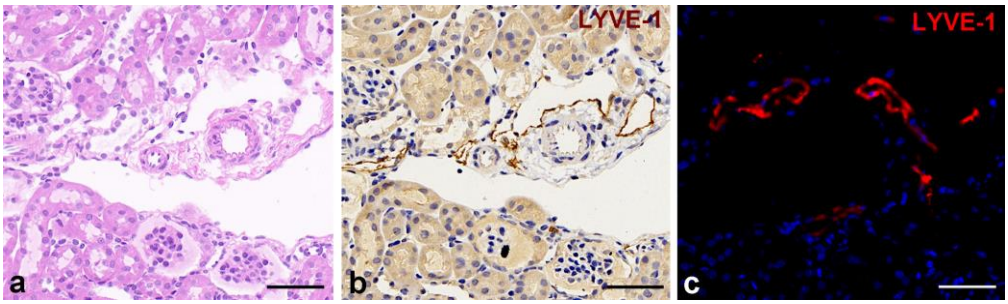

Figure 3: Lymphatic vessels in the kidney of the mouse. (A) HE staining. (B) IHC staining of LYVE-1. (C) IF staining of LYVE-1. Black and white bars = 50 µm

In SEM scanning

Lymphatic vessels in the cortex of the mouse kidney were well presented in the SEM image (Fig 4). Numerous endothelial microchannels were seen in the outer surface of vessels. The entrance of the endothelial microchannel contained a semi-circular "roof" and flat "floor" (Fig 4A and B white arrow). The size of the entrance was about 8 µm (height) x 7 µm (width) (Fig 4C). Numerous metabolites scattered around vessels, some of them gathered at entrances (Fig 4 yellow arrows).

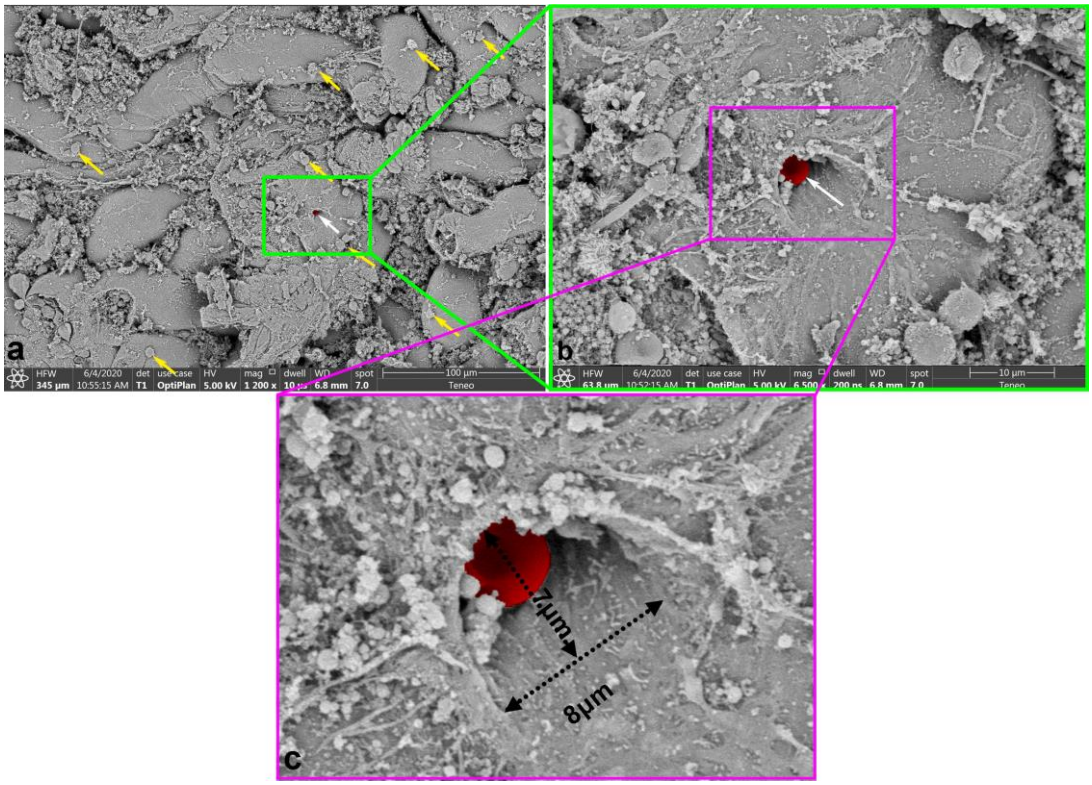

Figure 4: The endothelial microchannel in the wall of the lymphatic vessel of the kidney of the mouse. (A) A SEM image on a section of the mouse kidney. (B) A magnified image from the green boxed area in (A) showing an endothelial microchannel in the lymphatic vessel. (C) A magnified image from the purple boxed area in (B). Yellow arrows indicate metabolites gathering at the entrance of endothelial microchannels. White arrows indicate an erythrocyte (colored in red) entering the endothelial microchannel

DISCUSSION

The renal lymphatic vessel plays a very important role in health and diseases as the renal lymphatic vessel has one of three routes (the other two being the venous and urinary tract) where interstitial fluid and macromolecular metabolites can leave from the kidney (Katz *et al*., 1959; Russell *et al*., 2019). Clinical management needs to be supported by accurate and detailed anatomical knowledge of the renal lymphatics. The distribution and drainage of lymphatics around the kidney has been revealed in early mercury perfusion studies (Sappey, 1874) (Fig 5) as well as in subsequent studies by injecting the dye mixture indirectly (Rouvière, 1938), however it has still not been fully understood, especially the details of the intrarenal lymphatic vessel (Leak, 1971; Albertine *et al*., 1980; Yang *et al*., 1981; Ishikawa et al. 2006; Lee *et al*., 2011; Seeger *et al*., 2012; Tanabe *et al*., 2012). In this study, details of the 3D distribution and vasculature of the renal lymphatic vessel especially the endothelial microchannel was revealed in the thick renal tissue of the mouse after using techniques of IF staining, tissue clearing and scanning electron microscope (SEM).

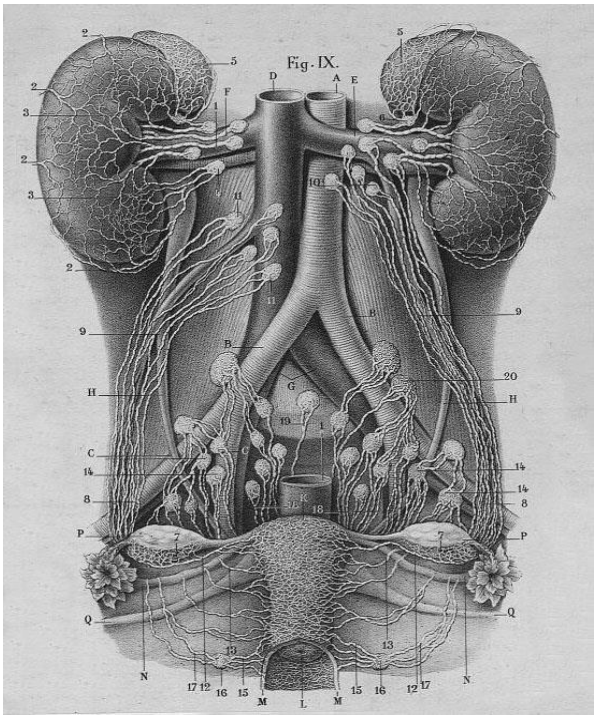

Figure 5: The lymphatic distribution of the kidney in man

(https://archive.org/details/BIUSante_01562/page/n231/mode/2up. Sappey, P. C. Anatomie, Physiologie, Pathologie des vaisseaux lymphatiques. Paris: Adrien Delahaye, 1874. pp 232)

It has been shown that intercellular gaps of the lymphatic capillary were considered as the major passage for removing interstitial fluid and macromolecular metabolites (Leak, 1971; Albertine *et al*., 1980; Yang *et al*., 1981; Mendoza *et al*., 2003; Baluk *et al*., 2007; Galie *et al*., 2009; Breslin *et al*., 2018; Russell *et al*., 2019), while we found that numerous lymphatic endothelial microchannels were seen in the wall of lymphatic vessels of the mouse kidney (Fig 2A), the entrance was formed by a semi-circular "roof" and flat "floor" (Fig 4B). We assumed that this special structure could be considered as a "door" of the endothelial microchannel for controlling the entry of the interstitial fluid and metabolites. When the lymphatic vessel was filled with lymph, the intraluminal pressure increased, the "floor" was raised and the "door" was closed (Figs 4 and 6A). On the contrary, after lymph was emptied to the larger lymph vessel, the intraluminal pressure decreased, the "floor" was descended, the "door" was opened and the interstitial fluid and metabolites entered the lumen of the vessel (Figs. 4 and 6B). This process might be the mechanism of the lymphatic vessel removing the interstitial fluid and metabolites from the kidney of the mouse.

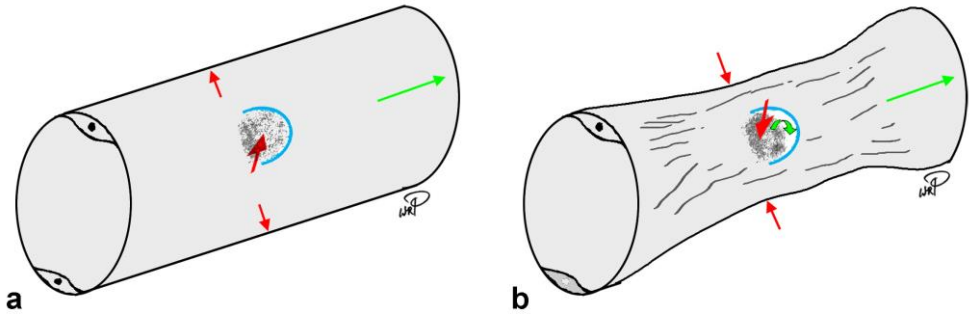

Figure 6: A sketch of the endothelial microchannel in the wall of the renal lymphatic vessel. (A) The lymphatic vessel is fully filled with lymph. (B) The vessel is emptied. Blue curved line indicates the "roof" of the endothelial microchannel; Grey area indicates the "floor" of the endothelial microchannel; Red arrows indicate the inner and outer pressure of the vessel; Green arrows indicate the direction of the lymph flow

Finally some questions and conjectures have been raised since the result from this study has been obtained and need to be carried out: (1) Whether the endothelial microchannel could be found in lymphatic vessels of other organs or tissues? (2) Would a similar structure be found in the human body? (3) Whether pathological conditions would result in structural changes?

CONCLUSION

The intrarenal lymphatic vasculartur in the renal cortex of the mouse has been presented and the lymphatic endothelial microchannel has been described in detail. The results will provide basic knowledge of animal experiment for further scientific study and clinical applications.

Declaration of interests: No competing financial interests exist.

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