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Communication between lymphatic and venous systems in mice

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ABSTRACT

The lymphatic system in mice consists of lymphatic vessels and 22 types of lymph nodes. Metastatic tumor cells in the lymphatic system spread to distant organs through the venous system. However, the communication routes between the lymphatic and venous systems have not been fully elucidated. Here, we identify the communication routes between the lymphatic and venous systems in the axillary and subiliac regions of MXH10/Mo-*lpr*/*lpr* inbred mice, which develop systemic swelling of lymph nodes up to 10 mm in diameter, allowing investigation of the topography of the lymph nodes and lymphatic vessels. Using a gross anatomy dissection approach, the efferent lymphatic vessels of the proper axillary lymph node were shown to communicate with the subclavian vein. Furthermore, we found that the thoracoepigastric vein, which connects the subclavian vein and inferior vena cava, runs adjacent to the subiliac and proper axillary lymph nodes, and receives venous blood from these lymph nodes routed through small branches. The direction of blood flow in the thoracoepigastric vein occurred in two directions in the intermediate region between the proper axillary lymph node and subiliac lymph node; one to the subclavian vein, the other to the inferior vena cava. This paper reveals the anatomy of the communication between the lymphatic and venous systems in the axillary and subiliac regions of the mouse, and provides new insights relevant to the investigation of the mechanisms of lymph node metastasis and cancer immunology, and the development of diagnostic and treatment methods for lymph node metastasis, including drug delivery systems.

1. Introduction

The lymphatic system plays a crucial role in immune surveillance and fluid homeostasis as well as in tumor formation and metastasis progression. The lymphatic system is somewhat different from the cardiovascular system in that it is a blind-ended network, and the flow in the system is unidirectional and not driven by a central pump (i.e. the heart). The flow of lymph eventually communicates with the venous system. As a result, tumor cells that enter the lymphatic system can communicate with the venous system. Therefore, elucidation of the communication routes between the lymphatic and venous systems is essential for the investigation of metastatic mechanisms and clinical treatment strategies. However, the anatomy of the communication routes between the lymphatic and venous systems has not been fully investigated in animal experiments. One reason has been the lack of a noninvasive imaging system capable of monitoring metastasis over time in an appropriate animal model. The overall anatomy of the murine lymphatic system has not been fully characterized, even though murine models have been used widely for immunology and cancer biology studies. The lack of data arises mainly because it is difficult to distinguish lymph nodes (LNs) from their surrounding adipose and connective tissue; this is due partly to their small size, and partly because of the difficulty visualizing the lymphatic vessels (LVs) that transport clear-to-white-colored lymph fluid.

We have previously reported that the lymphatic system in mice consists of LVs and 22 types of LN, and revealed the positional relationships of these LNs (Shao et al., 2013). This study was carried out using MXH10/Mo-*lpr*/*lpr* (MXH10/Mo/lpr) inbred mice that exhibit systemic lymphadenopathy, with some peripheral LNs being as large as 10 mm in diameter, but show the advantage of not developing severe glomerulonephritis and vasculitis seen in other murine models of lymphadenopathy (Shao et al., 2013). The basic structures of the LNs, including the medulla, paracortex and cortex, and the lymphatic channels are well maintained in MXH10/Mo/lpr mice. Furthermore, the anatomical results are essentially consistent with those previously obtained using BALB/cAnNCrl mice (Van den Broeck et al., 2006). In the present study, we clarified the communication routes between the lymphatic

Abbreviations: LN, lymph node; LV, lymphatic vessel; MXH10/Mo/lpr, MXH10/Mo-*lpr*/*lpr*; PALN, proper axillary lymph node; SiLN, subiliac lymph node; TV, thoracoepigastric vein.

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and venous systems in the axillary and subiliac regions of MXH10/Mo/lpr mice using anatomical and imaging methods. As pointed out previously (Van den Broeck et al., 2006), the way the murine LN nomenclature has been defined is unclear, resulting in confusion among researchers. In the present study, we used the murine LN nomenclature defined in two previous publications (Van den Broeck et al., 2006; Shao et al., 2013). The vein that communicates with the subclavian vein and the inferior vena cava, and runs adjacent to the subiliac LN (SiLN) and proper axillary lymph node (PALN), was named the thoracoepigastric vein (TV).

2. Materials and methods

All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of Tohoku University.

2.1. Mice

MXH10/Mo/lpr mice are a substrain of the recombinant inbred mouse strain, MXH/lpr (Shao et al., 2013). MXH10/Mo/lpr mice were produced using two different parental inbred strains as progenitors, MRL/MpJ-*lpr*/*lpr* (MRL/lpr) and C3H/HeJ-*lpr*/*lpr*, followed by an F1 intercross and more than 20 generations of strict brother–sister matings. MXH10/Mo/lpr mice are unique in that most of their peripheral LNs are up to 10 mm in size at 2.5–3 months of age. MXH10/Mo/lpr mice do not develop severe glomerulonephritis and vasculitis, in contrast to MRL/lpr mice (Murphy and Roths, 1978), and thus their life span is longer. The phenotype of the *lpr* (lymphoproliferation) gene is characterized by the accumulation of a large number of polyclonal $CD4^{-}CD8^{-}$ T cells in the LNs and spleen (Nakatsuru et al., 1999). The *lpr* gene is recognized as a 'promoting factor' for collagen disease in MRL mice due to Fas-mediated apoptotic insufficiency; the causative genes of collagen disease are considered to be the 'background' genes of MRL/lpr mice (Nose et al., 2013).

2.2. Identification of the lymphatic and venous systems

Mice ($n = 13$) were placed under deep general anesthesia with 2.5% isoflurane in oxygen (Abbott Japan Co., Ltd., Tokyo, Japan) using an inhalation gas anesthesia system for small laboratory animals, and the LNs and lymphatic and venous systems surgically exposed and carefully characterized. India ink (20 μL) was injected into the LNs, using a syringe with a 30G needle, to confirm the lymphatic routes. Lymphatic and venous routes were visualized with the aid of a stereomicroscope (SZX10, Olympus, Tokyo, Japan), and digital images were recorded (Camedia C-5060 Wide Zoom, Olympus).

2.3. Production of NDB-liposomes

1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) (MC8080; NOF Co.), 1,2-distearoyl-sn-glycerol-3-phosphatidylethanolamine-methoxy-polyethyleneglycol (DSPE-PEG[2000-OMe]) (DSPE-020CN, NOF Co.), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE) (FE6060, NOF Co.) (92:6:2 mol/mol) were dissolved in a sufficient quantity of chloroform, and the organic solvent then completely removed during flask rotation (60 hPa, at 65 °C for 2 h). The resulting thin film of liposomes was dissolved in phosphate-buffered saline to form multi-lamellar vesicles. After two freeze–thaw cycles to form large unilamellar vesicles, the diameter of the fluorescent liposomes was adjusted to approximately 600 nm using extruding equipment (Northern Lipids) and a filter (pore size, 600 nm). The size and zeta potentials of the NDB-liposomes were 642 nm and −1.5 mV, respectively, measured using a particle size and zeta potential analyzer (ELSZ-2; Otsuka Electronics, Osaka, Japan).

2.4. Visualization of flow in veins

Five mice were used for visualization of flow in veins. Under deep general anesthesia, an arc-shaped incision was made in the abdominal skin of the mouse from the subiliac to the proper axillary region, and 100 μL of 0.01 μM NDB-liposomes was then injected into the tail vein at a rate of 100 μL/min for 60 s ($n = 5$). Fluid flow in the veins was visualized using a fluorescence stereomicroscope (M165-FC; fluorescent filter: GFP2; excitation: 460–500 nm; emission: > 510 nm; Leica, Bensheim, Germany) that was connected to a high-speed camera (Cool SNAP HQ2; Photometrics, Tokyo, Japan) operating at 10 frames/s.

3. Results

3.1. Communication between lymphatic and venous systems in the axillary and subiliac regions

Fig. 1 shows the routes of communication between the lymphatic and venous systems in the axillary and subiliac regions of MXH10/Mo/lpr mice. The efferent LVs of the SiLN are connected to the PALN, and the efferent LVs of the PALN to the subclavian vein. The TV, which connects the subclavian vein and the inferior vena cava, runs adjacent to the SiLN and PALN, and receives venous blood draining from these LNs through small branches.

Fig. 2 shows magnified images of the right SiLN (Fig. 2A) and left SiLN (Fig. 2B). When India ink was injected into the SiLN, it ran towards the PALN in the efferent LVs of the SiLN. Two efferent LVs that run to the PALN may be seen in Fig. 2A and B. The TV runs adjacent to the SiLN, receives venous blood that drains from the SiLN, and then connects to the inferior vena cava.

Fig. 3 shows the communication between the efferent LV of the PALN and the subclavian vein, in the right PALN (Fig. 3A) and left PALN regions (Fig. 3B). These images were obtained by injecting India ink into the PALN, and monitoring its progression to the subclavian vein via the efferent LV of the PALN. Fig. 3C shows the venous system connected to the left PALN. The left TV that runs adjacent to the left PALN, and receives venous blood draining from the PALN, connects to the left subclavian vein. The efferent LVs that extend from the left SiLN make connections with the left PALN. The efferent LV (indicated by the white dotted arrow) connects to the left subclavian vein.

3.2. Direction of blood flow in the TV

The TV, which connects the subclavian vein and the inferior vena cava, runs adjacent to the SiLN and PALN and receives venous blood draining from these LNs, routed through small branches. Next, we investigated the direction of blood flow in the TV after injection of fluorescent solution into the tail vein of the mouse (Fig. 4). Fig. 4B shows a magnified view of the square region X between the PALN and SiLN, and Fig. 4E shows a magnified view of the square region Y over the SiLN. It should be noted that Fig. 4A is the same as Fig. 1, and does not correspond exactly with the *in vivo* images shown in Fig. 4B and E. Fig. 4C shows a frame obtained from Supplementary Video 1 which illustrates the square region X indicated in Fig. 4B. Fig. 4D shows the directions of flow in the vein network connected to the TV in this region; two veins are connected to the TV at points b and c, respectively. Flow in the TV occurred in two directions: one was oriented towards the subclavian vein at point b, the other towards the SiLN at point c. The flow stagnated or moved up and down in sections b–c (see Supplementary Video 1). The bidirectional flow always occurred in the central region between the SiLN and PALN where other veins connected to the TV. Fig. 4F shows a frame obtained from Supplementary Video 2, showing the square region Y indicated in Fig. 4E. Fig. 4G shows the directions of flow in the vein network that connected to the TV over the SiLN. Here, the direction in the TV was always oriented towards the inferior vena cava (see Supplementary Video 2).

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