Organization and developmental aspects of lymphatic vessels*

Osamu Ohtani and Yuko Ohtani

Department of Anatomy, Faculty of Medicine and Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

Summary. The lymphatic system plays important roles in maintaining tissue fluid homeostasis, immune surveillance of the body, and the taking up dietary fat and fat-soluble vitamins A, D, E and K. The lymphatic system is involved in many pathological conditions, including lymphedema, inflammatory diseases, and tumor dissemination. A clear understanding of the organization of the lymphatic vessels in normal conditions would be critically important to develop new treatments for diseases involving the lymphatic vascular system. Therefore, the present paper reviews the organization of the lymphatic vascular system of a variety of organs, including the thyroid gland, lung and pleura, small intestine, cecum and colon in the rat, the diaphragm in the rat, monkey, and human, Peyer's patches and the appendix in the rabbit, and human tonsils. Methods employed include scanning electron microscopy of lymphatic corrosion casts and tissues with or without treatment of alkali maceration technique, transmission electron microscopy of intact tissues, confocal microscopy in conjunction with immunohistochemistry to some lymphatic-specific markers (i.e., LYVE-1 and VEGFR-3), and light microscopy in conjunction with enzyme-

histochemistry to 5'-nucleotidase. Some developmental aspects of the lymphatic vessels and lymphedema are also discussed.

Introduction

The lymphatic system plays an important role in maintaining tissue fluid homeostasis by collecting and transporting protein-rich interstitial fluid *via* lymph nodes, larger collecting lymphatic vessels, lymphatic trunks including the thoracic duct, and — thereby — back to the blood vascular circulation. The lymphatic system also plays an important role in immune surveillance of the body by transporting activated immune cells from peripheral tissues to regional lymph nodes. In addition, central lacteals, or central lymphatic vessels, within the intestinal villi are involved in the uptake of dietary fat and of the fat-soluble vitamins A, D, E and K. The lymphatic system is, however, involved in many pathological conditions, including lymphedema, inflammatory diseases, and tumor dissemination.

Gasparo Aselli (1581-1626) discovered lymphatic vessels in the dog mesentery in 1622, a finding published in *De Lactibus* in 1627 (Classic Encyclopedia, <http://www.1911encyclopedia.org/Aselli>; Rusznyák *et al.*, 1967). Ever since his discovery of lymphatic vessels, many scientists have devoted themselves to unraveling the mysteries of the lymphatic system. In spite of the lack of specific markers for lymphatic vessels, their distribution and morphology was largely defined by the end of the 20th century (Ohtani *et al.*, 1997).

In the past decade, however, the discovery of specific markers for lymphatic vessels has provided new insights into the molecular mechanisms that control lymphatic system growth and function (Cueni and Detmar, 2006).

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Address for correspondence: Prof. Osamu Ohtani, MD & PhD, Department of Anatomy, Faculty of Medicine and Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, 930-0194 Japan
Tel: +81-76-434-7205, Fax: +81-76-434-5010
E-mail: osmotani@med.u-toyama.ac.jp

These markers include prox-1 (Wigle and Oliver, 1999; Petrova *et al.*, 2002), podoplanin (Wetterwald *et al.*, 1996; Breiteneder-Geleff *et al.*, 1999), LYVE-1 (Lymphatic Vessel Endothelial HA receptor 1; Banerji *et al.*, 1999), VEGFR-3 (Kaipainen *et al.*, 1995), macrophage mannose receptor (Irjala *et al.*, 2001), and CCL21 (Gunn *et al.*, 1998). Desmoplakin and plakoglobin are known to be present in complexus adhaerentes, i.e., zonula-like plaque-bearing junctions between LECs (Schmelz *et al.*, 1994). These two kinds of substances can be specific markers for lymphatic vessels (Ebata *et al.*, 2001; Hirakawa *et al.*, 2003), a current topic of controversy. Fedele *et al.* (2004) have reported that desmoplakin and plakoglobin antibodies are not specific marker of lymphatic vessels in the skin. Integrin $\alpha 9$, which is required for the normal development of the lymphatic system, can be also a specific marker for lymphatic vessels, but it is only transiently expressed in the developing thoracic duct at a given embryonic day (Huang *et al.*, 2000). Another novel marker, LA102 against mouse lymphatic endothelial cells, has been reported by Ezaki *et al.* (2006).

The present paper reviews the organization of lymphatic vessels in a variety of organs as revealed by various methods, including scanning electron microscopy, transmission electron microscopy, enzyme-histochemistry to 5'-nucleotidase (Werner *et al.*, 1987; Kato and Miyauchi, 1989), and confocal laser scanning microscopy in conjunction with immunohistochemistry using markers specific to lymphatic vessels such as LYVE-1 (rabbit polyclonal, Abcam, California) and VEGFR-3 (rabbit polyclonal, Snata Cruz Biotechnology, California). The organs reviewed are the thyroid gland, lung, costal pleura, small intestine, cecum, and colon in the rat, diaphragm in the rat, monkey, and human, Peyer's patches and appendix in the rabbit, and human tonsils surgically resected. The liver also contains voluminous lymphatic vessels, a finding reviewed elsewhere (Ohtani and Ohtani, 2008). The lymph node is representative of the lymphatic system, but we will review the node structure, function, and cancer metastasis in a separate paper. This paper also reviews some morphological events that occur during *in vivo* lymphangiogenesis and microenvironments that promote lymphangiogenesis *in vitro*.

Structure of lymphatic vessels in general

By the end of the 20th century, many scientists had made great efforts to demonstrate lymphatic vessels using a variety of methods, including dye injection, reconstruction of serial sections, corrosion cast, enzyme-histochemistry, among others. The lymphatic vascular system consists of

noncontractile initial lymphatic networks and collecting lymphatic vessels. Initial lymphatic vessels (or initial lymphatic capillaries) are tubulo-saccular and lack smooth muscle cells, valves, and any distinct continuous basement membrane (Kutsuna, 1968). The lymphatic network in the diaphragmatic pleura in the rat consists of probable initial lymphatic vessels with many valves (Fig. 1a). The initial lymphatic vessels — or "lymphatic endings" — in the bat wing have smooth muscle cells and valves (Webb and Nicoll, 1944). There are lymphatic lacunae or lymphatic sinuses with wide and irregular lumens and few valves in some tissues (Fig. 1b). Lymphatic endothelial cells (LECs) are strongly attached at the anchoring filaments to the surrounding collagen and elastin fibers (Leak and Burke, 1966; 1968). The main molecular component of the anchoring filaments is fibrillin, and the anchoring filaments connect the extracellular matrix and the endothelial cell cytoskeleton through the transmembrane integrin and focal adhesion kinase molecule (Gerli *et al.*, 2000). LECs show complexus adhaerentes (Schmelz *et al.*, 1994) and single contact (or overlapping) junctions. Hämmerling *et al.* (2006) showed that the complexus adhaerentes of mammalian lymphatic endothelia comprises zonula adherens proteins (VE-cadherin, α - and β -catenin, protein p120^{cas}, and afadin), desmosomal plaque components (desmoplakin and plakoglobin), and tight-junction proteins (claudin-5 and ZO-1). During the expansion of the initial lymphatic vessels, overlapping junctions can be opened, thus allowing fluid to flow from the interstitium into the lumen; during compression, overlapping junctions can be closed, thereby retarding the return of lymph flow into the interstitium.

Collecting lymphatic vessels are, on the other hand, located downstream of the initial ones and serve as a drainage system. Collecting lymphatic vessels are endowed with smooth muscle cells as well as valves (Fig. 1c–e). Lymphangions, first described by Mislin and Rathenow (1962), are the morpho-functional units of the lymphatic vessels that include the valve and adjacent portion of the lymphatic vessel extending to the next valve (Gashev, 2002). Lymph can be propelled centripetally by passive or extrinsic driving forces that include lymph formation, arterial pulsation, contractions of skeletal muscles, central venous pressure fluctuations, gastrointestinal peristalsis, and respiration (Gashev, 2002). Lymph can be also propelled centripetally by intrinsic or active lymph pumps, which are the coordinated contraction of the chains of lymphangions (Gashev, 2002). Contractions of the lymphangions are initiated by the pacemaker activity of the smooth muscle cells of the lymphangion wall (Ohhashi *et al.*, 1980; Allen

Fig. 1. General structure of lymphatic capillaries (**a**, **b**) and collecting lymphatics (**c**-**e**). **a**: Typical lymphatic capillaries in the pleural side of the rat diaphragm. Arrowheads indicate blind ends, and arrows indicate locations of valves. Enzyme-histochemical staining to 5'-nucleotidase. Scale bar = 200 μm (Micrograph from Ohtani *et al*, 1993). **b**: Lattice-like network of lymphatic vessels with wide and irregular shapes (i.e., lymphatic lacunae) in the peritoneal side of the rat diaphragm. Immuno-histochemistry to LYVE-1. Scale bar = 50 μm . **c**: Smooth muscle cells of the collecting lymphatic vessel in the rat diaphragm at postnatal week 6. Smooth muscle cells around the valves (arrowheads) tend to run circumferentially, while those between valves run longitudinally or spirally. Immunohistochemistry to α -smooth muscle actin. Scale bar = 50 μm (Micrograph from Ohtani and Ohtani, 2001). **d**, **e**: Scanning electron micrographs of collecting lymphatics with smooth muscle cells (yellow) in the adult rat diaphragm treated with a warm (60°C) KOH aqueous solution for 10 min. Initial segments of collecting lymphatics are sparsely surrounded with smooth muscle cell projections of various directions (**d**), while more proximal segments of collecting lymphatic vessels are circumferentially oriented smooth muscle cells (**e**). An abluminal side of a valve (arrowheads) can be seen in **d**. Scale bars = 25 μm (**d**, **e**). (Micrographs from Ohtani, 1992).

Fig. 2. Scanning electron micrograph (a) and confocal micrograph (b) of the rat thyroid glands. **a:** A thyroid gland treated with a KOH aqueous solution at 60 °C for 10 min, thereby eliminating connective tissue fibers. Lymphatic vessel (yellow, L) can be seen between follicles (F). There are also capillaries (red, C) around the follicles (F). **b:** Lymphatic vessels showing immunoreactivity to LYVE-1 (green) can be seen running between clusters of follicles. Scale bars = 50 μ m (a, b)

and McHale, 1986; Van Helden, 1993; von der Weid *et al.*, 1996; von der Weid and Zawieja, 2004).

Thyroid gland

Early studies by light and transmission electron microscopy revealed lymphatic capillaries in the thyroid gland (Rienkoff, 1931; Bargmann, 1939; Kuhlenkampff, 1950; Nakachi, 1972). Shimada (1981) in his scanning electron microscopy of the monkey thyroid gland treated by HCl-collagenase digestion technique clearly demonstrated that lymphatic vessels in the monkey thyroid gland vary in size from about 20 μ m to 100 μ m or more and form a loose network among follicles (Fig. 2). He also found that blood capillaries with pericytes form dense networks that surround individual follicles.

The thyroid gland in the rat also has a lymphatic network similar to that in the monkey. Scanning electron microscopy of the rat thyroid gland treated with the alkali-collagenase maceration technique has shown

that lymphatic vessels with a wide and irregular lumen run between clusters of follicles, each of which is surrounded by a dense network of blood capillaries (Fig. 2). Immunohistochemistry to LYVE-1 has also shown lymphatic vessels surrounding aggregates of follicles (Fig. 2).

Lung and pleura

It is now widely accepted that pulmonary lymphatic vessels are located within or below the pleura depending on species, in interlobular septa and perihilar spaces, and around bronchi and bronchiole, arteries, and veins (Albertine *et al.*, 1982; Leak and Jamuar, 1983; Marchetti *et al.*, 1994; Aharinejad *et al.*, 1995) (Fig. 3). The dense network of lymphatic capillaries beneath the visceral pleura in the rat can be visualized by confocal microscopy of the pleura several minutes after a simple intravenous injection of FITC-dextran (Fig. 3a). This presumably shows that FITC-dextran extravasated from the blood vessels quickly gathers into lymphatic vessels.

Fig. 3. Confocal (a, b, e) and light (c, d) micrographs of rat lungs. **a:** The subpleural lymphatic network filled with FITC-dextran. The picture was taken under a confocal microscope several minutes after an intravenous injection of FITC-dextran. Scale bar = 10 μm . **b:** Subpleural lymphatic vessels (arrowheads) immunohistochemically stained to LYVE-1. Scale bar = 50 μm . **c:** Lymphatic vessels (L) along the bronchial tree. 5'-nucleotidase staining. Scale bar = 100 μm . **d:** Lymphatic vessels (dark brown, arrowheads) along the bronchiole (B) and a branch of the pulmonary artery (A). Scale bar = 100 μm . **e:** LYVE-1 positive lymphatic vessels (arrowheads) along a branch of the pulmonary artery (A). B: bronchiole. Scale bar = 50 μm .

Our studies by enzyme-histochemistry to 5'-nucleotidase and immunohistochemistry to LYVE-1 show the lymphatic network beneath the visceral pleura and along the bronchial tree, arteries, and veins (Fig. 3).

India ink injected into the pleural cavity rapidly enters the lymphatic vessels in the costal pleura in the rat (Wang *et al.*, 1997). The lymphatic vessels in the costal pleura form a lattice-like network (Fig. 4a). Masada *et al.* (1992) have shown the lymphatic vessels in the parietal pleura of the monkey by enzyme-histochemistry to 5'-nucleotidase and light- and transmission electron- microscopy.

The lymphatic vessels in the costal pleura in the rat

extend funnel-like projections to the pores between mesothelial cells, thus forming lymphatic stomata through which lymphatic vessels directly open to the pleural cavity (Wang *et al.*, 1997) (Fig. 4b–d). No lymphatic stomata have been observed in the diaphragmatic pleura in the rat. The lymphatic stomata in the costal pleura are quite similar to those in the peritoneum of the diaphragm (see below) and those in the monkey parietal pleura reported by Masada *et al.* (1992). It is considered that pleural fluid is absorbed through lymphatic stomata into lymphatic vessels.

Fig. 4. Lymphatic vessels of the rat costal pleura. **a:** Lattice-like lymphatic network (black) that have taken up India ink injected into the pleural cavity. **b:** Scanning electron micrograph of the surface of costal pleura showing lymphatic endothelial cells (yellow) emerging into the gaps between mesothelial cells. Scale bar = 5 μ m. **c:** Transmission electron micrograph showing a lymphatic stoma connecting the pleural cavity (P) and lymphatic lumen (L). Mesothelial cells are colored in blue, and lymphatics in yellow. **d:** Scanning electron micrograph of a freeze-fractured rat diaphragm showing that lymphatic endothelial cells (yellow) extend to the gaps between mesothelial cells, thus forming a lymphatic stoma that directly connects the pleural cavity to the lymphatic lumen (L). Scale bars = 5 μ m (c, d)

Diaphragm

The diaphragm plays an important role not only in respiration but also in absorbing and transporting peritoneal fluid via lymph nodes back to the blood circulation. Von Recklinghausen (1863) noticed that milk injected into the peritoneal cavity entered lymphatic vessels in the diaphragm. It was, however, in the latter half of the 20th century that lymphatic stomata — through which peritoneal fluid enters the diaphragmatic lymphatic vessels — were clearly defined (Bettendorf, 1978; Leak and Rahil, 1978; Tsilibary and Wissig, 1983, Ohtani *et al.*, 1993; Nakatani *et al.*, 1996). Development of the lymphatic vessels in the diaphragm will be discussed later (see below).

There are three layers of lymphatic vessels in the diaphragm: the lattice-like network consisting of irregularly shaped lymphatic lacunae with a wide lumen and few valves within or below the peritoneum covering the diaphragm, the network of typical tubulo-saccular lymphatic vessels with many valves within or below the diaphragmatic pleura, and the lymphatic network in the muscular layer (Fig. 1a, b, 5a, b). The lymphatic lacunae within the diaphragmatic peritoneum extend many funnel-like projections of their endothelia towards the pored region of the mesothelium. On coming into contact with the mesothelium, many of the lymphatic projections are perforated at their ends, thus giving rise to stomata connecting the lymphatic lumen to the peritoneal cavity (Ohtani *et al.*, 1993) (Fig. 5c, d). The lymphatic stomata in the diaphragm form clusters, which are especially distinct

Fig. 5. Confocal micrographs (**a**, **b**), and scanning (**c**) and transmission (**d**) electron micrographs of the rat diaphragm. **a**, **b**: Subperitoneal lymphatic vessels show immunoreactivity to LYVE-1 and have a wide and irregular lumen as well as many projections towards the peritoneal surface. Scale bars 25 μm (**a**), 50 μm (**b**). **c**: Funnel-like projections emerge through the gaps between mesothelial cells, thus forming direct connections with the peritoneal cavity to the lymphatic lumen. Scale bar = 5 μm . **d**: Serial sections show that a gap (larger arrow) between the mesothelial cells (**M**) continues to the space between the mesothelial cells and lymphatic vessel (*); this space in turn continues to the lymphatic lumen (**L**) through a gap between lymphatic endothelial cells (smaller arrow). Thus the peritoneal cavity (**P**) connects directly to the lymphatic lumen. Scale bar = 1 μm

Fig. 6. Scanning electron micrographs of peritoneal surface of the monkey (**a, b**) and human (**c**) diaphragm and a subperitoneal collagen sheet of the human diaphragm prepared by the alkali-water maceration technique (**d, e**). (The tissue pieces of the human diaphragm were obtained from a cadaver donated for research and the teaching of anatomy at the Faculty of Medicine, University of Toyama.) **a:** There are clusters of cuboidal mesothelial cells among flat mesothelial cells. Scale bar = 100 μm **b, c:** A closer view shows openings (arrowheads) of the lymphatic stomata among the cuboidal mesothelial cells. Scale bars = 10 μm **d:** There are clusters of pores in the subperitoneal collagen sheet. Scale bar = 100 μm **e:** A closer view shows round or oval pores in the subperitoneal collagen sheet. Scale bar = 10 μm

in the monkey and human (Fig. 6). This has been clearly shown by scanning electron microscopy of collagen fibers processed by the alkali-water maceration technique (Ohtani, 1987a; Ohtani *et al.*, 1988). The collagen fiber sheets immediately below the mesothelium in the monkey and human diaphragm show many clusters of small pores, through which extensions of lymphatic vessels from the lymphatic lacunae pass and open through gaps between the mesothelial cells to the peritoneal cavity (Fig. 6d, e).

In spite of our extensive investigation of the pleural surface of the diaphragm by scanning electron microscopy, we could not find any pores suggestive of lymphatic stomata. India ink injected into the thoracic

cavity of the rat enters lymphatic vessels in the costal pleura, but not those in the diaphragmatic pleura (see above). These findings suggest that the diaphragmatic pleura has few, if any, lymphatic stomata that directly connect the pleural cavity with lymphatic lumen.

A substantial volume of fluid seems to enter the diaphragmatic lymphatic vessels through the lymphatic stomata. Indeed, fluid containing some dye injected into the peritoneal cavity rapidly enters the lymphatic vessels in the diaphragm and reaches the mediastinal lymph nodes. If the volume of fluid absorbed by the diaphragmatic lymphatic vessels exceeds the draining capacity of the collecting lymphatic vessels and lymphatic

trunk, part of the fluid may enter the pleural cavity, thus causing a hydrothorax as occasionally seen in patients treated with continuous ambulatory peritoneal dialysis (CAPD) as well as in many pathological conditions, such as adhesion during various inflammatory diseases. Our investigation has shown that ligation of the lymphatic trunks draining the diaphragmatic lymph causes peritoneal fluid to enter the pleural cavity (Ohtani and Ohtani, 1997). The lymphatic stomata can be routes through which cancer cells in the peritoneal cavity enter lymphatic vessels.

Small intestine

Although many authors (e.g., Mall, 1888; Ranvier 1896; Patzelt, 1936; Grau and Schlüns, 1962) described the microanatomy of lymphatic vessels in the small intestine, their three-dimensional organization was not clearly demonstrated. Our SEM of lymphatic corrosion casts, together with SEM of fractured tissues, has revealed the three-dimensional organization of the lymphatic system in the rat small intestine (Ohtani, 1987b); our findings correlate well with those obtained by immunohistochemistry to LYVE-1 (Fig. 7).

The villus possesses 1 - 10 or more central lacteals, depending upon the villous width (Fig. 7a-c). The lacteals in each villus have interconnections between adjacent ones. At the lower half of the villus the lacteals fuse and form a wide sinus. From the bottom of the sinus in the villus, 1 to 3 lymphatic vessels descend and lead into the dense network of lymphatic vessels in the submucosa. Interestingly, there are many longitudinally oriented smooth muscle cells associated with the central lacteals in the villi (Fig. 7d, e). Contraction of the smooth muscle cells in the villi propels the lymph in the central lacteals to the submucosal lymphatic vessels. In the muscularis externa there is a coarse network of lymphatic vessels which, together with the submucosal one, drains into collecting lymphatic vessels continuous with the mesenteric ones.

Cecum and colon

Whether or not lymphatic vessels in the cecum originate from the mucosa had been controversial. Our previous light and scanning electron microscopic (Ohtani and Ohtani, 2003) and present immunohistochemical studies in the rat have demonstrated that the lymphatic vessels originate in the superficial mucosa, some 50 μm below the basement membrane of the luminal epithelium, and descend between the intestinal glands into the lymphatic

network located around the bottom one fourth of the glands (Fig. 8). The mucosal lymphatic vessels drain into the polygonal network of collecting lymphatic vessels located in the submucosa (Fig. 8). Lymphatic vessels in the submucosa and in the muscularis externa drain into collecting lymphatics located between the inner and outer muscular layers, and finally lead into those in the mesentery. The organization of lymphatic vessels in the colon resembles that of the cecum.

In the rat, there is a collection of lymphoid follicles — termed the "cecal patch" — that responds to luminal antigens as does the appendix and Peyer's patches (Friedenstein and Goncharenko, 1965; Owen, 1977; Bockman, 1983). Lymphoid follicles of the cecal patch are surrounded by a dense plexus of lymphatic vessels — to be referred to as the perifollicular lymphatic plexus (Ohtani and Ohtani, 2003). There are many short blind-ended lymphatic vessels projected from the perifollicular lymphatic plexus toward the follicle periphery. From the perifollicular lymphatic plexus arise several thick lymphatic vessels which, while gathering lymphatic vessels from neighboring regions of the cecum *en route*, converge into thicker lymphatic vessels in the mesentery to lead into regional lymph nodes located near the cecum.

Peyer's patches

Peyer's patches are aggregates of lymphoid follicles in the small intestine and representative gut-associated lymphoid tissues (GALT) which sample antigens from the gut lumen by M cells, process them in dendritic cells, and present the processed antigens to lymphocytes to promote their proliferation, interaction, and differentiation to effector cells in their immune responses (Bockman and Stevens, 1977; Owen, 1977; Bhalla and Owen, 1982; Rouse *et al.*, 1984; Owen *et al.*, 1986).

We have reported on the organization of the reticular network of rabbit Peyer's patches (Ohtani *et al.*, 1991). Immediately beneath the patch dome epithelium, there are sheets of collagen fibrils with numerous pores. Within the patches, collagen fibers repeatedly divide and fuse, forming the reticular network. The reticular network within the follicle is looser than within the dome, the corona, or the inter- (or para-) follicular area. The latter three compartments show similar structures and consist of numerous intercommunicating small subcompartments within the reticular network. Reticular cell processes with numerous fenestrations embrace not only collagen fibers forming the reticular network, but also sheaths of high endothelial venules (HEVs) and lymphatic vessels. Sheaths of collagen fibers of HEVs and lymphatic

Fig. 7. Lymphatic vessels in the small intestine. **a:** Scanning electron micrograph of a corrosion cast of the upper small intestine in the rat. The central lacteals in each villus anastomose and form a sinus (s) at the level of the villous base. They also possess interconnections at the upper portion of the villus. From the bottom of the sinus, two or three perpendicularly oriented lymphatic vessels descend and lead into the submucosal lymphatic plexus (sl). Scale bar = 200 μm **b:** Scanning electron micrograph of a fractured villus of the upper small intestine in the rat showing central lacteals (yellow) which are well correlated with the corrosion cast seen in **a**. Scale bar = 50 μm **c:** Confocal laser scanning micrograph of a section of the rat small intestine showing LYVE-1 positive (green) central lacteals in the villi that were cut at a right angle to that in **b** and perpendicular to the serous membrane of the small intestine. Scale bar = 25 μm **d, e:** Scanning electron micrographs of the rabbit small intestinal villi macerated with warmed (60°C) alkali for 10 min. There are longitudinally oriented smooth muscle cells (brown) associated with the central lacteals (yellow). Scale bars = 10 μm

Fig. 8. Lymphatic vessels in the rat cecum. **a:** Scanning electron micrograph of a lymphatic corrosion cast. Blind-ended lymphatic capillaries beginning in the superficial mucosa descend and gather into slightly thicker lymphatic vessels (ml) in the deep mucosa. They then pour into thicker submucosal lymphatic vessels (sl). An arrowhead indicates a constriction indicative of a valve location. Scale bar = 500 μm . **b, c:** Confocal laser scanning micrographs of either tangential (**b**) or transverse (**c**) sections of the mucosa showing mucosal lymphatic vessels with LYVE-1 immunoreactivity (green). The distribution pattern in **b** correlates well with that seen in **a**. There are lymphatic vessels some 20 μm below the basement membrane of the epithelium (**c**). Scale bars = 50 μm .

vessels are also fenestrated, indicating the sites through which lymphocytes and other free cells migrate. Thus, the reticular network of Peyer's patches is organized so as to facilitate the migration and lodging of free cells and thereby enable antigen-to-antigen and cell-to-cell interactions during an immune response.

The lymphatic vessels exist in the villi projected on the parafollicular regions and in the parafollicular regions (Fig. 9a). In the parafollicular region, there are HEVs located in close proximity to the lymphatic vessels, both strongly expressing Aquaporin-1 (Fig. 9b, c). As in the lymph node, the net absorption of water may occur through the HEV endothelium (Ohtani *et al*, 2003). Further studies will be needed to settle this question.

In the superficial part of the parafollicular area, there

are many blind-ended lymphatic vessels interconnecting to form a plexus (Fig. 9d). The central lacteals within the villi located over the parafollicular area drain into the plexus (Fig. 9d). The lymphatic vessels of the plexus gradually fuse into flat but wide lymphatic vessels that also interconnect to form the perfollicular sinus surrounding the follicle (Fig. 9d). The perfollicular sinus is located in the parafollicular area. The sinuses are not continuous along the entire bottom of the follicle but possess many interruptions through which blood vessels travel (Ohtani and Murakami, 1990). From the bottoms of the perfollicular lymphatic sinuses, thin lymphatic vessels descend into the submucosal collecting lymphatic vessels.

Fig. 9. Confocal laser scanning micrographs of rat Peyer's patches (**a–c**), and scanning electron micrographs of lymphatic corrosion casts of the rabbit Peyer's patch (**d**) and rabbit appendix (**e**). **a:** LYVE-1 positive lymphatic vessels can be seen in the villi (v) and between follicles (F). Scale bar = 100 μ m. **b, c:** Lymphatic vessels (L) and HEV (H) show Aquaporin-1 immunoreactivity. HEVs are located in close proximity to lymphatic vessels in the parafollicular area. The rabbit anti-Aquaporin-1 antibodies were purchased from Chemicon International (Temecula). Scale bars = 25 μ m(b), 10 μ m(c). **d:** The interconnected central lacteals (cl) are linked with a lymphatic plexus in the parafollicular area (if). The plexus gradually converges into the perifollicular lymphatic sinus (s), which incompletely surrounds the follicle. The bottoms of the follicles are also partially covered with wide lymphatic sinuses. (Micrograph from Ohtani and Murakami, 1990). **e:** The lymphatic network (m) in the mucosa is connected across the isthmus by straight lymphatic vessels continuous with the lymphatic plexus (if) in the parafollicular area. The lymphatic plexus in the parafollicular area is continuous with the upper ends of the perifollicular lymphatic sinuses (s). The perifollicular lymphatic sinuses are not continuous along the entire wall of each follicle, but are much more voluminous than those of Peyer's patches. Scale bars = 500 μ m (d, e)

Appendix

The appendix is also a representative of GALT that responds to luminal antigens (Bockman, 1983). It is now generally accepted that in the parafollicular area, lymphocytes migrate into the lymphatic vessels and migrate out of the HEVs. Although some early authors did describe the lymphatic system of the appendix, we have examined its three-dimensional organization by corrosion casting/scanning electron microscopy (Ohtani *et al.*, 1986). As in the Peyer's patches and in human palatine tonsil (Fujisaka *et al.*, 1996), in the parafollicular area, there is a plexus consisting of anastomosing tubular lymphatic vessels that lead into the upper ends of the perifollicular sinuses (Fig. 9e). A polygonal network of lymphatic vessels in the glandular mucosa leads into perpendicularly oriented lymphatic vessels continuous with the lymphatic vessels in the parafollicular area. Each follicle is surrounded by a well-developed perifollicular sinus, which is much more voluminous than that in the Peyer's patch (Fig. 9e). From the bottoms of the sinuses, thin lymphatic vessels descend into the submucosal collecting lymphatic vessels. The voluminous perifollicular lymphatic sinuses seem to have great potential capacity as a reservoir and as a drainage route for fluid and lymphocytes. The well-developed lymphatic network in the glandular layer suggests extensive participation in fluid flux.

Human palatine tonsil

Human palatine tonsils are located in the fauces, i.e., in the front line of mucosa-associated lymphoid tissues. Being thus strategically placed for protection of the body against invasion by bacteria and foreign proteins, they must readily uptake and recognize antigens and start appropriate immune reactions (Oláh, 1978; Owen and Nemanic, 1978). As in the Peyer's patches and appendices, lymphocytes pass from the blood *via* the endothelium of HEVs into the parenchyma of the tonsil and then return to the blood *via* lymphatic vessels.

The microvascular organization of the human palatine tonsil has been revealed by scanning electron microscopy of microvascular corrosion casts (Ohtani *et al.*, 1989) (Fig. 10a). The tonsillar arteries travel in the connective tissue septa and give off many branches. They further branch into arterioles, which in turn enter the follicle and the parafollicular region. These arterioles, giving off capillaries *en route*, reach the subepithelial region where they break up into sinusoidal capillaries. The

subepithelial capillary network overlying the follicle protrudes hemispherically towards the crypt, while that overlying the parafollicular region has many switch-back loops of capillaries projected towards the crypt. The subepithelial sinusoids gather into the HEVs which, collecting capillaries in the follicle and the parafollicular region *en route*, course down into the parafollicular region alongside the follicle. The HEVs ultimately lead into the ordinary veins in the septa. The microvascular organization of the human palatine tonsil is schematically shown in Figure 10a.

The distribution of lymphatic vessels in human palatine tonsils has been studied by scanning electron microscopy of lymphatic corrosion casts and enzyme-histochemistry to 5'-nucleotidase (Fujisaka *et al.*, 1996) (Fig. 10b, c) and is schematically shown in Figure 10d. Tubular lymphatic vessels originating some 200–300 μm below the epithelium form a three-dimensional network in the parafollicular region. Some lymphatic vessels around the lower part of the follicle are flat, wide, and irregular in shape, and thus appear to be lymphatic sinuses — to be referred to as perifollicular lymphatic sinuses. The lymphatic vessels in the parafollicular region drain into those in the connective tissue septa, which run rather straight. The lymphatic vessels in the septum finally gather into the broader capsular lymphatic vessels. The voluminous lymphatic vessels in the palatine tonsil appear to have great potential to collect lymphocytes and fluids from the parenchyma of the tonsil.

Developmental aspects of lymphatic vessels

Our previous studies by enzyme-histochemistry to 5'-nucleotidase and transmission- and scanning-electron microscopy show that the lymphatic vessels of the rat diaphragm develop primarily by sprouting from pre-existing lymphatic vessels during fetal and early postnatal periods (Shao *et al.*, 1998). Our recent study by immunohistochemistry to a lymphatic specific marker, LYVE-1, shows that prior to the formation of tubular vessels, numerous LECs independently migrate in line to the place where lymphatic vessels are expected to be formed both in the subpleural and subperitoneal spaces (Fig. 11). The LECs sometimes form lymphatic islands independent from pre-existing lymphatic vessels (Ohtani and Ohtani, 2001) (Fig. 11). The islands and/or single LECs in line subsequently join together and connect with pre-existing lymphatic vessels, thereby developing a lymphatic network (Fig. 11). This may indicate that lymphatic vessels are formed by joining migrating LECs, in addition to simple sprouting. Rutkowski *et al.*

Fig. 10. Blood and lymphatic vascular organization of the human palatine tonsil. **a:** A schematic presentation of the microvascular organization of the human palatine tonsil. The area shaded with smaller dots represents a germinal center (GC), and the one with larger dots is a dark shell (or mantle zone) (M). The dense capillary network drawn in the upper one third of the picture is the subepithelial sinusoidal capillary network. Vessels colored in red are arteries or arterioles, and those in blue are veins and venules. Veins shaded with dots represent HEVs. **b:** A scanning electron micrograph of the lymphatic corrosion cast of the human palatine tonsil. The tubular lymphatic vessels, forming a network in the parafollicular area (P), are gathered into the broader lymphatic sinuses (S) surrounding the lower part of the follicle (F). (Continued on the next page ↗)

Fig. 11. Confocal micrographs showing the process *in situ* of the formation of lymphatic vessels in the pleural side of the rat diaphragm. Initially, LECs migrate in line (a) and later join to form lymphatic vessels (b, c). Lymphatic islands (arrowheads) can be seen in b. As the lymphatic vessels develop, isolated LECs gradually disappear. The lymphatic vessels seen in d are similar to those seen in adults. Immunohistochemistry for LYVE-1. **b:** Stereo-pair. Scale bars = 50 μ m

(2006) also report that LECs migrate in the direction of interstitial fluid flow as single cells that later join together into vessels during skin regeneration in adult mice.

Little attention has been given to the development of smooth muscle of lymphatic vessels, except for a few studies (Ohtani and Ohtani, 2001; von der Weid and Zawieja, 2004). Figure 12 shows some representative morphological changes during the formation of collecting lymphatic vessels endowed with smooth muscle cells (see also Fig. 1c–e). Towards the end of postnatal week

2, free cells expressing α -smooth muscle actin (α -SMA) immunoreactivity increase in the rat diaphragm. A coarse plexus of smooth muscle cells surrounding the lymphatic vessels first appear at postnatal week 2, and this plexus become denser with age. The smooth muscle cells of the lymphatic vessels seem to be differentiated from mesenchymal cells to form a plexus surrounding the lymphatic vessels, thereby forming the collecting lymphatic vessels.

Except for lymphangiogenic growth factors and their

✂ **Fig. 10. c:** A light micrograph of the human palatine tonsil reacting to 5'-nucleotidase: cryostat section of 40 μ m thick. The tubular lymphatic vessels (arrowheads) originate some distance below the epithelium (E) and partially surrounding the lymphoid follicles (F). They drain into the lymphatic vessels (arrows) in the septum. No lymphatic vessels can be seen in the subepithelial region. **d:** A schematic presentation of the lymphatic organization in the human palatine tonsil. E epithelium, LS epithelium infiltrated with lymphocytes (lymphoepithelial symbiosis), GC germinal center, M mantle zone (or dark shell), S septum. (Micrograph **a:** from Ohtani *et al*, 1989; **b–d:** from Fujisaka *et al*, 1996.)

Fig. 12. Confocal micrographs showing α -SMA immunoreactive cells in the rat diaphragm. **a:** At postnatal day 1, there are many fusiform α -SMA positive cells (green). **b:** Some α -SMA positive cells (arrows, yellow) are in contact with lymphatic vessels (L) labeled with DiI-acetyl LDL (red). DiI-acetyl-LDL labeled cells show immunoreactivity for rabbit polyclonal antibodies to VEGFR-3 (M-20, Santa Cruz Biotechnology, California), but not to mouse anti-rat dendritic cells (OX62, Serotec, Raleigh, UK) or mouse anti-rat macrophages/monocytes (ED1, BMA Biomedicals AG, Augst, Switzerland), suggesting that they are not macrophages but lymphatic endothelial cells. There are also isolated α -SMA positive cells (arrowheads). Postnatal day 10. **c:** Irregularly shaped α -SMA positive cells (S) are attached to a lymphatic vessel labeled with DiI-acetyl LDL (red). Postnatal day 14. **d:** A coarse network of α -SMA positive cells with long, slender processes (S, yellow) surrounding a lymphatic vessel labeled with DiI-acetyl LDL (red). Postnatal week 3. **e–f:** Different segments of collecting lymphatic vessel endowed with α -SMA positive cells. **e:** Initial segments of the collecting lymphatic vessels are surrounded by a sparse network of α -SMA positive cells. **f:** As the collecting lymphatic vessels become thicker, the network of α -SMA positive cells surrounding the vessels becomes denser. **e–f:** Postnatal week 6.

Fig. 13. Confocal (a–c, f–h) and scanning electron micrographs (d, e). **a–c:** LECs were harvested from the thoracic duct of either normal SD rats (a, b) or the green rats* (c). The LECs cultured on wells coated with type I collagen in an ordinary incubator containing 5% CO₂ and air (a) show a wide but thin configuration, and did not grow in layers, but those in a low oxygen condition (O₂ 5.0%) have proliferated in layers (b). The LECs cultured on Cell Culture Inserts® in an incubator with low (5% O₂) oxygen concentrations show tube-like aggregations. The LECs in a show LYVE-1 immunoreactivity (IR), those in b show VEGFR-3 IR, and those in c show GFP (green) and LYVE-1 IR (yellow). **d:** SEM of the LECs cultured on Cell Culture Inserts® in an incubator with low (5% O₂) oxygen concentrations show the LECs have actually formed tube-like lymphatic vessels. **e:** SEM view of a collagen fiber sheet of the human thoracic duct prepared by the alkali-water maceration technique. The collagen fiber sheet preserves the natural three-dimensional structure. (The thoracic duct was obtained from a human cadaver donated for research and the teaching of anatomy at the Faculty of Medicine, University of Toyama.) **f:** When the LECs are cultured on a collagen sheet from the human thoracic duct, they proliferate rapidly and form a three-dimensional network. The LECs are labeled with DiI-acetyl-LDL. **g, h:** When the LECs with GFP are implanted into the peritoneal cavity of newborn SD rats, they enter lymphatic vessels in the diaphragm. Some of them appear to be incorporated into the lymphatic vessels (g), while others form aggregations (h). The lymphatic vessels and implanted LECs show LYVE-1 IR. The antibodies used are rabbit polyclonal to LYVE-1 (ab 14917, Abcam, California) and rabbit polyclonal to VEGFR-3 (Flt-4) (M-20, Santa Cruz Biotechnology, California). Scale bars = 50 μm (a, b), 100 μm (c), 10 μm (d, e), 25 μm (f), 100 μm (g, h)

receptors, little is known about the microenvironment that promotes lymphangiogenesis both *in vivo* and *in vitro*. We harvested LECs from the thoracic ducts of either normal Sprague Dawley (SD) rats or SD-Tg(CAG-EGFP) rats (green rats; Amersham, Tokyo) and cultured them in culture media (HuMedia-EG2, Kurabo Industries, LTD, Osaka) in a variety of conditions. Western blotting and immunohistochemistry revealed that the cultured LECs express lymphatic specific markers, including LYVE-1, and VEGFR-3. LECs cultured in an incubator with air containing 5% CO₂ proliferate much more slowly than those in an N₂-O₂-CO₂ incubator with low oxygen concentrations (5% O₂) (Mizuno *et al.*, 2003). The former LECs are thin and large and in a single layer, while the latter are smaller and stratified (Fig. 13a, b). When cultured on Cell Culture Insert® (BD Biosciences, Billerica, MA) in an incubator with low oxygen concentrations, LECs proliferate in layers and sometimes form tube-like structures with LYVE-1 immunoreactivity (Fig. 13c). Scanning electron micrographs show the tube-like structures are in fact tubes (Fig. 13d). Interestingly, LECs cultured on collagen fiber sheets obtained from the human thoracic duct, aorta, or diaphragm rapidly form three-dimensional networks of lymphatic vessels (Fig. 13e, f). The structures have been confirmed to be tubes by SEM and to express LYVE-1 immunoreactivity. A collagen fiber sheet prepared by the alkali-water maceration technique preserves the three-dimensional organization of collagen fibers in their natural form (Ohtani, 1987a, Ohtani *et al.*, 1988). These findings suggest that appropriate scaffolds would play an important role in LEC proliferation and tube formation. When the LECs were implanted into the peritoneal cavity in neonatal SD rats, some of them passed through the lymphatic stomata into the lymphatic vessels in the diaphragm while others remained in the peritoneal cavity and proliferated there to form tubular vessels. Some of the LECs entering the diaphragmatic lymphatic vessels appeared to be incorporated into the developing lymphatic vessels of the host, but others formed granules within the host lymphatic vessels (Fig. 13g, h). These data appear to suggest a possibility that the implantation of LECs may be useful to promote lymphangiogenesis *in vivo*. As our experiments are preliminary, further extensive studies are mandatory to clearly define environments that promote lymphangiogenesis.

Lymphedema is defined as tissue swelling due to the accumulation of protein-rich interstitial fluid as a consequence of insufficient lymph transport owing to lymphatic hypoplasia, impaired lymphatic function, or an obstruction of lymph flow. Hereditary lymphedema is rather rare (Greenlee *et al.*, 1993). Recently, some genetic

bases for hereditary lymphedema have been revealed (Fang *et al.*, 2000; Irrthum *et al.*, 2000; 2003; Karkkainen *et al.*, 2001; Witte *et al.*, 2001; Saaristo *et al.*, 2002). There is, however, a growing prevalence of acquired lymphedema of the extremity, which is ascribable almost exclusively to the increasing numbers of patients submitted for breast and pelvic cancer surgery (Szuba and Rockson, 1998). Surgical removal of lymph nodes in the treatment of patients with cancer may lead, after a period of time, to the occurrence of lymphedema. Most patients with lymphedema are treated conservatively by means of various compression therapies, including massage, pneumatic compression, and compressive garments. Lymphatico-venular anastomoses by supermicrosurgery (Koshima *et al.*, 2000; 2003) have been reported to be effective in the treatment of lymphedema — though it remains to be fully determined why lymphatico-venular anastomoses are effective in lymphedema treatment. Boardman and Swartz (2003) have reported that interstitial fluid channels formed before lymphatic endothelial cell organization in a mouse tail lymphedema model, suggesting that interstitial fluid flow may play a role in lymphangiogenesis. Reportedly, injection of VEGF-C in a surgical lymphedema model in the rabbit ear successfully regenerates a lymphatic network (Szuba *et al.*, 2002). The reconstruction of interrupted lymphatic vessels, and thereby recruiting lymph flows sufficient to maintain interstitial fluid balance, is a great challenge for preventing the disabling condition of lymphedema.

Conclusion

The present paper reviews the general structure of lymphatic vessels and the organization of the lymphatic vascular system of a variety of organs, including the thyroid gland, lung and pleura, diaphragm, small intestine, cecum and colon, Peyer's patches, appendix, and tonsil. Immunohistochemistry to lymphatic-specific markers such as LYVE-1 has confirmed our previous findings obtained by SEM of lymphatic corrosion casts, SEM of tissues, TEM of intact tissues, and light microscopy of histochemically stained samples. This review serves for a better understanding of the organization of the lymphatic vessels in normal conditions, which is critically important to develop new treatments for diseases involving the lymphatic vascular system. This paper also reviews some developmental aspects of lymphatic vessels. In the diaphragm, lymphatic vessels develop primarily by sprouting, but prior to the formation of tubular vessels, numerous LECs independently migrate to the place

where lymphatic vessels will form. LECs harvested from the rat thoracic ducts proliferate faster in a low oxygen environment than in a normal one. When cultured on collagen fiber networks with natural organization, LECs rapidly form a lymphatic plexus. The reconstruction of interrupted lymphatic vessels, and thereby recruiting lymph flows sufficient to maintain interstitial fluid balance, is a great challenge for preventing the disabling condition of lymphedema.

Notes

The Transgenic Animals are covered by one or more of the following patents: 1) US 6 172 188, EP 851874, US 5 958 713, and EP 0815257, and under international patent application PCT/EP01/06848 and other pending and foreign applications; and 2) US 5 625 048, US 5 777 079, US 5 804 387, US 5 968 738, US 5 994 077, US 6 054 321, US 6 066 476, US 6 077 707, US 6 090 919, US 6 124 128, US 6 319 669, US 6,403,374, EP 0804457, EP 1104769, JP 3283523, and other pending and foreign patent applications.

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