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*Cellular and Molecular Biology*

## *Invited Review*

# Organ specificity of the structural organization and fine distribution of lymphatic capillary networks: Histochemical study

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**Summary.** Histochemical studies of the microcirculatory system were reviewed with regard to the organ specificity of the structural organization and fine distribution of the lymphatic capillary network. The lymphatics and blood vessels are characterized by an enzyme-histochemical method using 5'-nucleotidase (5'-Nase), alkaline phosphatase (ALPase) and/or diaminopeptidase (DAPase) staining in additon to an immunohistochemical method. The 5'-Nase-positive lymphatic vessels can be distinguished histochemically from arterial and venous vessels based on ALPase and DAPase activity, respectively. The specificity and localization of the enzyme reactions were confirmed by comparative histochemical studies of the same specimen with light microscopy and scanning or transmission electron microscopy. These histochemical methods are discussed in relation to their ability to demonstrate the organ specificity of vascular networks under normal and pathological conditions.

**Key words:** Lymphatics, Histochemistry, Alkaline phosphatase, 5'-Nucleotidase, Diaminopeptidase, Blood capillary, Microcirculation

## 1. Introduction

In the processes of ontogeny, the lymph vascular system play a secondary, later role to the blood vascular system in absorbing or draining substances and tissue fluids, as the system and organs become more complicated, and thereby plays an important role, along with the blood vascular system, in the maintenance of homeostasis in the body. The structural organization and fine distribution of lymhatic vessels in the tissues are very important in the pathological physiology of a variety of microcirculatory disorders, infectious diseases and cancer. For this purpose, it is necessary to identify lymphatics and blood capillaries and to understand the characteristics of their structural organization and fine distribution. Numerous light microscopical or electron microscopic observation methods have been reported in the morphological study of lymphatic vessels. However, none of them has clearly distinguished lymphatic capillaries from blood capillaries; therefore such difficulty in the identification of lymphatic capillaries is a bottle neck in the study of the lymphatic system. In this article, we shall give an outline of the organ specificity of the lymphatic capillary network within each organ, taking note of the relevance of the blood capillary network from our new histochemical approach that is different from conventional observation methods.

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## 2. Fine structure of lymphatics - Historical background

Since the terminal lymphatic capillaries (ie. initial lymphatics, Casley-Smith, 1970) of the lymph vascular system are not uniform in size and their lumens are shaped irregularly, it is difficult to distinguish them from tissue spaces or blood capillaries. While the lumens of blood vessels are not cloudy in the tissue where blood vessels have been perfused, because the blood components have been washed out, those of lymphatics are quite often accompanied with nebulous substances (lymph fluid) (Marchetti et al., 1985; Shimada et al., 1990). It has been shown three-dimensionally by scanning electron microscopical (SEM) observation methods such as lymphatic corrosion cast (Ohtani and Ohtsuka, 1985; Castenholz, 1986, 1987; Ohtani et al., 1986; Magari, 1990; Ohtani and Murakami, 1990, 1992; Yamanaka et al., 1995; Sugito et al., 1996) and alkaline digestion of connective tissue (Ushiki, 1990; Ohtani, 1992) that the lymphatic capillaries initiate from incomplete blind ends. The walls of lymphatic capillaries with monolayered flat endothelial cells are very thin except at nuclear sites. Regarding the boundaries of endothelical cells, there are such modalities as 1) end-to-end, where both ends of the cells are adhered; 2) overlapping, where one end overlaps the other; and 3) interdigitating, where adhered parts intervene mutually like forks, and the adherence is

characterized by the fact that there are many parts where the cells are more or less separated (Shimada et al., 1990; Ji et al., 1996). Marking substances such as carbon particles (Noguchi et al., 1988; Yasunaga et al., 1991a; Ji et al., 1996), fluorescent beads (Fig.1a) and low viscosity resins (Ohtani et al., 1986; Ohtani and Murakami, 1992; Fujisako et al., 1996), introduced by intra-parenchymal puncture injection into tissue spaces can be absorbed readily into lymphatic capillaries. Also, silver nitrate solution (Mori, 1969) or low viscosity resins (Ohtani and Murakami, 1987) injected into blood vessels leak out and enter into surrounding lymphatic capillaries through connective tissue spaces. Such phenomena are more remarkable in younger individual, since the blood vessel structure is still underdeveloped.

Lymphatic capillaries are deficient of pericytes, with the immediate basal sides of endothelial cells being very rough, and lacking the basement membranes or with only undeveloped basement membranes(Casley-Smith,1980; Oh et al., 1997). Microfibrils called anchoring filaments (Leak and Burle, 1968; Leak, 1970; Lauweryns et al., 1976), ca. 8nm in diameter, exist in the sites where the basement membranes are lacking. These microfibrils, unlike collagen fibers in the surrounding connective tissues, consist of oxytalan fibers and continue as elastic fibers around lymphatic vessels (Bock, 1978; Solito et al.,1997). It is said that the amount of anchoring filaments varies by tissue (Fraley and Weiss, 1961; Fawcett et al., 1969), and it is believed that they play a role of fixing lymphatic capillaries to the surrounding connective tissues, and protecting walls from excessive swelling, as well as supporting lymphatics so they are not flattened when tissue pressure is enhanced (Casley-Smith, 1968, 1970).

Valves are available to prevent reverse flow of lymph fluid in the collecting lymphatics with a diameter more than ca.150nm, which collect lymph fluid from lymphatic capillaries. At the adhering part of the valves, there are intervening layers with thin connective tissues, entering between the endothelial layers to support valves. As lymphatics are a little swollen at the location of valves, they show an appearance of rosary beads. Elastic fibers exist on the outer periphery of lymphatic capillaries, but smooth muscles appear like rings only after collecting lymphatics (Ohtani, 1992). Incidentally, it is believed that most lymphatics seen in the sub-membranous tissues of intestinal tracts are collecting lymphatics which possess valves and smooth muscles.

## 3. Enzyme histochemistry

### *Light microcsopy*

When studied histochemically, the enzyme activities of phosphatases in the walls of lymphatics are clearly different from those in the blood vessels, with the activities of 5'-nucleotidase (5'-Nase) (Fig. 1b), adenylate cyclase and guanylate cyclase being remarkably high in the lymphatics but low in the blood vessels (Vetter, 1970; Ohkuma and Nishida, 1987; Werner et al., 1987). On the other hand, the activities of alkaline phosphatase (ALPase) and dipeptidyl aminopeptidase (DAPase) are low in the lymphatics but high in the blood vessels. Especially, ALPase activity is high in arterioles and arterial capillaries (Kato et al., 1993a, 1996; Ushiki and Abe, 1998), while DAPase

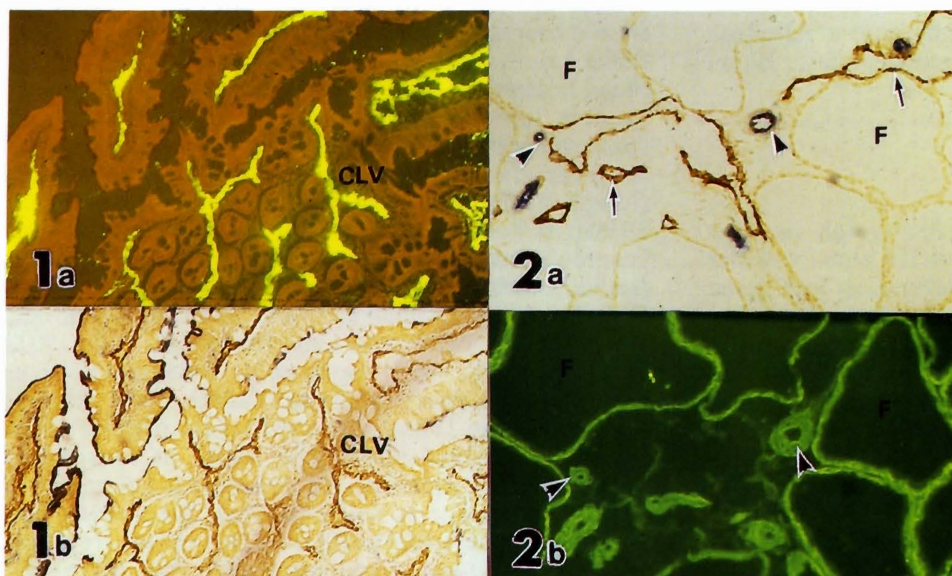


**Fig.1.** Light micrographs of a cryosection of the monkey ileum stained with 5'-Nase staining after intra-parenchymal puncture injection with fluorescent beads. **a.** Fluorescence micrograph. Central lymphatic vessels (CLV) are colored green due to the fluorescent beads absorbed. **b.** 5'-Nase activity of the same preparation stained with 5'-Nase staining, as in Figure 1a. This shows that the CLV in the intestinal villi, which absorbed the fluorescence, revealed 5'-Nase activity. x 150

**Fig. 2.** Serial cryosections of the monkey thyroid gland stained with enzyme-histochemical (a) and immuno-histochemical (b) staining. **a.** 5'-Nase-ALPase double staining. 5'-Nase-positive lymphatics (dark brown, arrows) are clearly distinguished from ALPase-positive blood capilllaries (blue, arrowheads) in the interfollicular connective tissues. F: Follicle. **b.** FITC-immunofluorescence staining for type IV collagen in the basement membranes. The lymphatics, which lack distinct continuous basement membranes, are not colored, whereas blood capillaries (arrowheads) and follicular epithelial cells surrounding follicles are stained green at the basement membrane. x 150.

activity is high in venules and venous capillaries (Lojda, 1979; Grim et al., 1986; Kato et al., 1993a, 1996). As seen in these examples, although these enzymatic activities are variable depending on the thickness and property of vascular vessels in both the lymphatic capillaries and blood capillaries (Kato et al., 1993a, 1996), the difference in 5'-Nase activity is the most remarkable and distinct, as far as the lymphatic capillaries and blood capillaries are concerned. Both vascular vessels can be stained distinctively by 5'-Nase-ALPase double staining (Figs. 2a-4), which takes advantage of the difference in the enzymatic activities (Werner et al., 1987; Kato and Miyauchi, 1989; Kato, 1990a,b; Werner and Schunke, 1989; Kato et al., 1991). Furthermore, the identification of the lymphatics, arteries and veins can be achieved to some extent through 5'-Nase- DAPase-ALPase triple staining (Kato et al., 1996).

An important point regarding 5'-Nase reaction is to add to the reaction medium, *L*-tetramisole, an inhibitor

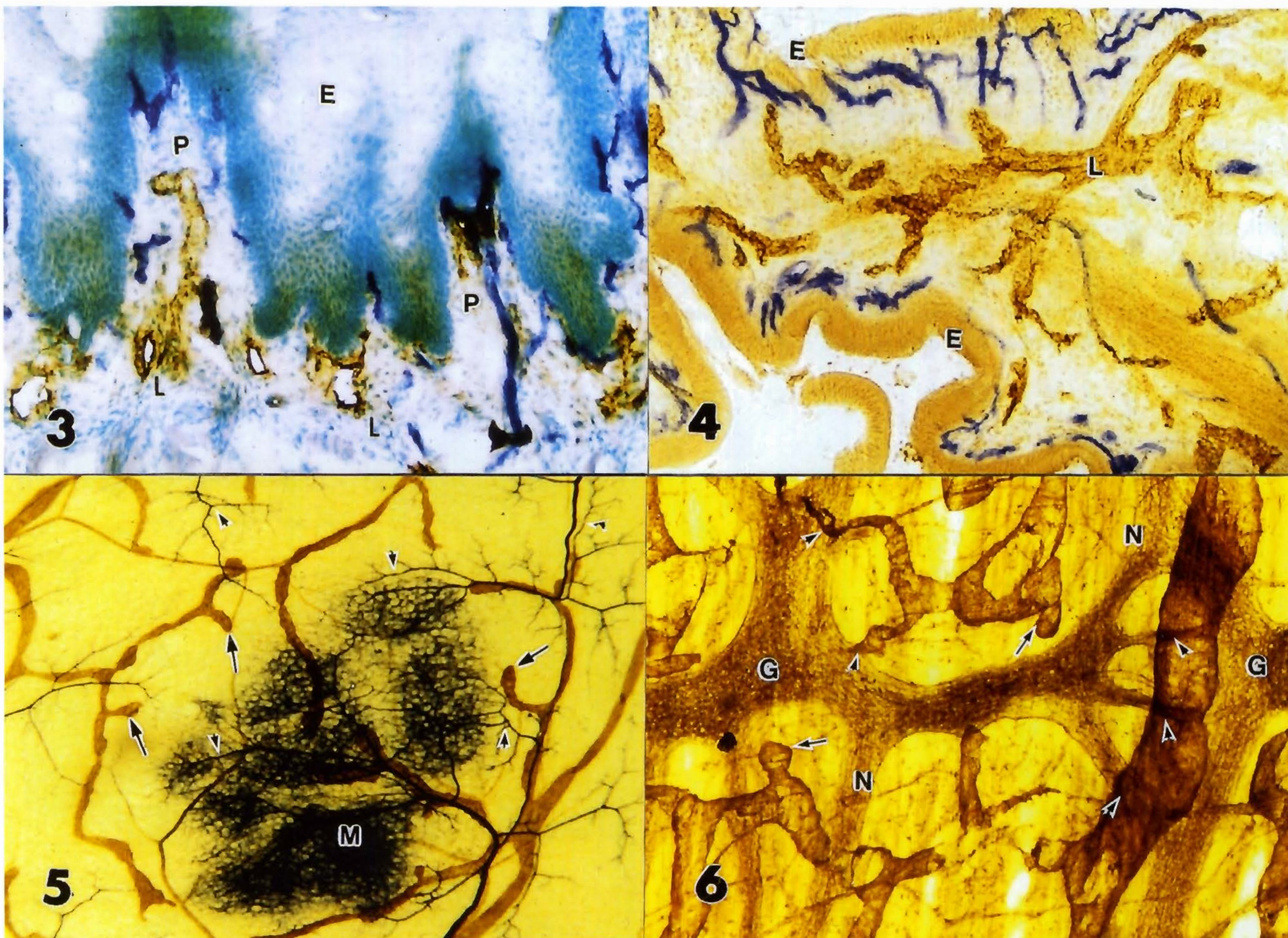


**Fig. 3.** Cryosection of the monkey tongue stained with 5'-Nase-ALPase double staining followed by methyl green-counterstaining. Note ALPase-positive blood capillaries (blue) and 5'-Nase- positive lymphatics (L, dark brown) in the papillary layer (P) of the lamina propria mucosa. E: epithelium. x 150

**Fig. 4.** Cryosection of the human gallbladder stained with 5'-Nase-ALPase double staining. The 5'-Nase-positive lymphatics ( L, dark brown) are clearly seen surrounded by ALPase-positive capillary networks (blue) in the villous-like subepithelial layer. E: epithelium. x 300

**Fig. 5.** Whole-mount preparation of the mesenterium of the monkey colon stained with 5'-Nase-ALPase double staining. Each 5'-Nase-positive initial lymphatics appears tubulo-saccular shaped (brown, arrows), whereas blood vessels (arteriols and arterial capillaries) are colored dark blue (arrowheads). M: milky spot. x 45

**Fig. 6.** Whole-mount preparation of the myenteric layer of the monkey jejunum treated with 5'-Nase-AChE double staining. A well-developed network of 5'-Nase-positive lymphatics (dark brown) is demonstrated in addition to a dense nerve plexus (N: Auerbach's plexus, light brown) with AChE activity. Many blind endings (arrows) in the apical parts of the lymphatics cluster around the ganglia (G). AChE-positive nerves attach to the wall of a 5'-Nase-positive lymphatics (arrowheads). x 50

of non-specific ALPase, at a low concentration not influencing 5'-Nase activity (DePierre and Karnovsky, 1974), for the purpose of detecting specific 5'-Nase activity, because 5'-adenosine monophosphate used as a substrate can be a substrate of non-specific ALPase reactions, too (Kato et al., 1991). Since it is generally known that there are tissue differences or species differences, including humans, in enzymatic activities (Kato et al., 1993a, 1996), it is important to conduct staining under as uniform conditions as possible in consideration of these factors when visualization of lymphatics is attempted using this method.

This enzyme histochemical method can be applied to frozen tissue sections (Werner et al., 1987; Kato and Miyauchi, 1989; Kato, 1990a,b) or embedded tissue sections using soft paraffin melting at low temperatures (44-48 °C) (Okada, 1992, 1994; Kato et al., 1993a) or low temperature polymers (JB-4) (Kato et al., 1991, 1993a, 1996). The resin section method is excellent in the preservation of tissues. It is convenient in not mistaking lymphatic lumens for tissue spaces, and excellent in terms of sample preparation and reproducibility (Kato et al., 1991, 1993a). As the resin embedding gives clear tissue structures, toluidine blue staining of its semi-thin sections can identify lymphatic capillaries to some extent (Kubo et al., 1989), but, more reliable results are obtained in the same field of light microscopy by using the enzymatic double staining distinction method of both vascular vessels. When thick frozen tissue sections are stained for 5'-Nase activity, profiles appearing like leaves of an oak tree (Kato, 1990c), which are peculiar to the lymphatic endothelial cells, become clear, resembling those obtained by the conventional intra-arterial injection of India ink plus silver nitrate (Mori, 1969).

On the other hand, in the extension method (whole-mount preparation), unfixed membranous tissues (mesenterium, pleura and diaphragma) or walls of digestive organs are extended as they are on a plastic plate, fixed and immersed in 5'-Nase reaction medium and ALPase reaction medium, respectively (Fig. 5). The distribution of both vascular vessels in the subserosal layer are visualized over a wide range and the distribution conditions can be observed well by light microscopy (Masada et al., 1992; Kato et al., 1993b, 1994a,b; Shao et al., 1998). The lymphatic capillary network thus visualized often has valve-like constrictions, and appears like beads of a rosary. Further study is necessary to determine if such constrictions are the same as the valves of collecting lymphatics.

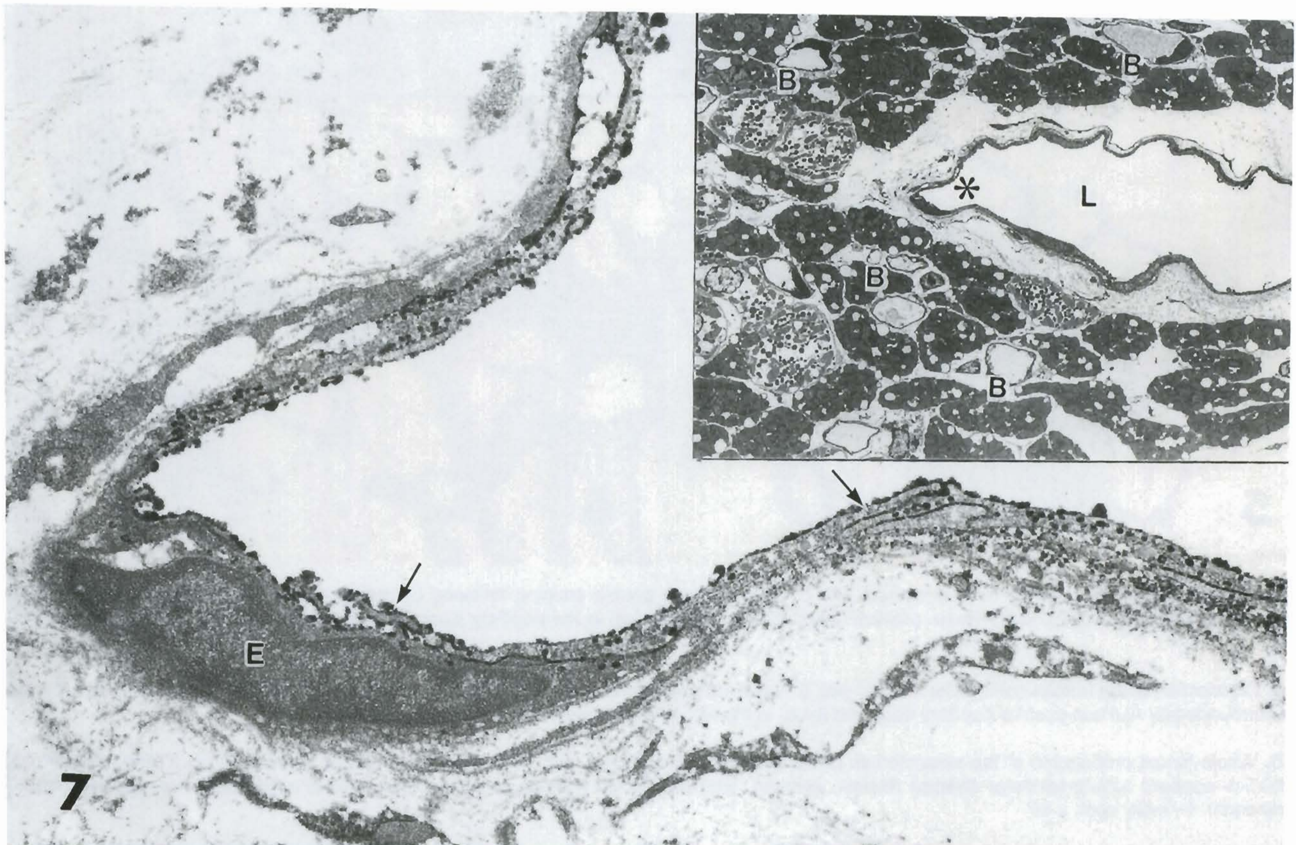


**Fig. 7.** TEM views of 5'-Nase-positive lymphatics (L) and 5'-Nase-negative blood capillary (B) on the lingual side of the monkey epiglottis treated with the cerium based 5'-Nase reaction medium. Higher magnification of the area indicated with asterisk in lower magnification (inset, x 1200 ). The dense granular precipitations (arrows) are noticed on the luminal and abluminal surfaces of lymphatic endothelial cells (E) and pinocytotic vesicles. x 18,000

However, such a valve-like structure is presumed to play a role in preventing not only reversed flow of lymph fluids from occurring in the thin wall layers or membranous organs but also excessive expansion or closing of lumens due to outer pressure. Also, extended and fixed tissues, which are ablated into mucous membranes, muscular layers and serous membranes by a manual method or by immersion in 2N sodium bromide solution, and double stained as above to find out the structural organization and fine distribution of vascular vessels and nerve plexus in the luminal wall layers can be seen very well (Fig. 6) (Ji et al., 1996; Ji and Kato, 1997a; Shimoda et al., 1997, 1998; Kato et al., 1998).

*Electron microscopy*

Reaction products in the endothelial layer of the lymphatics recognized as 5'-Nase-positive images in light microscopy are detected on the surface of cell membranes of the luminal side of the endothelial cells by transmission electron microscopy (TEM), and also often in pinocytotic vesicles or on the surface of membranes of the basal side. In light microscopy of 5'-Nase activity by the lead method, lead phosphate, a reaction product, is detected as lead sulfide in the color reaction using ammonium sulfide. On the other hand, as cerium phosphate produced in the cerium method is not colored with ammonium sulfide, it is necessary to convert it into a lead compound by using silver sulfide. However, the reaction image of 5'-Nase obtained by the cerium method is finer than that by the lead method, consisting of granular-like continuous reaction products that appear on the membranous surface of the endothelial cells (Fig. 7). Accordingly, the cerium method is more convenient in showing the localization of reactions by TEM (Kato, 1990c).

In the observation of the tissue sections, tissue blocks and extended specimens using the related SEM backscattered electron images (BEI) of 5'-Nase staining (metal method) subsequent to freeze drying and vacuum evaporation of carbon, the reaction positive sites of the lymphatic endothelia can be seen shining brightly due to strongly backscattered electrons (BEs) from the metals of the reaction products (Fig. 8). As non-reactive sites by 5'-Nase are visualized only dimly because of very weak BEs, the stereoscopic localization of reactions can be

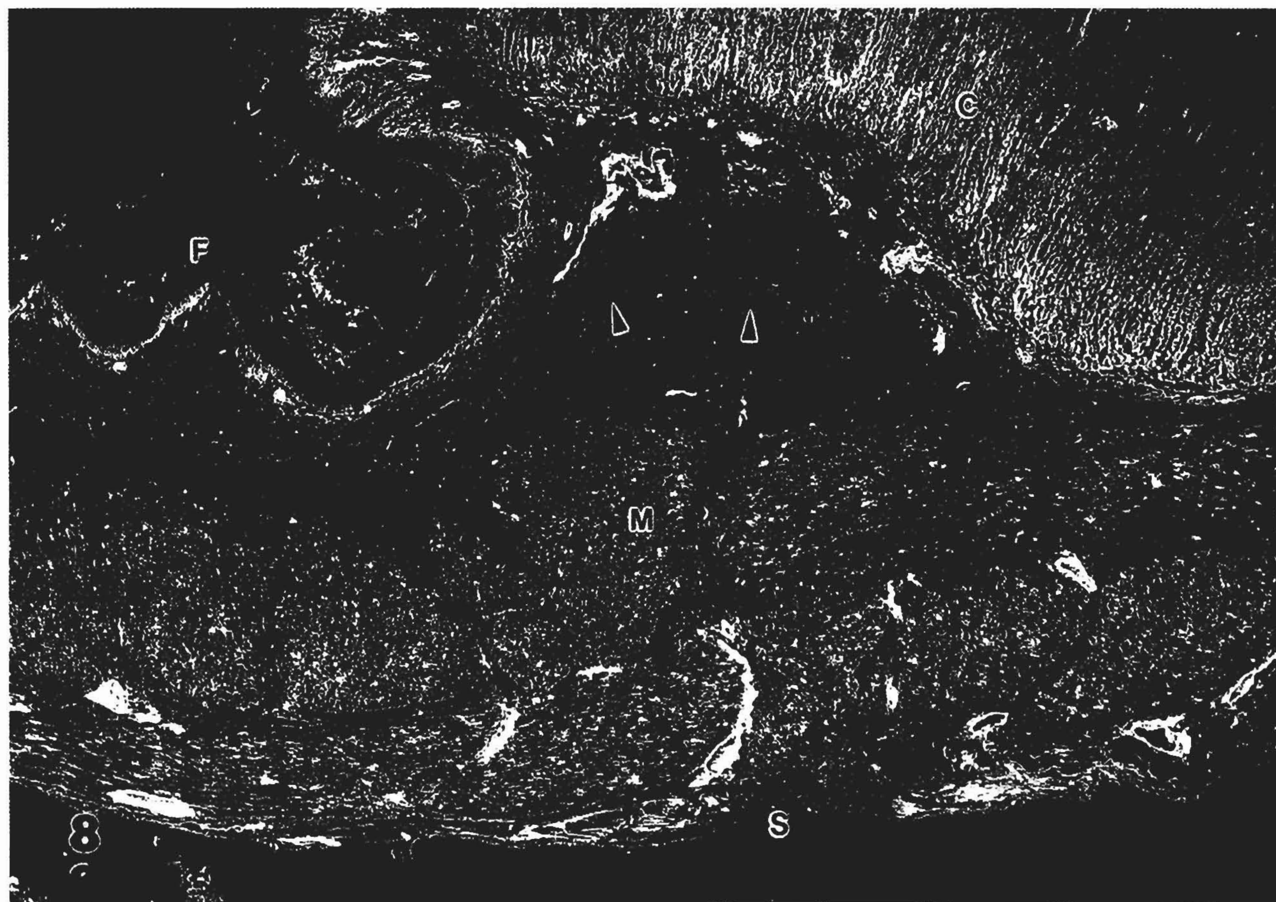


**Fig. 8.** Backscattered imaging (BEI)-SEM of the residual tissue block of the rat stomach, after cutting a cryosection, stained with 5'-Nase staining (lead method). The 5'-Nase-positive lymphatics are strongly highlighted as compared with 5'-Nase-negative blood vessels (arrowheads) in each layer of the gastric wall. F: Fundus; C: Corpus; M: Muscle layer; S: Serosa. x 700

estimated by comparative observation of the secondary electron images (SEI) in the same field and from synthetic profiles of the SEI and BEI. Incidentally, because the strengh of BEs is parallel to the atomic numbers, lead (Pb82, mass 207.19) has stronger BE energy than cerium (Ce58, mass 140.12), the observation of 5'-Nase reaction using the lead method is more effective (Fig. 9). Thus, the method is convenient for observation of enzymatic reactions, etc., of cells and tissues, as it is possible to observe the surface structure of the SEI and the metallic sites seen by the BEIs in the stained SEM specimens with a heavy metal according to the histochemical SEM method. Up to date, comparative observation of 5'-Nase stained electron profiles of lymphatics of light microscopic sections and the adjacent SEM-BEI of the remaining tissue block have been performed, as well as comparative observations of light microscopic profiles of 5'-Nase-positive lymphatic network and those of SEM-BEI in the same field of thick sections and extended specimens (Kato, 1990a; Kato and Gotoh, 1990; Kato et al., 1993b, 1998).

## 4. Immunohistochemistry

The immunohistochemical method is one of the most reliable methods in terms of specificity and manual procedures. As lymphatic capillaries are deficient of clear continuous basement membranes, while most blood vessels, including capillary vessels, possess clear basement membranes, it is possible to distinguish non-reactive lymphatics by staining and visualizing the blood vessels using ant-type IV collagen antibody (Fig. 2b) or laminin, a constituent of basement membranes. So far, factor XIII antigen (FXIII), *Ulex europaeus* lectin (UEL) or angiotensin converting enzyme (ACE) which is a marker of several endothelia, has been studied histochemically as to the extent of expression in the lymphatic endothelia (Gnepp, 1987; Nagle et al., 1987; Kubo et al., 1989; Otsuki et al., 1990). Histochemical observations have been performed on actin and vimentin in the microfilament of lymphatic endothelial cells (Ito, 1991). However, since the expression amount of these markers is not uniform by sites, and the expression in the

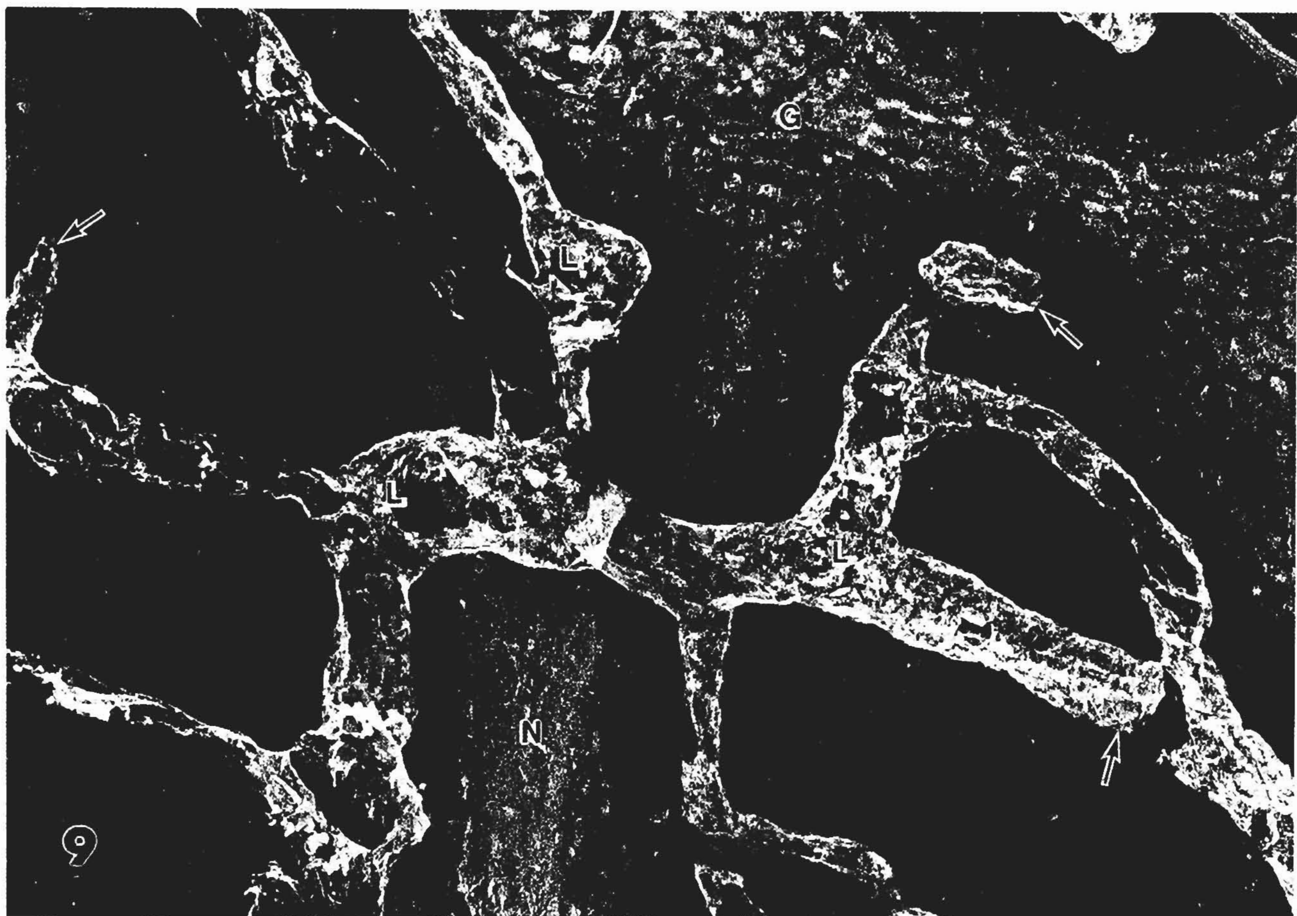


**Fig. 9.** Backscattered imaging (BEI)-SEM of the whole-mount preparation from the myenteric layer of the monkey jejunum treated with 5'-Nase-AChE double staining for lymphatics and nerve plexus, respectively. Some segments of 5'-Nase-positive initial lymphatics (L) appear to run along thick nerve strands (arrows) and gather with the blind endings (arrowheads) around the ganglia (G), corresponding to the light microscopic profiles shown in Figure 6. x 350

lymphatic endothelia is weaker than that in the endothelia of the blood vessels (Fig. 10), they are not necessarily suitable for identification in the lymphatic endothelia. Thus, no satisfactory distinction between these vascular vessels has been achieved. At present, no specific marker for identification of lymphatics has yet been found. Recently, attempts have been made to prepare lymphatic endothelial monoclonal antibodies with endothelial cells from thoracic ducts of rats used as antigens. According to the reports (Ezaki et al., 1990; Ezaki, 1992), it has been shown that distinction of both vascular vessels is possible by double staining of this monoclonal antibody to detect the lymphatic endothelia and anti-type IV collagen antibodies or anti-laminin antibodies in the blood vessel basement membrane (Fig. 11).

## 5. Fine distribution of lymphatics

It is said that location relationship between lymphatic capillaries, blood capillaries and surrounding tissues is usually determined by organs. In the tissues possessing free surfaces with abundant material exchanges, blood capillaries are located near the surface layer and lymphatic capillaries in a deep location. In the lingual mucosa as well as the skin dermis, there are more blood capillaries than lymphatic capillaries in the proper papillary layer, and more lymphatic capillaries in a little deeper location. Lymphatic networks in the lingual body are well developed on the ventral and lateral sides as compared with those on the dorsal side (Castenholz, 1987; Kato and Miyauchi, 1989). In the epiglottis, there is a lower tissue density on the lingual side than on the laryngeal, and many 5'-Nase-positive lymphatics are found on the lingual side. Thus, the magnitude of acute edema in the epiglottis seems to be related to the density of the subepithelial layer (lamina propria) including the covering epithelium (unpublished data). In the central location of intestinal villi, there are several central lymphatic vessels (central lacteals), surrounded by a blood capillary network (Ohtani and Ohtsuka, 1985). Such three-dimensional structures of both vessels are similar to those in the mucosal plicae of the monkey and human gallbladders (Fig. 4, Kato et al., 1994c; Kudo et al., 1998).

It is widely believed that the lymphatics in the large intestine are not prominent in the lamina propria mucosae but can be identified lying in proximity to the lamina muscularis mucosae (Kveitys et al., 1981). Light microscopic observation of Berlin blue and India ink (Hirasima et al., 1984) and SEM observation of corrosion casts (Ohtani and Murakami, 1992) made by intra-parenchymal puncture injection have revealed that rod-like lymphatics originate in the superficial mucosa of the large intestine. In their histochemical studies, Yasunaga et al. (1991a,b) demonstrated that 5'-Nase-positive lymphatics begin in the superficial mucosa and lead into the lymphatic network in the deep mucosa of monkey and human large intestines, although the upper-third of the gastric lamina propria mucosae is normally devoid of lymphatics (Listrom and Fenoglio-Preiser, 1987; Ohtani and Murakami, 1992; Sugito et al., 1996; Ji and Kato, 1997a).

Recently, the intimate association of peptidergic

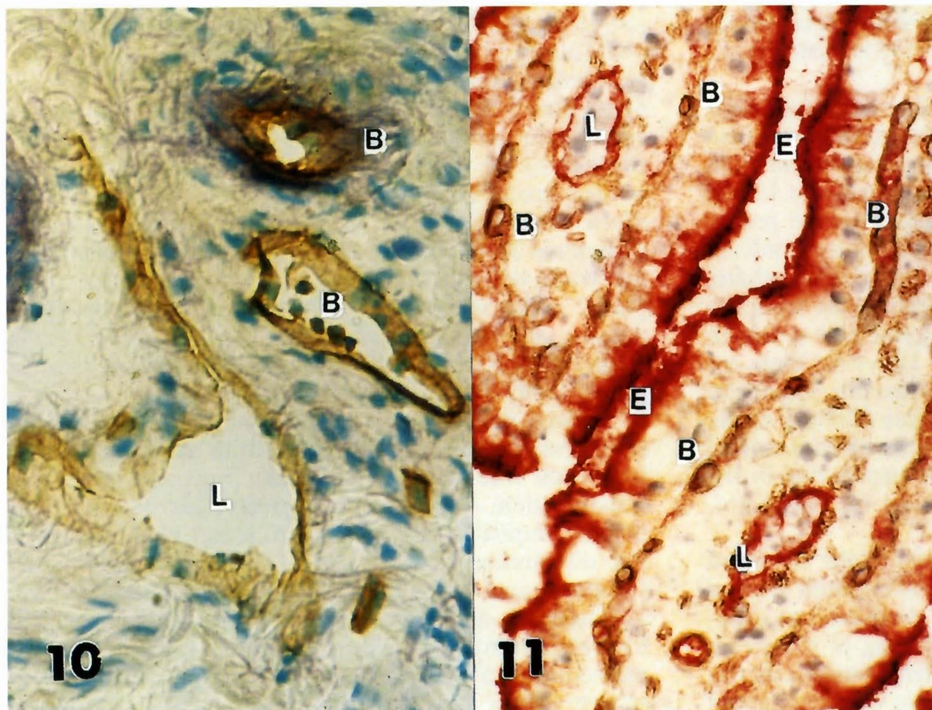


**Fig. 10.** Immunoperoxidase staining for UEA-1 lectin binding of vascular vessels in the monkey tongue. The blood vascular walls (B) are strongly stained with UEA-1, whereas the walls of the lymphatics (L) are weakly stained. x 480

**Fig. 11.** Double immunostaining of the rat small intestine by using anti-type IV collagen antibody for blood capillary and monoclonal antibody for rat lymphatics. The central lymphatic vessels (L) in the intestinal villi are colored only red by the monoclonal antibody, whereas sub-epithelial blood capillries (B) are stained dark blue at the basement membrane (micrograph courtsey of Dr. T.Ezaki ). E: epithelium. x 600

nerve fibers with the central lymphatic endothelium was demonstrated in the intestinal villi of dogs (Ichikawa et al., 1990, 1991a,b) and the rat liver (Ito et al., 1990) by TEM and immunohistochemistry. When the muscular layer of intestinal walls is ablated and its thin-layer extension specimens are subjected to double staining by 5'-Nase reaction and acetylcholinesterase(AChE) reaction, 5'-Nase-positive lymphatic networks and AChE-positive nerve networks (Auerbach's plexus) are clearly visualized (Fig. 6). Furthermore, when the electron microscopic histochemical method is used, lymphatics and nerve fibers are seen close to each other. This finding suggests a mutual relationship (Kato et al., 1998; Shimoda et al., 1998).

In the mesenterium and falciform ligaments of liver, etc, the lymphatic capillaries are noted in the subserosal layer (Fig. 4). In the diaphragm, immediately below the abdominal methothelial cells, the initial lymphatics are seen as sinus-like lymph lumens continuing to the macula cirbriformis, and deep-seated blood vessels (Kato et al., 1993c; Oya et al., 1993; Abu-Hijleh et al, 1995; Shao et al., 1998). Also, in the arthro-synovial membranes, where the blood capillary network is in the shallow layer of the tunica propria of synovial membranes, lymphatics initiate in the deeper layer and become well developed in the fibrous layer and in the myoepithelial membranes in the deep layer (Fujiwara et al., 1995; Itonaga et al., 1997). In the muscular tissues, the lymphatics are not directly adjacent to myofibers and initiate from connective tissues of the fascia. In the connective tissue, the lymphatic capillary network is well developed in the transitional zone between muscles and tendons (fascia and lacertus). In the grandular tissues, the blood capillaries are distributed adjacent to the proper secretory tissue region, but the lymphatics are distributed a little apart (Shimada, 1981; Kato et al., 1993a).

In the parenchymal organs, the blood capillaries are well developed in lobules, whereas the lymphatic capillaries appear in the connective tissues between lobules, but not in lobules. In general, the blood capillaries form a dense network adjacent to the proper tissues or cells, and are involved in material metabolism. On the other hand, it can be said that the lymphatic capillaries form a loose network a little apart from the blood capillaries over a wider range, and play a role in draining tissue fluids in the intersitia. However, there are reports that, in the pancreas of the guinea pig, lymphatic capillaries have been seen in the lobules (Bertelli et al., 1993; Navas et al., 1995). Our recent histochemical observations, have shown lymphatic capillaries in the neighborhood of the pancreatic acini (Ji and Kato, 1997b) .

Also, according to light microscopy of the atrophied thymus of mice, lymphatics are seen along with arteries and veins in the medulla; these are described as intra-thymic lymphatics (Smith, 1955; Hwang et al., 1974; Kato, 1988). Furthermore, we have demonstrated that in histochemical and electron microscopic observations of the atrophied thymus, lymphatics are adjacent to the surrounding lumens of the blood vessels and continue partially to the lumens. We have also reported the possibility that lymphatics initiate from the sites continuing to the perivascular spaces (Kato, 1990c, 1997).

At the location where the cerebral and spinal nerves pass through the medulla, the dura mater continues into the epineurium. The lymphatics located in the root region of this dura mater and spinal nerves initiate in the neighborhood of the intervertebral foramen and run along the spinal nerve root (Magari, 1983a,b). According to a recent histochemical study of the subarachnoid-lymphatic connection in the distribution mode by Miura et al. (1998), it was shown that lymphatics are more remarkably developed in the cervical region, especially, in the brachial plexus, than in the thoracic or lumbo-sacral regions. This confirmed that the cerebrospinal fluid is absorbed not only from sinus venosus through arachnoidal granulation, but absorbed into the epidural lymphatic network from the sheath of upper spinal nerves.

### 6. 5'-Nase activity and lymph absorption

In general, 5'-Nase activity exists in various cells, and is known and used widely as a biochemical marking enzyme of plasmalema. Because 5'-Nase shows high activity in the intestinal villi and microvilli in the absorptive epithelial cells of renal tubules, etc., it is presumed to enhance membranous permeability and adjust material absorption. Interestingly, as reported previously (Kato et al., 1996), while 5'-Nase activity is remarkably high in the lymphatic capillaries absorbing tissue fluids, its activity is reduced slightly in the collecting lymphatics, which transport and store lymphatic fluids, and in the cisterna chyli and thoracic duct, etc. 5'-Nase activity in the central lymphatic vessels in the intestinal villi is higher when fat is aborbed by the administration of olive oil than in a non-treated group (unpublished data). On the other hand, when a lymphatic flow is closed by the ligation of the abdominal thoracic duct, expansion of the lymphatics in the walls of intestinal tracts and mesenterium takes place, with a tendency to reduce the 5'-Nase activity of the central lymphatic endothelia (Fig. 12). These results suggest that 5'-Nase activity in the lymphatic endothelia plays mainly a role of material absorption.

Dental pulp is loose connective tissue enclosed by solid tissue, and its microcirculation system plays an important role in draining tissue fluid and spreading inflammation. A longtime controversy has been whether or not lymphatics are present in dental pulp. Recently, fine distribution has been shown by electron microscopy (Bishop and Malhotra, 1990; Marchetti, 1992). Furthermore, most recently, lymphatics were visualized by light microscopy using the enzyme-histochemical method with 5'-Nase activity as a marker. Here, it was shown for the first time that the lymphatic capillaries,

positive to 5'-Nase reaction, initiate from the neighborhood of the odontoblast layer in the periphery of dental pulp (Aoyama et al., 1995; Matsumoto et al., 1997).

## 7. 5'-Nase activity and lymphatic development

Because 5'-Nase activity in the lymphatics of fetal tissue during the genetic process of lymphatics in the walls of digestive tracts is remarkably low, similar to ALPase activity in the blood vessels, both vessels cannot necessarily be stained distinctively. However, 5'-Nase activity of lymphatics in neonatal tissue is not so different from that in adults (Ji and Kato, 1997a). Whereas lymphatics of the diaphragma are not well developed in the fetus, they do develop quickly following postnatal respiratory exercises (Poggi, 1991). In the histochemical studies by Shao et al. (1998), the status of postnatal development of the rat diaphragma is shown quite well. Also, it has been reported that, with aging, the walls of the lymphatics in the tissues become thinner, the profiles of lumens more irregular, and the lymphatic network more deformed (Jdanov, 1966).

Regarding lymphangiogenesis, the development of lymphatics has only rarely been studied. This is mainly due to the fact that specific markers for lymphatic endothelial cells are not available during embryonic development. Thus, questions like "where lymphatics come from and how they develop?" or "how existing lymphatics extend themselves?" continue to be asked. Concerning the growth of lymphatics, hypotheses such as sprouting from endothelial cells of the vein, originating in the mesenchymal cells of the tissues, or both are discussed, but, without certain evidence for

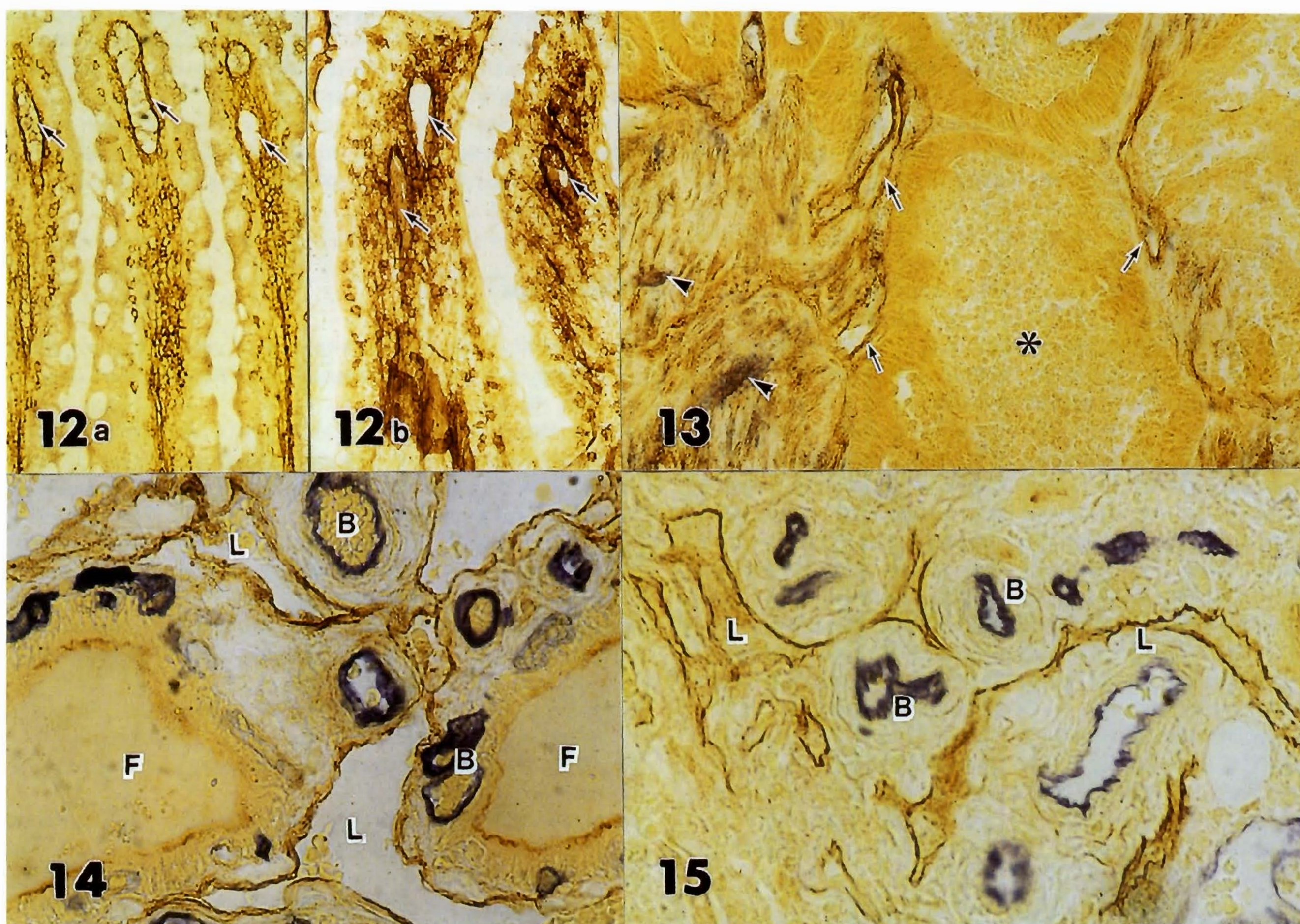


**Fig. 12.** Cryosections of the 5'-Nase-staining intestinal villi of the rat ileum with **(a)** or without **(b)** thoracic duct ligation. The central lymphatic vessels (arrows) reveal weak 5'-Nase activity 5 days after lymphatic obstraction as compared with strong 5'-Nase activity of controls. x 500

**Fig. 13.** Resin (JB-4) section of the large intestinal carcinoma stained with 5'-Nase-ALPase double staining. On the well differentiated site, 5'-Nase-positive lymphatics (dark brown, arrows) and ALPase-positive blood vessels (blue, arrowheads) are clearly noticed. Asterisk shows carcinoma. x 300

**Figs. 14, 15.** Cryosections of the human thyroid tumors including hyperthyroidism **(14)** and follicular adenoma **(15)**. Both lymphatics (L) and blood vessels (B) reveal strong 5'-Nase activity and ALPase activity, respestively. F: follicle. x 500

either of them, the question remains unanswered (Kotani, 1990; Wilting et al., 1997).

In our series histochemical studies, isolated cystic or tubular island-like 5'-Nase-positive profiles, so-called "lymphatic islands" (Castenholz et al., 1991), are occasionally noticed apart from the network in the neighborhood of the initiating region of the lymphatic capillaries consisting of a capillary network. These are all lumens surrounded by endothelial cells, and there are those which continue to the projected region of the initiating lymphatics by partial fine bridging. This profile suggests the possibility of a formation process in the lymphatic capillary network at an advanced stage, and is throught to be an interesting finding in understanding the development of lymphatics (Kato et al., 1993b, 1994a-c).

## 8. 5'-Nase activity under pathological condition

In the subcutaneous healing granulation tissues, following experimental injuries, such as skin incision, we find lymphatics with higher 5'-Nase activity than in normal tissues and those sinus-like expanded lymphatics suggest active absorption and exchange of tissue fluids. Also, in the human large intestine a pedunculated adenoma that proliferates while maintaining a relatively normal structure, exhibits 5'-Nase activity of the lymphatic capillaries in the head and stem parts that is higher than that in the normal submucosa (Fig. 13, Yasunaga et al., 1991c). This tendency is more remarkable especially since the lymphatics are considered to perform active absorption in the interfollicular, subepithelial connective tissues of the human thyroid glands including hyperthyroidism (Fig. 14) and follicular adenoma (Fig. 15). At the same time, the blood capillaries were also noticed that present remarkable meandering and narrowing with high ALPase activity, suggesting angiogenesis. On the other hand, weak 5'-Nase activity of the lymphatics in angiosarcomatous lesions in the dermis is noticed because of long-standing edema (Anan et al., 1998).

In early stage epithelial cancer tissue of induced tongue cancer in the hamster, the lymphatic capillary network having high 5'-Nase activity is found in the proper membranous layer immediately below the epithelia, suggesting active hyperergasia (Endo, 1993). However, in infiltrated type cancer, the expanded lymphatics are seen along with infiltrate and the proliferation of cancer. 5'-Nase activity is reduced in each case (Nakayama, 1995).

## 9. Concluding remarks and perspectives

As one of the effective methods in observing a lymphatic networks in the organs, organ specificity of the lymphatic capillary network is clarified on a light microscopic level by the histochemical method using 5'-Nase activity as a marker. From experimental observations regarding physiological functions of 5'-Nase activity in the endothelia of lymphatics, it is suggested that this enzyme is deeply involved with the permeation and absorption activity of materials in the endothelical cells of the lymphatics. The link between the development of the organs and their physiological functions should be considered when evaluating the characteristics of the fine distribution of the lymphatic capillary network in various organs by this method. Also, in pathological tissues, changes in the enzymatic activity in the tissues should be considered as well. Furthermore, with regard to cancerous tissues, it will be interesting in the future to study how the differences in 5'-Nase activity in the lymphatic endothelia relate to the degree of differentiation of cancer and lymphatic metastasis in cancerous cells.

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