Development of the Lymphatic Network in the Muscle Coat of the Rat Jejunum as Revealed by Enzyme-Histochemistry

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Summary. The process of lymphangiogenesis was studied in the muscle coat of the rat small intestine by light and scanning and transmission electron microscopy; identification of lymphatic vessels was made by 5'-nucleotidase staining.

Light and scanning electron microscopy demonstrated that the intramuscular lymphatic network formation, which started only postnatally, was attributable to the vascular sprouting of slender lymphatic endothelial projections and to a splitting of the vessels, causing intervascular meshes of various sizes. The growing lymphatics were consistently closed by the endothelial cells, which were characterized by an abundance of cell organelles and prominent cytoplasmic processes. The cells often revealed close contacts with the processes of developing smooth muscle cells in the jejunal muscle coat, suggesting a possible role for the latter cells in the guidance of the lymphatic extension.

The present study is the first to suggest the closed nature of lymphatics persisting throughout their development, even at the initial stage of lymphangiogenesis.

The gastrointestinal lymphatic system plays an important role in the transport of tissue fluid, including cellular elements (ALLEN, 1967). The fine structure and distribution of lymphatic vessels in the gut have been investigated by various methods in several mammalian species (MORI, 1969, SATOMURA et al., 1978; OHTANI and OHTSUKA, 1985; OHTANI, 1987, 1992; OHTANI and MURAKAMI, 1987; USHIKI, 1990), but the manner of the growth of the intestinal lymphatics remains to be elucidated. For this reason, an investigation of the development of the intestinal lymphatic network holds critical importance for understanding lymphangiogenic events — not only during normal growth, but also under pathological conditions.

We previously demonstrated the usefulness of enzyme histochemistry for 5'-nucleotidase (5'-Nase)

in whole-mount preparations for visualizing the structural organization of lymphatics in the gastrointestinal tract (SHIMODA et al., 1996, 1997; SHIMODA, 1998). In the present study, we applied this method to various tissue preparations of the jejunum of rats from birth to 10 weeks of age in order to demonstrate the process of the postnatal lymphatic growth at both the light and electron microscopic levels.

MATERIALS AND METHODS

Wistar rats of either sex were allocated to six groups consisting of 3 animals each: newborn (less than 24 h), 1, 2, 4, 8 and 10 weeks old, the last group representing adult rats. The animals were kept under standard laboratory conditions, and all experiments were carried out according to the Guidelines for Animal Experimentation, Oita Medical University.

Tissue preparation

After the animals of each group were sacrificed by exsanguination under deep anesthesia with ether, the jejunal segments were removed and placed in a cold cacodylate buffer (0.1 M, pH 7.2) containing 7% sucrose. These segments were cut along the mesenteric border, and their luminal surfaces were washed with the same buffer.

Light microscopy

The specimens were stretched on a plastic plate with the serosal side up and immersed in 4% paraformaldehyde solution containing 7% sucrose for 4 h at 4°C. Enzyme-histochemical staining for 5'-nucleotidase (5'-Nase) activity was performed using whole-mount preparations; these comprised the entire jejunal segments in the newborn and 1 and 2-week-old rats, whereas they comprised tissue strips including the serosa, longitudinal muscle, the myenteric layer and

Fig. 1. Light micrograph of a whole-mount preparation of the muscle coat of the newborn rat jejunum treated with 5'-Nase staining. View from the abluminal side. Tubular lymphatics (brown, *arrows*) with 5'-Nase activity are sparsely distributed in the subserosa of the mesenteric part of the intestinal wall. *Arrowheads* indicate bridging tubules connecting neighboring lymphatics. *BV* blood vessel, *M* mesenterium. $\times 40$

a small amount of circular muscle in the cases of 4, 8 and 10-week-old rats.

After a washing with the same buffer, these preparations were incubated in the reaction medium for 5'-Nase activity for 30–40 min at 37°C, as reported previously (SHIMODA et al., 1996, 1997, SHIMODA, 1998). The reaction medium contained 5'-adenosine monophosphate (AMP) (Sigma Chemical, St. Louis, MO, USA) as a substrate, lead citrate (TAAB Laboratories Equipment, Berkshire, England) as a capture agent, and 2 mM L-tetramisole (Sigma) as an inhibitor of nonspecific alkaline phosphatase (KATO, 1990). After a rinsing in distilled water, the tissues were treated with 1% ammonium sulfide solution for 2 min at room temperature.

Controls were prepared as follows: 1) omission of the substrate (AMP) from the reaction medium; 2) addition of 50 mM NiCl_2 into the reaction medium; and 3) incubation of the specimens for 60 min at 60°C before treatment of the reaction medium.

All the specimens were mounted on glass slides with Crystal/mount (Biomed, Foster, CA, USA) and examined with a light microscope (BX-60, Olympus, Tokyo, Japan).

Scanning electron microscopy (SEM)

Several preparations stained for 5'-Nase activity were immersed in a Karnovsky's fixative for longer than one day at 4°C, followed by a rinse in distilled water. These specimens were processed for the maceration technique originated by TAKAHASHI-IWANAGA and FUJITA (1986) to remove the extracellular matrices from the tissues. Briefly, the tissues were placed in 6 N NaOH at 60°C for 10–15 min and rinsed in warm phosphate buffered saline (0.1 M, pH 7.4). They were then stained, to enhance conductivity, by the tannin-osmium method (MURAKAMI, 1974), dehydrated through a graded ethanol series, and dried according to the t-butylalcohol freeze-drying method described by INOUE and OSATAKE (1988). The

Fig. 2. Light (a) and scanning electron (b–e) micrographs of a whole-mount preparation of the muscle coat of the postnatal week 2 rat jejunum with 5'-Nase staining. View from the abluminal side. **a.** 5'-Nase-positive lymphatics (brown) show many sprouting profiles (*arrowheads*) and extend along the intestinal muscle bundles. The myenteric nerve plexus (*MP*) appears as a weakly stained network. The *double-headed arrow* indicates the longitudinal direction of the intestine. $\times 55$. **b** and **c.** Secondary emission (**b**) and backscatter (**c**) images of the red boxed area in **a**. Serosal tissues and longitudinal muscles have been removed. Endothelial cells of 5'-Nase-positive lymphatic in **b** are colored yellow, while those in **c** are highlighted by backscattered imaging. Many endothelial spindle processes (*arrowheads*) are seen in a lymphatic sprouting. *CM* circular muscle. **b, c:** $\times 1,500$. **d** and **e.** Secondary emission (**d**) and backscatter (**e**) images of the blue boxed area in **a**. Serosal tissue and longitudinal muscles have been removed. Endothelial cells of 5'-Nase-positive lymphatic in **d** are colored yellow, while those in **e** are highlighted by backscattered imaging. An extended lymphatic vessel shows many slender processes (*arrowheads*) and a bulbous projection (*arrow*) of the endothelium at its leading end. *CM* circular muscle. **d, e:** $\times 1,500$

Fig. 2. Legend on the opposite page.

Fig. 3. Light micrographs of whole-mount preparations of the muscle coat of the postnatal week 4 rat jejunum with 5'-Nase staining. Views from the luminal side. **a.** The 5'-Nase-positive lymphatics (brown) are distributed two-dimensionally throughout the myenteric layer to form a network equipped with multiple intervacular meshes. The *double-headed arrow* indicates the longitudinal direction of the intestine. $\times 55$. **b.** Higher magnification of tiny holes (*arrowheads*) in the myenteric lymphatic network. $\times 80$

dried specimens were mounted on aluminium stubs, coated with carbon at approximately 20 nm thickness, and observed under a scanning electron microscope (S-800, Hitachi, Tokyo) equipped with a GM type 30 backscattered electron detector. Micrographs of secondary emission and backscatter imagings were taken at an accelerating voltage of 10 and 20 kV, respectively (KATO and GOTOH, 1990).

Transmission electron microscopy (TEM)

Small tissue pieces from each rat for TEM were immersed in 0.1% glutaraldehyde and 4% paraformaldehyde dissolved in 0.1 M cacodylate buffer for 1 h, followed by a paraformaldehyde solution (4% w/v in the same buffer) for an additional 4 h at 4°C.

After rinsing in the cacodylate buffer containing 7% sucrose, the specimens were incubated for 15 min at 37°C in a cerium-based medium for 5'-Nase reaction that comprised a 0.1 M Tris-maleate buffer (pH 7.4), 1 mM 5'-AMP, 2 mM MgCl_2 (Nakarai Chemicals, Kyoto, Japan), 2 mM CeCl_2 (Sigma), and 1 mM L-tetramisole, as reported previously (KATO, 1990). Following a rinse in the same buffer, the tissues were postfixed in 2% osmium tetroxide in a 0.1 M cacodylate buffer for 2 h at 4°C. They were then dehydrated through a graded ethanol series and embedded in Epok 812 (Oken, Tokyo). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed using a JEM-1200EX transmission electron microscope (JEOL, Tokyo).

Fig. 4. Light micrographs of whole-mount preparations of the muscle coat of the postnatal week 8 rat jejunum with 5'-Nase staining. Views from the luminal side. **a.** A dense maze-like network of 5'-Nase-positive lymphatics (brown) is seen throughout the myenteric layer. $\times 55$. **b.** A segment of a lymphatic (*arrow*) of a mesh is tapered, and another (*arrowhead*) is segregated. $\times 80$

RESULTS

Enzyme reaction of lymphatic vessels

The enzyme-histochemical staining for 5'-Nase activity demonstrated, both under the light and electron microscopes, significant reaction products localized in the lymphatic vessels in all tissue preparations of the rat small intestine (Figs. 1-8). Little or no 5'-Nase activity was recognized in the blood vessels. Controls for the enzyme reaction showed no positively reacting structures. The present histochemical staining was thus successful in identifying the lymphatics as in our previous studies (KATO, 1990; KATO et al., 1993; SHIMODA et al., 1996, 1997; SHIMODA, 1998).

Light microscopy

In newborn rats, several slender lymphatics with 5'-Nase activity — extending from thick mesenteric

lymphatics — were distributed accompanying large blood vessels in the subserosa of the mesenteric part of the intestinal wall, but were absent in the proper muscle coat (Fig. 1). These lymphatics took a circular course towards the side opposite the mesenterium attachment and exhibited distally tapered tips (Fig. 1). They were occasionally connected to each other by bridging tubules (Fig. 1).

By postnatal week 2, the subserosal lymphatics gave off many lateral projections *en route* (Fig. 2a). These processes extended along the longitudinal muscles to enter the myenteric layer (Fig. 2a). Many sprouts with rounded or tapered tips were further issued from various sites of the lymphatic extensions (Fig. 2a).

At 4 weeks postnatally, multiple intervascular spaces of irregular dimensions (in the range of 10–200 μm) appeared within the myenteric lymphatic network, and were often located at triple or quadruple branching points of the network (Fig. 3). Many

Fig. 5. TEM views of a lymphatic vessel (*L*) in the muscle coat of the postnatal week 2 rat jejunum with 5'-Nase staining. Lymphatic endothelial cells (*E*) positive for 5'-Nase show cytoplasmic extensions containing abundant mitochondria (*m*), Golgi apparatus (*g*), smooth (*ser*) and rough (*rer*) endoplasmic reticula, thin filaments (*asterisks*), and caveolae (*arrowheads*). Small lateral spikes (*arrows*) of the endothelial cells are also seen. *SM* smooth muscle cell. *a*, *b*: $\times 18,000$

Fig. 6. TEM views of a lymphatic vessel (*L*) in the muscle coat of the postnatal week 2 rat jejunum with 5'-Nase staining. **a.** A leading tip of a growing lymphatic is closed by 5'-Nase-positive endothelial cells (*E*). A macrophage (*M*) is seen within the lymphatic. *FB* fibroblast, *SM* smooth muscle cell. $\times 5,500$. **b.** Higher magnification of the area indicated by the *asterisk* in **a**. A lymphatic endothelial cell (*E*) shows a filopodium-like projection (*arrow*). Close contact (*arrowhead*) between a bulbous process of a smooth muscle cell (*SM*) and the endothelial cell of a lymphatic is also seen. $\times 18,000$

Fig. 7. TEM views of a lymphatic vessel (*L*) in the muscle coat of the postnatal week 4 rat jejunum with 5'-Nase staining. **a.** Collagenous tissue covered with 5'-Nase-positive lymphatic endothelium (*E*) is seen within the lymphatic. $\times 4,500$. **b.** Higher magnification of the area indicated by the *asterisk* in **a**. Stromal elements are enveloped in the lymphatic endothelium. $\times 18,000$

Figs. 6 and 7. Legends on the opposite page.

Fig. 8. TEM views of a lymphatic (*L*) in the muscle coat of the postnatal week 8 rat jejunum stained with 5'-Nase. **a.** The 5'-Nase-positive lymphatic endothelium (*E*) is lengthened to form a large vessel. $\times 4,000$. **b.** Higher magnification of the area indicated by the asterisk in **a**. The endothelial cells showing interdigitation intensely react to 5'-Nase. $\times 18,000$

smaller holes with diameters of less than $10\ \mu\text{m}$ — probable precursors of such meshes — also occurred not only at branching points of the vessels, but also within their dilated segments (Fig. 3b).

The intervascular spaces grew larger with maturation, and some segments of the lymphatics forming these often tapered off to be segregated (Fig. 4b), probably resulting in novel lymphatic extensions. By week 8, the lymphatics elongated preferentially along the circular or longitudinal smooth muscle bundles and developed a dense maze-like network — corresponding to that of the adult rats (SHIMODA et al., 1996) — throughout the myenteric layer (Fig. 4a).

Scanning electron microscopy (SEM)

Following NaOH maceration of the surrounding stromal elements, SEM examination with secondary electron imaging (SEI) of the 5'-Nase-stained tissues allowed a precise analysis of the abluminal aspects of the growing lymphatic vessels in the intestinal muscle coat (Fig. 2b, d). The lymphatic endothelial cells were highlighted by backscattered electron imaging

(BEI) due to the 5'-Nase reaction (Fig. 2c, e); thus, these could be clearly identified by contrasting the BEI with the SEI.

The lymphatic vessels revealed cobblestone-like arrangements of irregularly-shaped endothelial cells endowed with round or elliptical nuclear regions, but not any periendothelial cells such as smooth muscle cells (Fig. 2b, d).

The three-dimensional structures of the small sprouts of the subserosal lymphatics, demonstrated in the 2-week-old rat intestine by light microscopy, were directly observed by SEM: these comprised endothelial cells equipped with spindle-shaped cytoplasmic processes ($3\text{--}5\ \mu\text{m}$ in caliber) (Fig. 2b). At this stage, the endothelial cells of the longitudinally-oriented lymphatic extensions displayed many cord-like cytoplasmic processes, and appeared to be loosely connected to each other (Fig. 2d). Small, bulbous filopodia from the endothelial surfaces were also demonstrated at the distal blunt ends of the extensions (Fig. 2d).

Fig. 9. Schematic presentation of the process of postnatal lymphatic growth in the muscle coat of the rat jejunum based on the present histochemical study.

Transmission electron microscopy (TEM)

The fine structure of the lymphatic vessels in the rat small intestine was clearly demonstrated by TEM. The lymphatic endothelial cells displayed dense granular precipitates due to 5'-Nase reaction on their surfaces, allowing the lymphatic vessels to be easily identified under the TEM. The enzyme activity of the lymphatic endothelium appeared to increase with age (Figs. 5-8).

In the 2-week-old animals, the lymphatic endothelial cells disseminated long cytoplasmic extensions (Fig. 5) that corresponded to the structures visualized by SEM (Fig. 2d), and revealed many microspikes projected into the interstitial spaces (Fig. 5). The endothelial cells were rich in mitochondria, cisternae of the endoplasmic reticulum, Golgi apparatus, free ribosomes, 7-10 nm thick filaments, and caveolae (Fig. 5). The lymphatic extensions were closed by endothelial cells interdigitating or overlapping each other, but not endowed with basal laminae (Figs. 5, 6).

The blunt ends of the growing portions of lymphatics in the 2-week-old rats revealed filopodia-like projections budding distally from the endothelial cells (Fig. 6), possibly corresponding to those observed in SEM (Fig. 2d). TEM of this region further disclosed frequent close contacts (less than 20 nm) between bulbous projections of coursing smooth muscle cells and the lymphatic endothelial cells (Fig. 6). Occa-

sional occurrences of macrophages were also shown within the lumina of the lymphatics at this stage (Fig. 6).

The myenteric lymphatics of the 4-week-old rats often demonstrated transcapillary tissues composed of collagenous materials enveloped in the lymphatic endothelial cells within their lumina (Fig. 7).

The lymphatic endothelial cells in the 8-week-old rats were lengthened and attenuated in their cytoplasm, causing them to assume the typical appearance of large capacity vessels (Fig. 8).

DISCUSSION

The present findings were able to demonstrate precisely the development of the lymphatic network in the rat jejunal muscle coat. A few reports have described the development of large submesothelial lymphatics in the diaphragm (POGGI et al., 1991; SHAO et al., 1998), but no paper, to our knowledge, has dealt with the development of visceral lymphatic vessels and capillaries. The present paper is the first to demonstrate the process of the initial lymphatic growth in a visceral organ. The intestinal muscle coat employed in our study was advantageous for investigating the formation of a lymphatic network because it was richly supplied with extrinsic lymphatics.

The manner of the intestinal lymphatic growth as revealed by the present study is schematically presented in Figure 9. The lymphatic network formation in the jejunal muscle coat was attributed to two remarkable events: one was the vascular sprouting from pre-existing lymphatics, while the other was the subsequent formation of intervascular meshes by vascular splitting. Both events contributed to enlarging lymphatic vascular beds. The splitting of existing vessels featuring intervascular hole formation has been documented in the postnatal development of blood vessels by previous investigators (CADUFF et al., 1986; BURRI and TAREK, 1990; VAN GRONINGEN et al., 1991; BURRI, 1992; PATAN et al., 1992); some termed it intussusceptive vascular growth. Thus, the postnatal lymphangiogenesis as demonstrated in the intestinal muscle coat corresponds, in its essential mode, to hemangiogenesis as described in previous reports (HUDLIKA and TEYLER, 1986; RISAU et al., 1988; PARDANAUD et al., 1989).

The present study also was first to succeed in three-dimensionally visualizing the growing lymphatics using SEM aided by enzyme-histochemistry. Application of the alkali maceration technique to the 5'-Nase-stained whole-mount tissues permitted direct SEM observation of both the spatial distribution of the reaction products and the three-dimensional structure of the labeled lymphatic endothelial cells. Thus, this SEM method holds promise for a precise morphological analysis of the cells histochemically defined by light microscopy.

The numerous cytoplasmic processes of the lymphatic endothelial cells involved in the vascular sprouting, which were demonstrated by the present SEM observation, probably indicate a high migratory potential of the cells. Furthermore, the dynamic structures of lymphatic endothelial extensions including filopodium-like projections are presumed to contribute to the formation of new vascular channels.

The present study further provided ultrastructural profiles of the postnatally-growing lymphatic vessels in the muscle coat of the rat small intestine. The unusual abundance of cell organelles, including numerous thin filaments in those lymphatic endothelial cells, suggests that the cells require high levels of cellular activity to protrude their processes and to migrate.

Several previous studies have demonstrated blind endings closed by the endothelium at terminal parts of developed lymphatic vessels, including central lacteals in intestinal villi (OHTANI and OHTSUKA, 1985; OHTANI, 1987; USHIKI, 1990), submucosal and myenteric lymphatics in the digestive tract (OHTANI

and MURAKAMI, 1987; SHIMODA et al., 1997; SHIMODA, 1998), and mesenteric lymphatics (KATO et al., 1993). The leading tips of the growing lymphatics observed in the present study revealed the same closed structure. Thus, the nature of lymphatics being closed by the endothelial cells is apparently preserved during the process of their growing, even at its earliest stage.

The occurrence of macrophages in the extending lymphatics of 2-week-old rats may indicate that the lymphatics already serve as pathways for cellular elements migrating into the general circulation at this stage. Such free cells as macrophages presumably have penetrated the lymphatic wall to enter the lymphatics, since they have been closed by the endothelial cells at their leading tips.

Of special note is the occurrence of adhesion-like close contacts between the endothelial cells and the cytoplasmic projections of the smooth muscle cells of the myenteric coat at the leading point of the extending lymphatics. This structural device possibly facilitates the endothelial cell migration navigated by the muscle cells. Postnatal lymphatic growth in the intestinal muscle coat, therefore, is suggested to be intimately associated with the development of the intestinal muscle cells due to the intake of food.

A tendency for the 5'-Nase activity of the lymphatic endothelial cells to increase with the vascular growth presumably indicates that the enzyme activity correlates to the functional maturation of the cells. This possibility remains to be explored in future studies.

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