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ORIGINAL PAPER

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Cytoarchitecture of the lamina muscularis mucosae and distribution of the lymphatic vessels in the human stomach

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Abstract The aim of the present study was to clarify the anatomical structure of the lamina muscularis mucosae (LMM) in the human stomach and to correlate it with the lymphatic spread of gastric cancer cells. Human stomachs taken at operation or autopsy were used. The specimens derived from these stomachs were examined by light microscopy immunohistochemistry and scanning electron microscopy (SEM). In the cardia and pyloric wall, bundles of smooth muscle cells of the LMM were relatively loose and thin and formed a reticular configuration. Small lymphatic capillaries (approximately 10–30 μm in diameter) were present directly above the LMM, and relatively large lymphatics (approximately 80–100 μm in diameter) were observed in the submucosal layer and within the LMM. In contrast, the LMM in the fundus, body, and antral wall was composed of tight, thick bundles of smooth muscle cells that ran straight. Large lymphatics were found directly beneath the LMM, but they were few in the lamina propria mucosae. In addition, lymphatics adjacent to veins were also found in the submucosa of the fundus. Structural differences in the LMM of the stomach wall might depend on physiological function. In this study, the relationship between the cytoarchitecture of the LMM or the distribution of lymphatic vessels and cancer invasion is discussed.

Key words D2-40 antibody (D2-40) · Human stomach · Lamina muscularis mucosae (LMM) · Lymphatic vessels · Scanning electron microscopy (SEM)

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Introduction

It is well known that cancer invasion depth closely correlates with lymphatic spread. Lehnert et al.[1] reported that the risk of lymph node metastasis in gastric cancer with submucosal invasion was four times higher than that in mucosal gastric cancer. Histologically, the lamina muscularis mucosae (LMM) separate the lamina propria mucosae from the submucosa.

So far, there have been few reports concerning the microscopic structure of the lamina muscularis mucosae (LMM) in the human stomach. Only some textbooks on histology (Ham,[2] Rhodin,[3] and Leeson and Leeson[4]) described that the LMM in the human stomach was composed of a few smooth muscle layers, including collagen and elastic fibers, based on light microscopic observations from a histological or surgical point of view. It is very important to clarify the cytoarchitecture of the LMM.

However, the LMM is not visible by scanning electron microscopy (SEM) without chemical digestion. Recently, the cytoarchitecture of the LMM in the human esophagus was clearly demonstrated by SEM by utilizing the elastase-NaOH digestion method.[5] Their architecture varied depending on location in the neck, thorax, and abdomen.

In addition, no studies have examined the correlation between the lymphatic vessels and the morphological configuration of the LMM, although Lehnert et al.[1] demonstrated the presence of small lymphatic vessels over the LMM and large lymphatic vessels in the submucosal layer in the human stomach. Furthermore, regional differences in the distribution of lymphatic vessels in the human stomach remain unclear, although the distribution and architecture of lymphatic vessels in the rat stomach varied in the different zones.

In the present study, the structure of the LMM and the lymphatic vessels in the human stomach were analyzed using light microscopy and scanning electron microscopy (SEM), and its correlation with the lymphatic spread mode of gastric cancer was considered anatomically.

In this study, the three-dimensional architecture of the LMM was visualized by SEM combined with the

elastase-NaOH digestion method. On the other hand, lymphatic vessels were identified by utilizing the immunohistochemical method,[6] in addition to hematoxylin and eosin (H&E) staining.

Materials and methods

Human stomachs taken at operation or autopsy were used as materials. Normal stomach tissues observed with the naked eye were also obtained with informed consent from 45 adult patients who underwent surgery for gastric cancer at Oita University. Specimens were longitudinally cut into 5-mm-thick slices with razor blades from the cardia, fundus, corpus, pyloric part, and pylorus, and processed for light microscopic and scanning electron microscopic examination according to the procedures described below. The gastric epithelium in all stomachs examined by us had histologically a normal appearance.

Light microscopy

Tissue blocks were fixed with 10% formalin, dehydrated in an ethanol series, cleaned by xylene, and embedded in paraffin. The sections (about 5 μm thick) were cut and stained with H&E. The formalin-fixed paraffin sections in five stomachs were also stained immunohistochemically with monoclonal mouse anti-human D2-40 antibody (D2-40) (Dako North America, Carpinteria, CA, USA)[7] to identify the lymphatic vessels. D2-40 is a new selective marker of lymphatic epithelium. D2-40 to a *O*-linked sialoglycoprotein (MW, 40 kDa) was purified from ascitic fluid, as previously described.[8] After epitope retrieval with citrate buffer (pH 6.0), sections were treated with 0.3% hydrogen peroxide and incubated sequentially with D2-40 (1:2000) for 12–18 h at room temperature. Then, they were subjected to Dako Envision System-HRP Labelled Polymer Anti-mouse (Dako North America) for 30 min. Immunoactivity was visualized with a DAB solution; sections were counterstained with Mayer's hematoxylin and examined under a light microscope.

Scanning electron microscopy (SEM)

The tissue blocks fixed in Karnovsky's fixative in ten stomachs were used for SEM. The blocks were treated with 6 N NaOH at 60°C for 10–15 min[9] or with 8 N HCl at 37°C for 3 h[10] for the digestion of collagen fibers. After being rinsed thoroughly in physiological saline, the blocks (about 2 cm × 2 cm) were placed in an elastase solution (1–5 mg/ml; Tohri, Tokyo, Japan) at 37°C for 12–18 h. The remaining paraffin blocks were used for SEM. They were deparaffined with xylene, hydrated, fixed again in Karnovsky's fixative, and immersed in 2 N NaOH at 37°C for 3 h to digest proteoglycan.[11] All specimens were then washed thoroughly in distilled water and placed in cacodylated-buffered 1% osmium tetroxide, 1% tannic acid solution, and 1% osmium tetroxide for 1 h each. The specimens were then dehydrated through a graded ethanol series, dried by the *t*-butanol drying method, coated with gold, and observed under an SEM (S-800; Hitachi, Tokyo, Japan) at an acceleration voltage of 15 kV.

Results

No individual variations of morphology in either the LMM or lymphatic vessels were found.

Light microscopic findings of the lamina muscularis mucosae (LMM)

Figure 1 a–e shows light micrographs of H&E-stained longitudinal sections of the human stomach. The LMM, separating the lamina propria mucosa from the submucosa, had a thickness of 50–300 μm and consisted of slender, spindle-shaped smooth muscle cells. The smooth muscle cells formed three to seven muscle bundles. The thickness and cytoarchitecture of the LMM varied considerably at different sites in the stomach.

In the cardia, the muscle bundles, consisting of three to six smooth muscle cells, were separated by wide spaces, were relatively sparse, and had a reticular arrangement (Fig. 1a). In the fundus, nearer to the fundic cardia, the spaces between the muscle bundles were narrower, with a more dense reticular arrangement (Fig. 1b). More distally, the muscle bundles had a linear arrangement. In the corpus, the LMM differed in structure between the lesser curvature and the greater curvature. In the lesser curvature, the LMM was about 100 μm thick, and the muscle bundles had a linear arrangement (Fig. 1c). In the greater curvature, the LMM was about 200 μm thick (Fig. 1d). In the pylorus, the structure of the LMM was similar to that in the cardia, namely, sparse muscle bundles with wide separation and a reticular structure. The LMM was about 200 μm thick (Fig. 1e). In all areas of the stomach, blood vessels and lymphatic ducts passing through the muscle bundles of the LMM were observed.

SEM findings of the lamina muscularis mucosae (LMM)

Treatment of the gastric tissue with NaOH or HCl at 60°C for 10 min followed by elastase treatment resulted in the removal of collagen and elastic fibers, thus exposing the stromal surface of smooth muscle cells in the LMM.

SEM observation of the cardia LMM from the side of the submucosa showed aggregates of groups of smooth muscle cells at random or in a reticular pattern (Fig. 2). The smooth muscle cells were slender, with spaces of 20–30 μm between the cells. In the fundus and corpus LMM, the smooth muscle cells were dense and generally arranged longitudinally. The LMM was composed of smooth muscle cells with a linear arrangement, with almost no space between the cells (Fig. 3a,b). The antral LMM was basically similar to that of the corpus. Slender smooth muscle cells

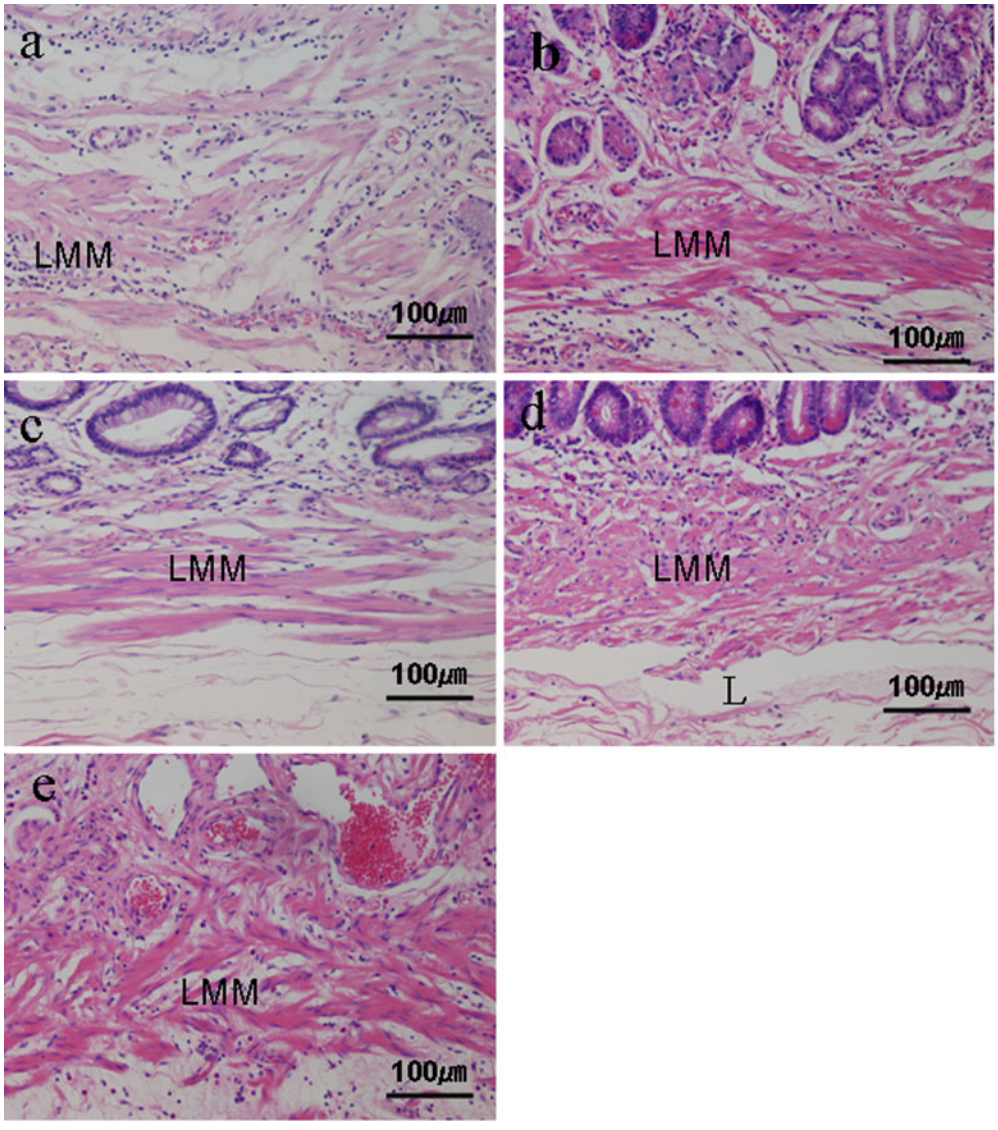

Fig. 1. **a** In the cardia, the muscle bundles of the lamina muscularis mucosae (*LMM*), consisting of three to six smooth muscle cells, are separated by wide spaces, are relatively sparse, and have a reticular structure. **b** Nearer to the cardia, the spaces between the muscle bundles are narrower, with a more dense reticular structure. **c** More distal to the cardia, the muscle bundles have a parallel structure. In the gastric corpus, the LMM differs in structure between the lesser curvature and the greater curvature. In the lesser curvature, the LMM is about 100 μm thick, and the muscle bundles have a parallel structure. **d** In the greater curvature, the LMM is about 200 μm thick, with a reticular structure. *L*, lymphatics or lymphatic capillaries. **e** In the pylorus, the structure of the LMM is similar to the cardia, namely, sparse muscle bundles with wide separation and a reticular structure. Thickness is about 200 μm

with a linear arrangement extended to the pylorus (Fig. 4a). In the LMM from this area, longitudinal lymphatic vessels were also observed (Fig. 4b). The pylorus LMM closely resembled that of the cardia. Smooth muscle cells had a reticular arrangement, with spaces between the cells.

Lymphatic vessels

Lymphatic capillaries distributed in the gastric tissue were relatively easily identified on light microscopy with H&E: these were identified by their size, somewhat larger than that of blood capillaries, irregular shape, and a thin layer of epithelial cells. The connective tissue around the lymphatic capillaries was sparse. To clarify more clearly the identification of lymphatic vessels and their distribution, we immunostained specimens with D2-40, which specifically reacted with endothelial cells of lymphatic capillaries and lymphatics. In the cardia, lymphatic capillaries were abundant above (lamina propria) and below (submucosa) the LMM (Fig. 5a). In the submucosa, the lymphatics had a relatively large diameter. Lymphatics were identified by the presence of valves. Lymphatic capillaries were also distributed just below the esophageal stratified squamous epithelium of the cardia (Fig. 5b). No lymphatic capillaries were seen just below the gastric simple columnar epithelium, but they were seen around the gastric glands.

In areas of the stomach other than the cardia, relatively small lymphatic capillaries (approximately 10–30 μm in diameter) were observed above the LMM in the lamina propria, and fairly large lymphatic capillaries or lymphatics (approximately 80–100 μm in diameter) were observed just below the LMM in the submucosa (Fig. 6a–c). In addition, in the fundus, large lymphatic capillaries or lymphatics adjacent to veins coursing through the submucosa were noted (Fig. 6a). In the gastric angle, the lymphatic capillaries in the

LMM ran parallel to the smooth muscle in a longitudinal direction (Fig. 6c). In the pylorus, as in the cardia, the lymphatic capillaries traversed the LMM in a nearly perpendicular direction (Fig. 6d).

The most interesting finding in the present SEM study was that the lymphatics ran not only within the LMM but also just below the LMM in all regions (Figs. 4b, 7a,b).

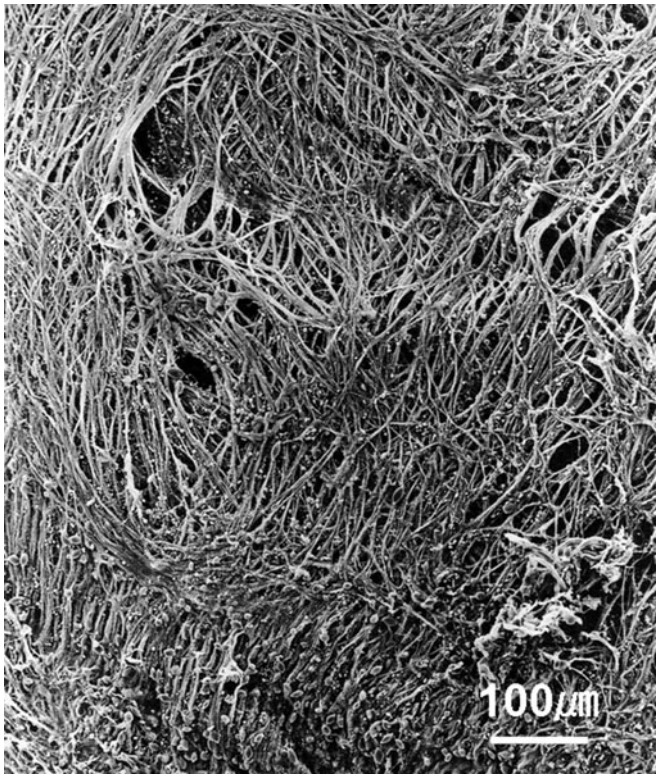

Fig. 2. Scanning electron micrograph (SEM) of the cardia LMM from the side of the submucosa shows aggregates of smooth muscle cells at random or in a reticular pattern. The smooth muscle cells are slender, with spaces of 20–30 μm between the cells

Discussion

The Japanese Research Society for Gastric Cancer[12] defined gastric cancer confined to the mucosa and/or the submucosa as early gastric cancer, regardless of the presence of lymph node metastasis. Lehnert et al.[1] suggested that lymphatic spread was closely related to cancer invasion into the submucosal layer in early gastric cancer. Thus, studying the microscopic structure of the LMM and the distribution of the lymphatic vessels of the stomach wall might provide a clue to the mechanism of gastric cancer spread in the early phase.

The structural variation of the LMM in the human esophagus was reported by Nagai et al.[5] Based on light microscopic findings, a few authors[3,4] reported that the LMM in the human stomach consisted essentially of two or three layers of smooth muscle cells, but they did not describe that the structure of the LMM varied depending on the location in the stomach. The present light microscopic and scanning electron microscopic studies in the human stomach revealed that the thickness and arrangement of the LMM varied considerably in different regions. Especially, the cardia of the stomach is located next to the esophagus, and it is of interest to clarify the structure to analyze the mode of cancer spread. In fact, the classification and the surgical approach for cardia cancer and Barrett's esophageal cancer have become increasingly controversial because of their complicated mode of lymphatic spread, despite the Siewert[13] classification as to cardia cancer. Our morphological results demonstrated that the cytoarchitecture of the lamina muscularis mucosae depended on the location in the human stomach, and a few smooth muscle bundle layers showed a reticular arrangement in the cardia or pylorus and a linear arrangement in the other parts. In addition, the smooth muscle bundles showed not a regular arrangement but a complicated reticular one. Intracardiac pressure has been reported to be 15–40 mmHg, which is higher than in the

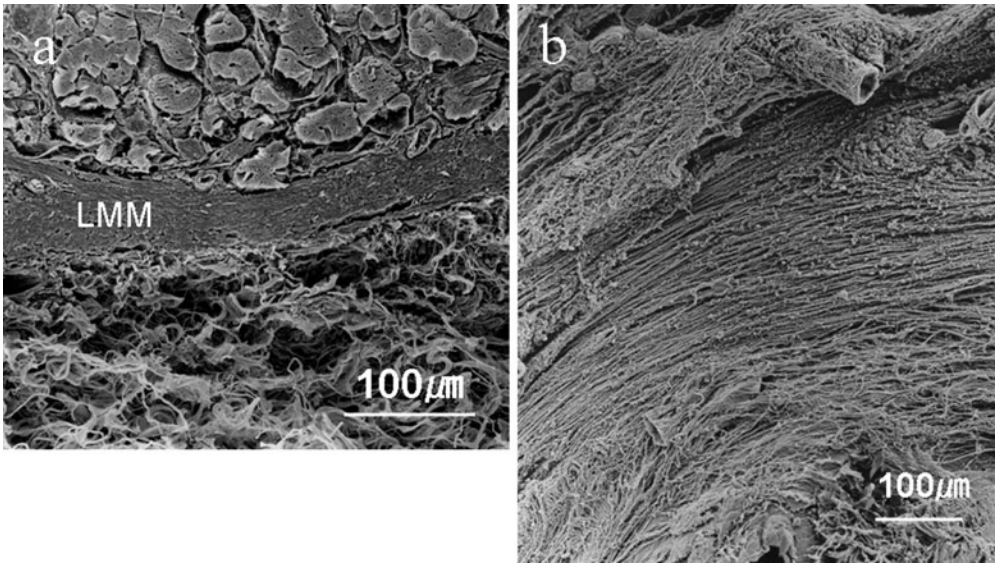

Fig. 3. **a** SEM of the corpus *LMM*. The smooth muscle cells are dense and generally arranged longitudinally. The LMM is composed of smooth muscle cells with a parallel structure, with almost no space between the cells. **b** SEM of the corpus LMM from the side of the submucosa. The smooth muscle runs relatively parallel

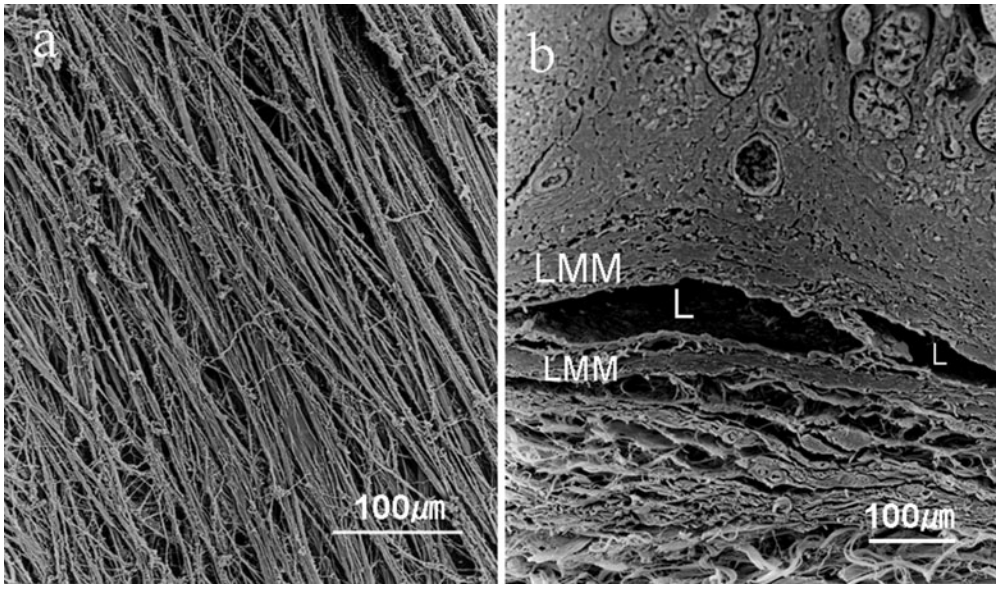

Fig. 4. **a** The antral LMM is basically similar to that of the corpus. Slender smooth muscle cells with a parallel structure extend to the pylorus. **b** Longitudinal lymphatics (*L*) in the *LMM* are also observed

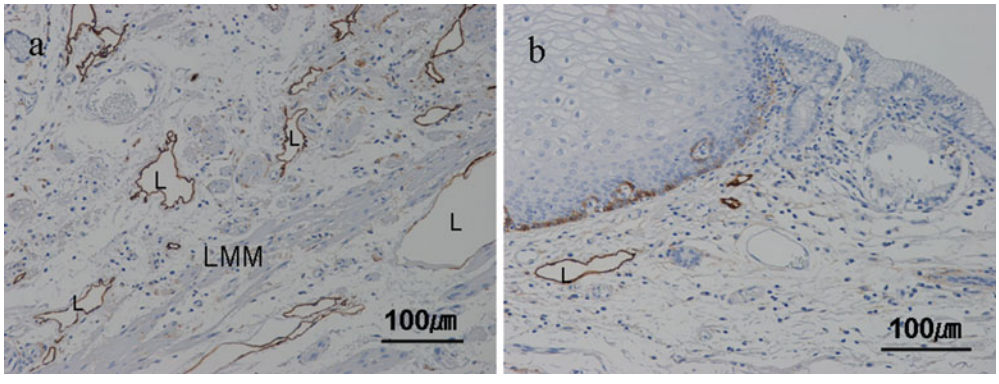

Fig. 5. **a** In the cardia, lymphatic (*L*) capillaries are abundant just above (lamina propria) and below (submucosa) the *LMM*. **b** Lymphatic capillaries are distributed just below the esophageal stratified squamous epithelium of the cardia. Lymphatic capillaries are not seen just below the gastric simple columnar epithelium, but they are seen around the gastric glands

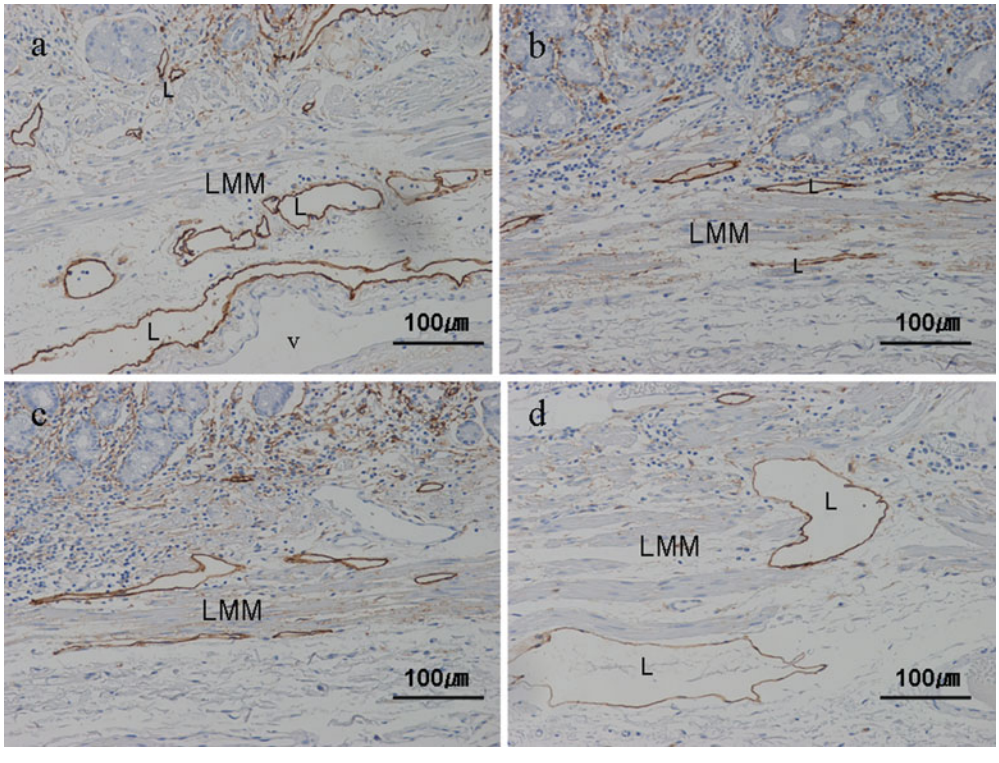

Fig. 6. **a** In the fundus, fairly large lymphatic capillaries or lymphatics are observed below the *LMM* in the submucosa. *V*, vein. **b** In the gastric angle, lymphatic capillaries are seen above and below the LMM. **c** In the gastric angle, lymphatic capillaries in the LMM and submucosa run parallel to the smooth muscle in a longitudinal direction. **d** In the pylorus, as in the cardia, the lymphatic capillaries traverse the LMM in a nearly perpendicular direction

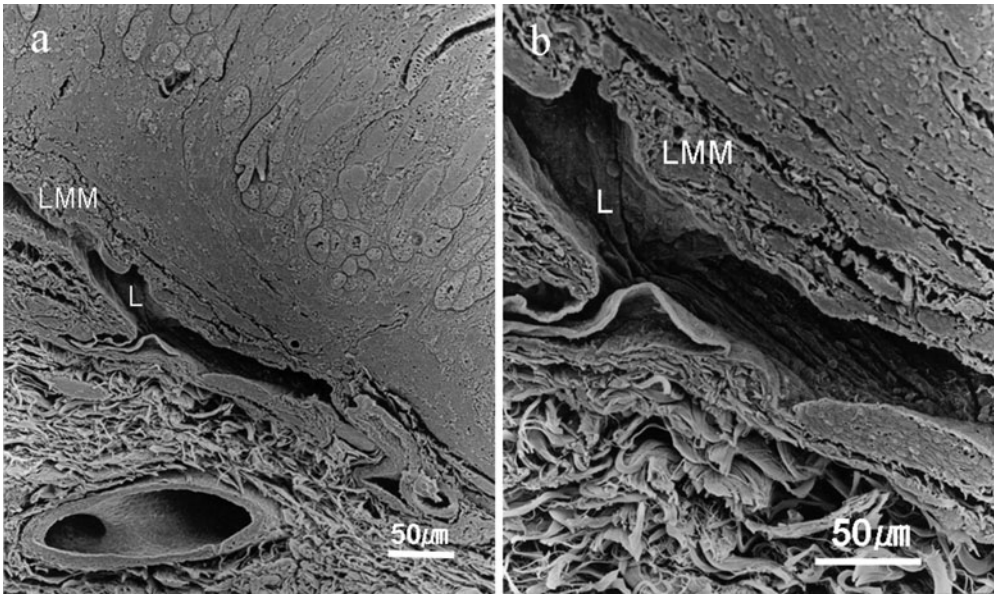

Fig. 7. **a** In the greater curvature of the corpus, SEM shows an interesting finding, namely, lymphatics (*L*) running longitudinally through the *LMM*. Some of these branches extend into the submucosa. **b** Magnified view of **a**

other parts.[14] Nagai et al.[5] reported that the reticular arrangement of the LMM in the cardia might be related to the need to maintain higher pressure. Furthermore, its relatively thin structure and large spaces between reticular lymphatic vessels might correlate with the high incidence of lymphatic spread of cancer cells. It is likely that the reticular arrangement of the LMM in the pylorus is essentially similar to that of the cardia.

In contrast to the reports by Nagai et al.[5] and Hashimoto et al.[15] concerning the distribution of lymphatic vessels in the lamina propria of the esophagus, several studies[16–20] reported on the lymphatic flow in the stomach. Because the gastric epithelium in the present specimens showed a normal appearance, it appeared that the structure and distribution of lymphatic vessels were not influenced by the disease. Our immunohistochemical results agreed with the previous report by Lehnert et al.[1] that lymphatic vessels could be seen in the lamina propria mucosae and the submucosal layer in the human stomach. It is worthy of notice that lymphatic capillaries in the human cardia were abundantly distributed beneath the stratified squamous epithelium, although those in the rat forestomach were not present beneath stratified squamous epithelium.[21] Recent advances have made it possible to observe lymphatic capillaries directly above the LMM and relatively large lymphatics in the submucosal layer of the cardia and pylorus immunohistochemically using D2-40. Especially, the present SEM study clearly demonstrated that large lymphatics were distributed within and directly beneath the LMM in all the regions in the human stomach. These findings might suggest that gastric cancer spreads easily via the lymphatic route in the relatively early stage, when cancer cells invade into the LMM in the stomach. Thus, the LMM might function as a kind of critical barrier against direct invasion of cancer cells. The SEM findings of the present study, showing that the lymphatic vessels above the LMM passed through to the submucosal lymphatic vessels, strongly support the previous report by Yoffey and Courtice.[22] In addition, the presence of a lymphatic network, which is in close proximity to veins, stimulates interest in the relationship with invasion of cancer cells.

In conclusion, the microscopic observations of the present study might provide a clue to explain the mechanism of lymphatic spread of gastric cancer in all parts of the stomach.

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