

Immunohistochemical Examination on the Distribution of Cells Expressed Lymphatic Endothelial Marker Podoplanin and LYVE-1 in the Mouse Tongue Tissue

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The clinical study for lingual disease requires the detailed investigation of the lingual lymphatic network and lymphatic marker-positive cells. Recently, it has been reported that several tissue cells and leukocytes express lymphatic markers, LYVE-1 and podoplanin. This study was aimed to clarify the lingual distribution of cells expressing LYVE-1 and podoplanin. In the mouse tongue, podoplanin is expressed in nerve sheaths, lingual gland myoepithelial cells, and lymphatic vessels. LYVE-1 is expressed in the macrophage marker Mac-1-positive cells as well as lymphatic vessels, while factor-VIII was detected in only blood endothelial cells. α -SMA was detected in vascular smooth muscle and myoepithelial cells. Therefore, identification of lymphatic vessels in lingual glands, the combination of LYVE-1 and factor-VIII, or LYVE-1 and Mac-1 is useful because myoepithelial cells express podoplanin and α -SMA. The immunostaining of factor-VIII on lymphatic vessels was masked by the immunostaining to LYVE-1 or podoplanin because lymphatic vessels express factor-VIII to a far lesser extent than blood vessels. Therefore, except for the salivary glands, the combination of podoplanin and α -SMA, or factor-VIII is useful to identify lymphatic vessels and blood vessels with smooth muscle, or blood capillaries.

Key words: podoplanin, LYVE-1, lymphatic vessels, macrophages

I. Introduction

The recent discovery of lymphatic endothelial cell markers, such as PROX1 [37], lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) [1], and podoplanin, made lymphology progress greatly at the molecular biology level [2]. Homeobox gene PROX1 induces the lymphatic development independent of the vascular system development from embryonic veins, and homozygous PROX1-null mouse embryos die at birth because of multiple developmental defects. PROX1 is a master regulator of podoplanin expression and enables vascular endothelial cells to reprogram to become lymphatic endothelial cells. However, PROX-1 is difficult to use for the clinical identification of lymphatic vessels because of the transient expression in a nucleus

[6, 11, 25, 37]. Podoplanin is a mucin-type 38-kDa transmembrane glycoprotein negatively charged by extensive O-glycosylation and has a high content of sialic acid [8, 16, 18, 29, 31, 33]. Podoplanin was first identified as the antigen of kidney glomerular epithelial cells, the podocytes [2], and is also homologous to T1 α -2 gene which encodes the type I alveolar cell specific antigen and to the oncofetal antigen M2A recognized by the D2-40 antibody [4, 17, 20, 26–28, 38]. Podoplanin is first expressed at around E11.0 in Prox1-positive lymphatic progenitor cells. Podoplanin (–/–) mice die at birth because of the respiratory failure and have defects in lymphatic formation with diminished lymphatic transport and congenital lymphedema, but do not have defects in blood vessel pattern formation [30]. We have recently reported that the lymphatic endothelium significantly increases podoplanin expression by the recognition of lipopolysaccharide and lipoteichoic acid through Toll-like receptors 2 and 4 [19]. In other somatic tissue cells, the expression of podoplanin is found in myoepithelial

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cells of salivary glands [9]. The podoplanin expression is rarely found in acini of the parotid gland but clearly found at the basal portion of acini in the submandibular and sublingual glands. Strong expression of podoplanin is also found at the basal portion of the intercalated duct, striated duct, and interlobular duct in all major salivary glands. Podoplanin could be a marker protein of salivary gland myoepithelial cells because podoplanin detection levels coincide with the distribution of myoepithelial cells specific for major salivary glands. In the tooth germ, podoplanin is strongly expressed in inner and outer enamel epithelia at the early bell stage. The strong expression of podoplanin in odontoblasts is sustained during the dentin formative period and diminished at the root formation stage [29].

Recently, it was shown that lymphatic vessels have the ability to express diverse leukocyte adhesion molecules in inflamed lingual tissue [13]. Clinical study for the infectious disease of the tongue requires detailed investigation of the lymphatic vessel network of the apex linguae to the submental lymph nodes and of the radix linguae to jugular vein digastric muscle lymph nodes, and on the initial lymphatic distribution in the tunica muscularis and lamina propria mucosae of the tongue. On the other hand, there have been reports for the expression of the lymphatic marker podoplanin in somatic cells including dendritic cells [20, 39], osteoblasts [8, 35], and epidermal cells [21–23, 26, 28], as well as salivary gland myoepithelial cells [9]. Therefore, in order to precisely identify the lingual lymphatic vessels, it is necessary to investigate the distribution of cells expressing lymphatic markers except for podoplanin. However, there has been no report that has investigated the correlation between podoplanin-positive cells and cells expressing other lymphatic markers like LYVE-1. LYVE-1 is selectively expressed in lymphatic endothelial cells [1, 14] in a subset of infiltrating macrophages found in tumors and inflamed tissues [3], and in the sinusoidal endothelium of liver and spleen. LYVE-1 knockout mice display an apparently normal phenotype with no visible alterations in hyaluronan metabolism, lymphatic vessel development and maintenance, or macrophage trafficking [5, 12]. LYVE-1 is also upregulated in macrophages by a variety of growth factors and cytokines in inflammatory conditions [32]. Therefore, the utility of lymphatic endothelial antigens may vary between tissues. This study aimed to investigate the correlation among cells expressing podoplanin and LYVE-1 as the lymphatic vessel markers, factor-VIII related antigen (factor-VIII) and α -smooth muscle actin (α -SMA) as the blood vessel markers, and Mac-1 as a macrophage marker.

II. Materials and Methods

Immunohistochemistry

The lingual tissue of 8-week-old closed colony ICR mice (male, $n=5$; Charles River Japan Inc., Yokohama, Japan) was used. Tissue was obtained after euthanasia by intraperitoneal injection with sodium pentobarbital (10 ml/kg, Nembutal, Abbott Laboratories, North Chicago,

IL, USA). The protocol for animal use was reviewed and approved by the animal experiment committee of Fukuoka Dental College, Fukuoka, Japan. Frozen 8 μ m sections were cut in a cryostat and fixed in 100% methanol for 2 min at -20°C . After treatment with 0.1% goat serum, the sections were treated with a cocktail of first antibodies: 0.5 $\mu\text{g/ml}$ of hamster anti-mouse podoplanin (monoclonal; AngioBio Co., Del Mar, CA, USA), rabbit anti- α -smooth muscle actin (monoclonal; Epitomics, Inc., Burlingame, CA, USA), rat anti-mouse CD11b/Mac-1 a chain (monoclonal; BD Biosciences, San Jose, CA, USA), rabbit anti-factor-VIII (polyclonal; Biogenesis Ltd., Poole, UK), and rat anti-mouse LYVE-1 (monoclonal; R&D systems Inc., Minneapolis, MN, USA) for 8 hr at 4°C . After reacting with first antibodies, the sections were immunostained for 0.5 hr at 20°C with a cocktail of second antibodies (0.1 $\mu\text{g/ml}$): AlexaFluor (AF) 488 or 568-conjugated goat anti-hamster, rabbit or rat IgGs (Molecular Probes, Invitrogen Com., Eugene, OR, USA), and examined by fluorescence microscopy BZ-8100 (Keyence Corp., Osaka, Japan) [7, 10, 13, 24, 32].

III. Results

In the double immunostaining for podoplanin and LYVE-1 of the tunica muscularis of the mouse apex linguae, there were nerve sheaths stained with only anti-podoplanin antibody, single cells with only anti-LYVE-1 antibody, and lymphatic vessels with both anti-podoplanin and anti-LYVE-1 antibodies (Fig. 1). In the double immunostaining for LYVE-1 and Mac-1 of the lamina propria mucosae of the mouse apex linguae, there were lymphatic vessels stained with only anti-LYVE-1 antibody, macrophages with only anti-Mac-1 antibody, and macrophages with both anti-LYVE-1 and anti-Mac-1 antibodies (Fig. 2). In the double immunostaining for podoplanin and α -SMA of the lamina propria mucosae of the mouse radix linguae, lymphatic vessels and blood vessels were stained with only anti-podoplanin antibody and anti- α -SMA antibody, respectively (Fig. 3). It was also observed that in the lingual gland, which is a mixed minor salivary gland composed of mucous and serous acini, the myoepithelial cells surrounding acinar cells were all stained with both anti-podoplanin and anti- α -SMA antibodies (Fig. 3). In the double immunostaining for LYVE-1 and factor-VIII of the mouse sublingual gland which is a mixed major salivary gland mainly composed of mucous acini, lymphatic vessels and blood vessels were stained with only anti-LYVE-1 antibody and anti-factor-VIII antibody, respectively, and there were no myoepithelial, duct and acinar cells, expressing LYVE-1 or factor-VIII (Fig. 4). In the double immunostaining for podoplanin and α -SMA of the tunica muscularis of the mouse radix linguae, lymphatic vessels and blood vessels were stained with only anti-podoplanin antibody and anti- α -SMA antibody, respectively, and there were nerve sheaths stained with only anti-podoplanin antibody (Fig. 5). In the double immunostaining for LYVE-1 and factor-VIII of

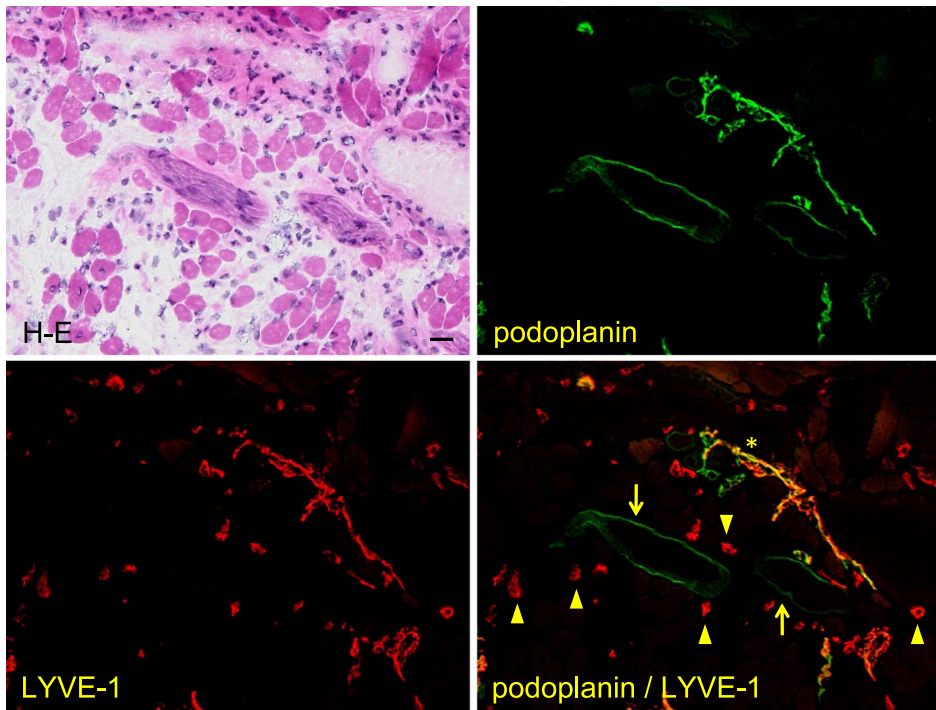


Fig. 1. Immunostaining for podoplanin and LYVE-1 of mouse apex linguae sections. H-E staining shows the intrinsic lingual muscle and nerve sheaths in the tunica muscularis. Nerve sheaths are stained with only anti-podoplanin antibody in green (arrows), single cells with only anti-LYVE-1 antibody in red (arrowheads), and lymphatic vessels with both anti-podoplanin and anti-LYVE-1 antibodies in yellow (asterisk). Bar=100 μ m.

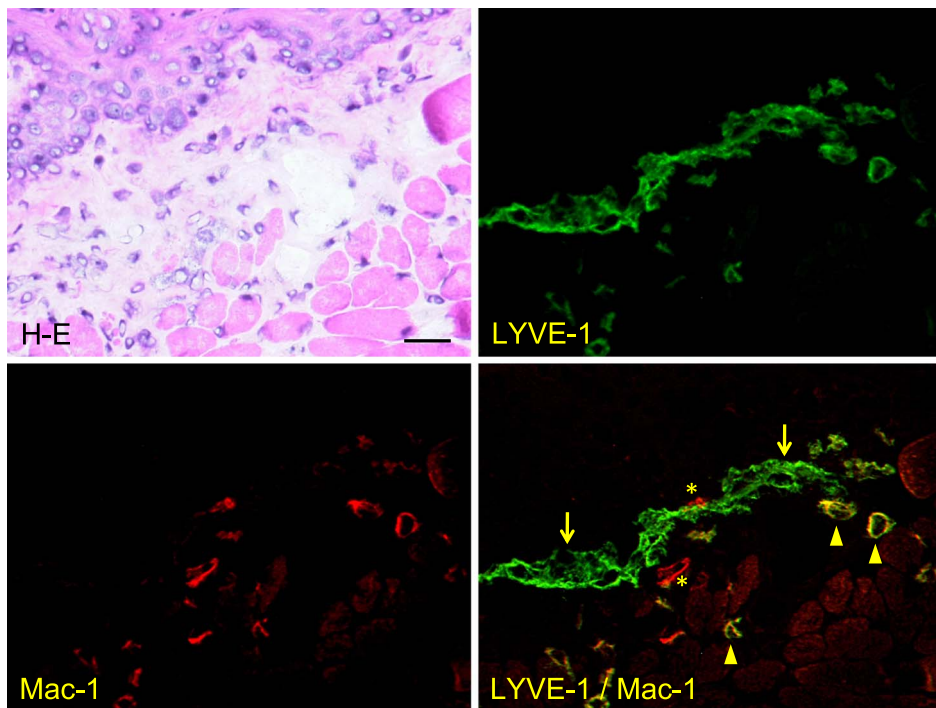


Fig. 2. Immunostaining for LYVE-1 and Mac-1 of mouse apex linguae sections. The H-E staining shows the lamina propria mucosae and the intrinsic lingual muscle in the lamina propria mucosae. Lymphatic vessels stained with only anti-LYVE-1 antibody in green (arrows), macrophages with only anti-Mac-1 antibody in red (asterisks), and macrophages with both anti-LYVE-1 and anti-Mac-1 antibodies in yellow (arrowheads). Bar=100 μ m.

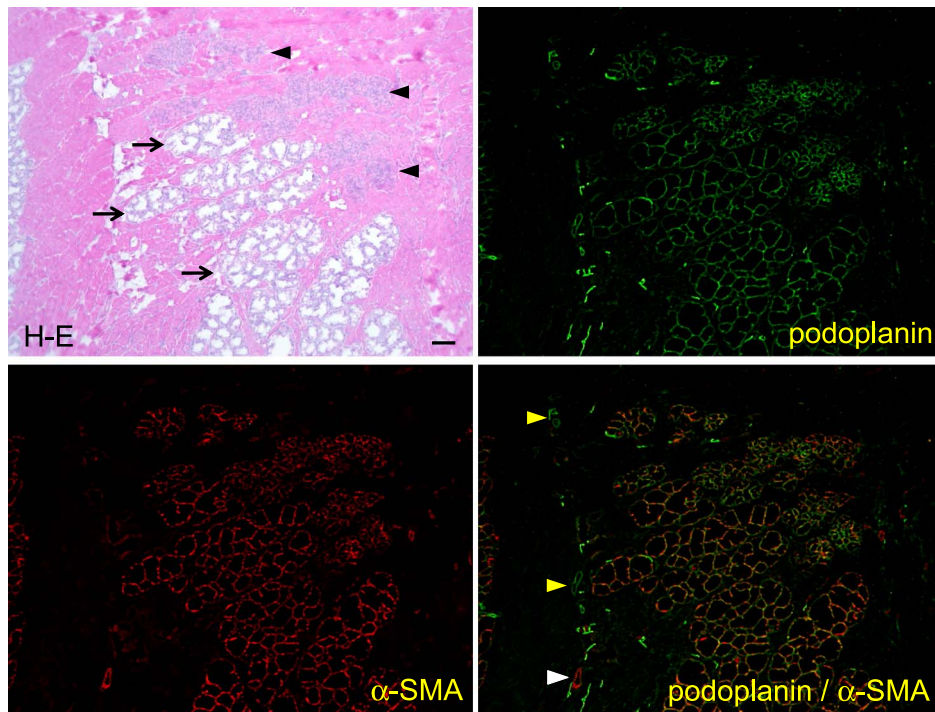


Fig. 3. Immunostaining for podoplanin and α -SMA of mouse radix linguae sections. H-E staining shows that the lingual gland is a mixed minor gland composed of mucous acini (arrows) and serous acini (arrowheads) in the radix linguae. In the lamina propria mucosae lymphatic vessels are stained with only anti-podoplanin antibody in green (yellow arrowheads) and blood vessels with only anti-SMA antibody in red (white arrowhead). In the lingual gland myoepithelial cells, the surrounding acinar cells are all stained with both anti-podoplanin and anti- α -SMA antibodies. Bar=100 μ m.

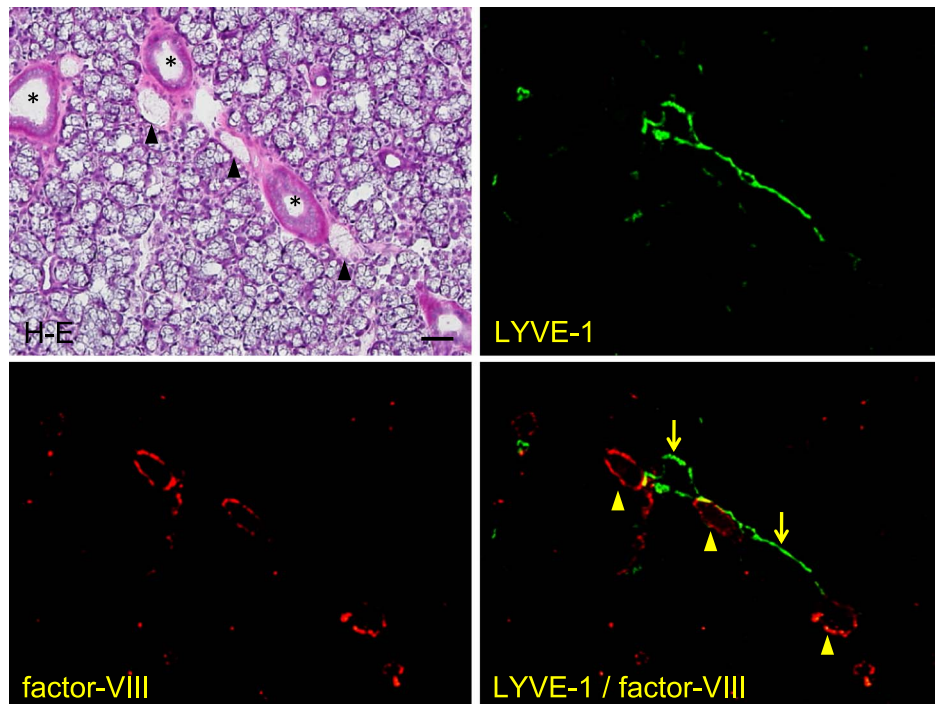


Fig. 4. Immunostaining for LYVE-1 and factor-VIII of the mouse sublingual gland. H-E staining shows that the intercalated ducts, having a narrow lumen surrounded by cuboidal cells, are interposed between acinar cells, and that the striated ducts (asterisks) are composed of columnar cells with acidophilic cytoplasm. Lymphatic vessels are stained with only anti-LYVE-1 antibody in green (arrows) and the blood vessels with only anti-factor-VIII antibody in red (arrowheads). Duct and acinar cells are stained by neither anti-LYVE-1 nor anti-factor-VIII antibodies. There are few LYVE-1-positive macrophages compared with tongue tissue. Bar=100 μ m.

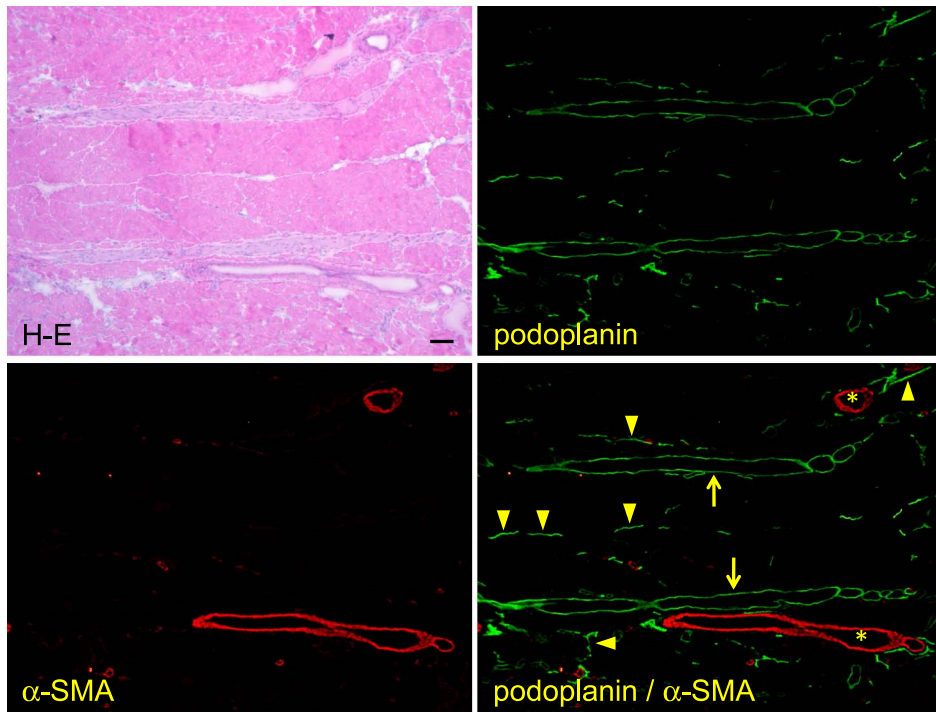


Fig. 5. Immunostaining for podoplanin and α -SMA of mouse radix linguae sections. The H-E staining shows the intrinsic lingual muscle and nerve sheaths in the tunica muscularis. Nerve sheaths (arrows) and lymphatic vessels (arrowheads) are stained with only anti-podoplanin antibody in green, and blood vessels with vascular smooth muscle cells are stained by anti-SMA antibody in red (asterisks). Bar=100 μ m.

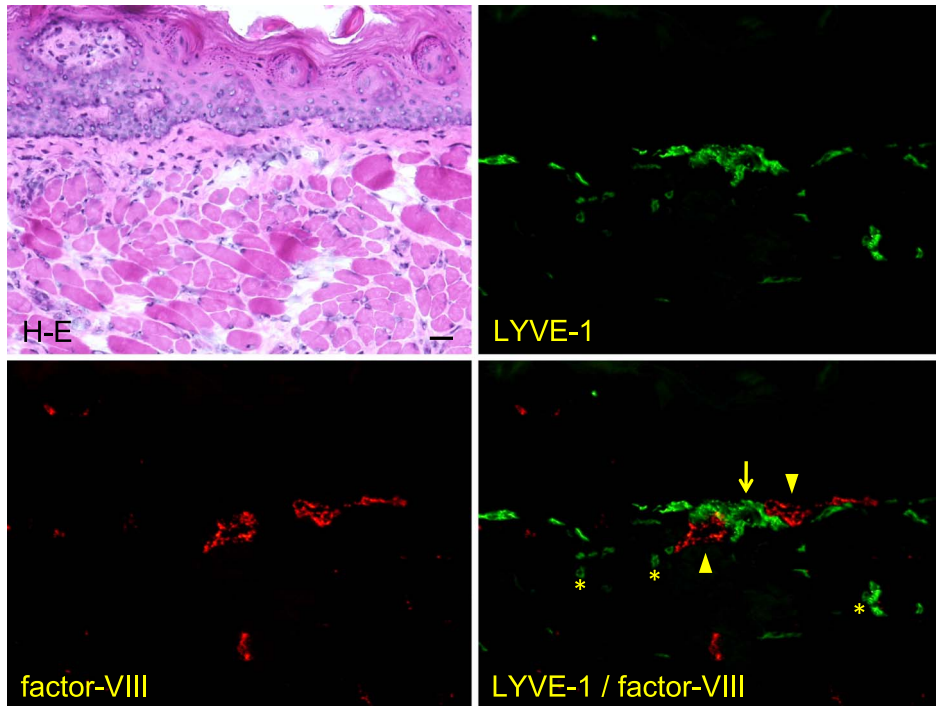


Fig. 6. Immunostaining for LYVE-1 and factor-VIII of mouse apex linguae sections. The H-E staining shows the lamina propria mucosae and the intrinsic lingual muscle in the tunica muscularis. In the lamina propria mucosae, lymphatic vessels (arrow) and single cells, which could be identified as macrophages with the unstained nucleus (asterisks), are all stained with only anti-LYVE-1 antibody in green, and the blood capillaries are stained with only anti-factor-VIII antibody in red (arrowheads). Bar=100 μ m.

the lamina propria mucosae of the mouse apex linguae, lymphatic vessels and blood vessels were stained with only anti-podoplanin antibody and factor-VIII antibody, respectively (Fig. 6).

IV. Discussion

In the comparison of podoplanin-positive and LYVE-1-positive cells in the tunica muscularis and lamina propria mucosae of mouse apex linguae, it was shown that podoplanin is expressed in nerve sheaths as well as lymphatic vessels, and that LYVE-1 is expressed in macrophages as well as lymphatic vessels (Figs. 1, 2). There is no report on the podoplanin expression in nerve-associated cells under normal conditions except for schwannoma [15]. The LYVE-1 and CD44 are the two most abundant receptors for the extracellular matrix glycosaminoglycan hyaluronan while LYVE-1 is the lymph-specific hyaluronan receptor on the luminal side of the lymphatic vessel wall [1, 5, 14]. CD44 and LYVE-1 bind both soluble and immobilized hyaluronan; however, unlike CD44, LYVE-1 is completely absent from blood vessels. LYVE-1 is a type I integral membrane protein and consists of a 322-residue polypeptide of which the sequence is 41% similar to CD44 having a 212-residue extracellular domain containing a prototypic hyaluronan-binding region. A copolymer of GlcNAc and GlcUA, glycosaminoglycan hyaluronan, is an abundant component of extracellular matrix and facilitates cell migration in inflamed mesenchymal tissues. Most of the hyaluronan in the tissue are turned over, collected in lymphatic vessels, and degraded in the medullary sinuses of lymph nodes [1, 5, 12, 14]. Recently, it has been reported that LYVE-1 is expressed by Mac-1-positive macrophages in malignant tumor and wound healing tissues. The CD44 is expressed on leukocytes and contributes to the traffic through tissues, while LYVE-1 regulates not only hyaluronan transportation but also macrophage migration by the binding function between LYVE-1 and hyaluronan [3, 14, 32]. The lymphatic vessels may play a role to control edema through hyaluronan transportation by LYVE-1. Podoplanin may be more useful than LYVE-1 to identify initial lymphatics in the oral mucosa tissue because of the difficulties of discriminating initial lymphatic endothelial cells from macrophages without Mac-1.

In the comparison of podoplanin-positive and α -SMA-positive cells in the lamina propria mucosae of the mouse radix linguae, it was shown that podoplanin and α -SMA are expressed in lymphatic vessels and in blood vessels, respectively. It was also observed that both podoplanin and α -SMA are expressed in the lingual gland (Fig. 3). In an earlier study, we initially investigated the distribution of lymphatic vessels in major salivary glands by immunostaining for the lymphatic endothelial cell marker, podoplanin, and discovered that most podoplanin-positive cells in major salivary glands were myoepithelial cells rather than lymphatic endothelial cells by immunostaining for podoplanin and myoepithelial cell marker P-cadherin [9]. In

the present study on the minor salivary gland of the tongue it was shown that podoplanin-positive cells are myoepithelial cells because the podoplanin-positive region accorded with the α -SMA-positive region in lingual glands. In the mouse sublingual gland, it was shown that LYVE-1 and factor-VIII are expressed in lymphatic vessels and in blood vessels, respectively, and that there were no myoepithelial cells expressing LYVE-1 or factor-VIII (Fig. 4). There were also LYVE-1-positive macrophages at a lesser extent than in the lingual tissue. On the identification of the lymphatic vascular distribution in both minor and major salivary glands, it is thought that the combination of LYVE-1 and factor-VIII, or LYVE-1 and Mac-1 are more useful than podoplanin alone because myoepithelial cells also express podoplanin.

In the tunica muscularis of mouse radix linguae, it was shown that podoplanin and α -SMA are expressed in lymphatic vessels and in blood vessels, respectively (Fig. 5). It is thought that the combination of podoplanin and α -SMA is useful to distinguish between lymphatic vessels and blood vessels having vascular smooth muscle cells in the oral tissues except for salivary glands [7, 34]. In Figures 1 and 5, the podoplanin-positive organ was identified as nerve sheaths based on haematoxylin-eosin (H-E) findings and on the expression of neither LYVE-1 nor α -SMA in the tunica muscularis of both apex and radix linguae. The general expression of podoplanin in nerve sheaths should be investigated. Recently, podoplanin upregulation has been reported in oral squamous cell carcinomas [26, 36]. Sixty percent of oral tongue cancers express high levels of podoplanin. Patients with high levels of podoplanin have a statistically significantly higher rate of lymph node metastasis and those with lymph node metastasis high-levels of podoplanin show the shortest disease-specific survival than other patients. These reports suggest that podoplanin serves as a predictor for the risk for lymph node metastasis and poor clinical outcome [17, 21, 40]. Therefore, it would be important to investigate the podoplanin expression in oral cancer but it is necessary to discriminate between oral cancer cells and initial lymphatic endothelium by using other markers like LYVE-1.

In the lamina propria mucosae of mouse apex linguae, it was shown that LYVE-1 and factor-VIII are expressed in lymphatic vessels and in blood vessels, respectively (Fig. 6). Factor-VIII but not α -SMA should be targeted to identify blood capillaries because there are no vascular smooth muscle cells in the capillaries. It is known that factor-VIII exists not only in blood vessels but also lymphatic vessels, and that the expression level is extremely lower in lymphatic vessels than blood vessels [24]. Since the immunostaining of factor-VIII on the lymphatic vessels was completely masked by the strong immunostaining to LYVE-1 (Fig. 6), it seems that factor-VIII is useful to distinguish blood capillaries from initial lymphatics immunostained with anti-podoplanin and anti-LYVE-1 antibodies. However, in order to identify initial lymphatics by LYVE-1, it is necessary to distinguish LYVE-1-positive vessels from macrophages by a marker

like Mac-1.

In conclusion for the molecules to identify the lingual initial lymphatics, podoplanin should be chosen rather than LYVE-1 because of LYVE-1-positive macrophages in the normal oral mucosa whereas LYVE-1 should be chosen rather than podoplanin because of podoplanin-positive myo-epithelial cells and oral cancer cells in the salivary glands and oral squamous cell carcinomas.

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