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Immunohistochemical co-localization of lymphatics and blood vessels in oral squamous cell carcinomas

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BACKGROUND: Differentiating lymphatic vessels from blood vessels is difficult, partly due to the lack of a specific method for identifying lymphatics. A new lymphatic vessel-reactive antibody, D2-40 has recently become commercially available. We examined the selectivity of D2-40 for lymphatics in oral neoplastic lesions for discrimination from blood vessels.

METHODS: Formalin-fixed, paraffin-embedded sections of oral lymphangiomas ($n = 3$), oral hemangiomas ($n = 7$), and oral squamous cell carcinomas (OSCC, $n = 46$) were double immunostained with D2-40 and anti-CD34 monoclonal antibodies (MoAb) using ENVISION-polymer technique with 5-bromo-4-chloro-3-indoxyl-phosphate (BCIP)/nitroblue tetrazolium chloride (NBT) and 3,3′-diaminobenzidine (DAB) as color reagents, respectively.

RESULTS: In the oral lymphangiomas and hemangiomas D2-40 was detected in all lymphatics, while all blood vessels were positive for CD34. In OSCC, number of vessels for lymphatics ($P < 0.01$) and for blood vessels in the perineoplastic areas were significantly greater than those in intratumoral areas.

CONCLUSIONS: These results indicate that lymphatic proliferation might be much more extensive in the peritumoral area than intratumoral.

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Keywords: blood vessels; double immunostaining; lymphatics; oral squamous cell carcinoma

Introduction

Lymphatic vessels are well known to be a major route for metastasis in many types of carcinoma. However, despite the obvious roles of the lymphatic circulation under both physiological and pathological conditions, compared with blood vessels, the lymphatic system has been relatively poorly studied until recently. This was at least partly due to difficulties in recognizing these vessels within tissues because there were no specific antibodies. Fortunately, several relatively specific antibodies for lymphatic endothelium, such as VEGR3 (1), podoplanin (2), lymphatic vessel endothelial HA receptor-1 (LYVE-1) (3), and Proxl (4), have been identified in recent years. By use of these antibodies, the presence of some intratumoral lymphatic vessels has been confirmed (5), despite the long-held belief that lymphatic vessels would be absent, become collapsed, or be unable to penetrate through the dense interiors of solid tumors (6). Therefore, lymphangiogenesis inside or around tumors, which would increase the potential for lymphatic metastasis of cancer, is increasingly being regarded as a possibility.

In addition to the factors mentioned above, M2A, a MW 40,000 O-linked sialoglycoprotein, has recently been found to be expressed specifically on the endothelium of lymphatic vessels, thus acting as an indicator in tissue sections. A monoclonal antibody (MoAb) against M2A, D2-40, has now become commercially available; however, its selectivity for lymphatics requires further confirmation (7). The objectives of this study were to confirm the selectivity of MoAb D2-40 for lymphatic endothelium and to establish a reliable double immunostaining method by which both lymphatics and blood vessels could be viewed in the same section under the microscope. In the present study, we examined the distribution of lymphatics and blood vessels within resected specimens of oral lymphangiomas, hemangiomas, and squamous cell carcinomas (OSCCs) using a double immunostaining method combining the MoAb D2-40 with anti-CD34, a well-known specific monoclonal antibody for vascular endothelium.

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Materials and methods

Specimens

Formalin-fixed, paraffin-embedded sections from three lymphangiomas, seven hemangiomas, and 46 OSCCs

Table 1 Clinical data and intratumoral and peritumoral positivity in oral squamous cell carcinomas

					D2-40 positivity		*CD34 positivity*	
No.	*Gender*	*Age*	*Site*	*Type*	*Intra*	*Peri*	*Intra*	*Peri*
1	F	71	Cheek	Moderately	+2	+3	+3	+3
2	F	71	Palate	Poorly	+	+2	+3	+3
3	M	57	Tongue	Poorly	+	+2	+2	+2
4	M	60	Gingiva	Moderately	+2	+2	+2	+3
5	F	68	Gingiva	Moderately	+2	+2	+2	+3
6	M	53	Tongue	Well	+	+2	+	+2
7	F	67	Tongue	Well	+	+	+2	+2
8	M	59	Gingiva	Moderately	+	+2	+2	+2
9	M	62	Gingiva	Well	+	+2	+2	+2
10	M	49	Gingiva	Well	+2	+2	+2	+3
11	M	64	Palate	Moderately	+2	+2	+2	+2
12	M	68	Tongue	Well	+	+2	+2	+2
13	M	72	Tongue	Well	+	+2	+2	+2
14	F	57	Tongue	Well	+2	+2	+2	+2
15	F	82	Gingiva	Moderately	+2	+2	+2	+2
16	M	71	Tongue	Poorly	+	+2	+2	+3
17	F	53	Gingiva	Moderately	+	+2	+2	+2
18	M	71	Tongue	Well	+	+2	+2	+2
19	M	54	Cheek	Poorly	+	+	+2	+2
20	F	53	Gingiva	Well	+2	+2	+2	+2
21	F	70	Gingiva	Well	+	+2	+2	+2
22	M	73	Gingiva	Moderately	+	+2	+	+2
23	M	57	Floor	Moderately	+	+2	+	+2
24	M	48	Floor	Well	+	+2	+2	+2
25	F	72	Floor	Well	+	+2	+	+2
26	F	57	Tongue	Moderately	+2	+2	+2	+2
27	M	58	Tongue	Well	+	+2	+2	+2
28	F	54	Gingiva	Well	+	+	+	+
29	M	47	Cheek	Well	+	+	+2	+2
30	F	38	Tongue	Poorly	+	+2	+	+2
31	F	66	Gingiva	Well	+2	+2	+2	+2
32	M	61	Floor	Poorly	+	+2	+2	+2
33	F	54	Cheek	Moderately	+2	+2	+2	+2
34	M	70	Floor	Well	+	+2	+2	+2
35	M	46	Tongue	Moderately	+	+2	+	+2
36	F	66	Gingiva	Well	+	+2	+	+2
37	M	58	Gingiva	Moderately	+	+2	+2	+2
38	M	74	Gingiva	Moderately	+	+2	+2	+2
39	M	64	Cheek	Poorly	+	+2	+2	+2
40	M	72	Cheek	Poorly	+2	+2	+2	+2
41	F	66	Gingiva	Well	+	+2	+2	+2
42	F	65	Gingiva	Moderately	+	+2	+2	+2
43	F	68	U-Sinus	Well	+	+2	+2	+2
44	M	76	Floor	Well	+	+	+2	+2
45	M	49	Gingiva	Moderately	+	+2	+2	+2
46	F	72	Gingiva	Well	+	+	+	+2

No., case number; Intra, intratumoral area; Peri, peritumoral area; Well, well differentiated; Moderately, moderately differentiated; Poorly, poorly differentiated; +, slight quantity; +2, moderate quantity; +3, severe quantity.

were obtained from archival paraffin blocks at Osaka Dental University Hospital. The lymphangioma specimens were from two males and one female ranging in age from 23 to 67 years (mean age, 45 years), hemangioma specimens were from one male and six females from 31 to 67 years (55 years) and OSCC specimens were from 26 males and 20 females from 38 to 82 years (62 years). OSCCs examined were histologically classified as well ($n = 22$), moderately ($n = 16$), or poorly ($n = 8$) differentiated (8) (Table 1).

Immunohistochemistry

These sections were dewaxed and successively rehydrated in a graded ethanol series for 5 min at each concentration. After being immersed in 3% H_2O_2 solution for 5 min to block endogenous peroxidase, the sections were incubated for 30 min at room temperature (RT) with anti-CD34 MoAb (Nichirei Co., Tokyo, Japan) to detect blood vessels, reacted with ENVISION-horseradish peroxidase (Dako Cytomation, Carpenteria, CA, USA) for 30 min at RT, and visualized with 3,3′diaminobenzidine (Dojin Chemicals, Kumamoto, Japan) with 0.01% H_2O_2. The sections were then subjected to autoclaving for 15 min at 121°C to retrieve the antigen, after which they were incubated for 1 h at RT with D2-40 MoAb (Signet Laboratories Inc., Dedham, MA, USA) to detect lymphatics, reacted with ENVISION-alkaline phosphatase (Dako Cytomation) for 30 min, and visualized with

BCIP/NBT (Dako Cytomation). Nuclear fast red was used for counterstaining as the occasion demands. Finally, the sections were mounted on Aquatex® (Merck, Darmstadt, Germany) and observed by light microscopy. The quantity of lymphatics and blood vessels was divided into three degrees of slight, moderate, and severe under light microscope of ×200 magnification.

Results

All of the hemangioma, lymphangioma, and OSCC sections were positive for D2-40 and CD34. D2-40-positive vessels, which were stained blue, did not contain erythrocytes, whereas all of the CD34-positive vessels, which were stained brown, did. Thus, the D2-40-positive vessels were identified as lymphatic vessels, while the CD34-positive vessels were identified as blood vessels. Furthermore, many more CD34-positive vessels than D2-40 positive vessels were observed in the hemangiomas (Fig. 1a). On the other hand, many more D2-40-positive vessels than CD34-positive vessels were present in the lymphangiomas (Fig. 1b). Thus, lymphatics could be easily differentiated from blood vessels in the same section, because of the different colors on immunostaining.

Degree of the localization of lymphatic and blood vessels in OSCC

The degree of D2-40 positivity for lymphatic vessels was classified as slight (+): fewer than five positive

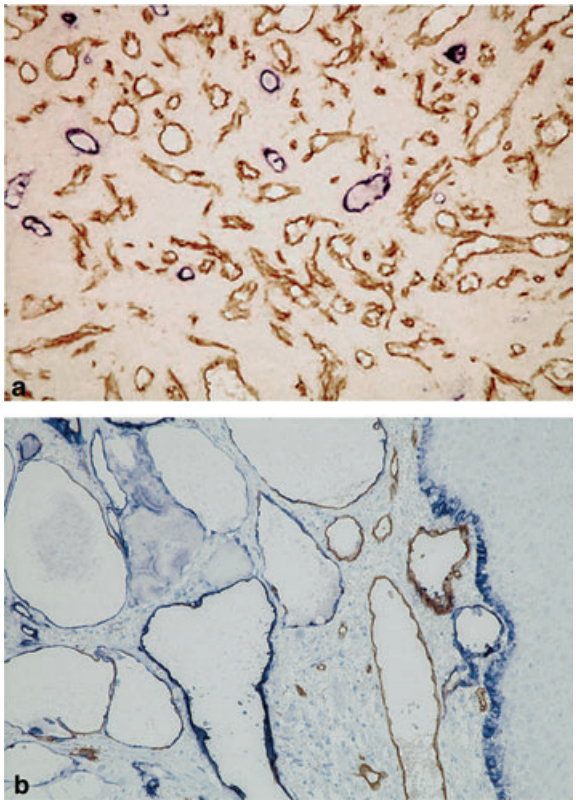

Figure 1 (a) Double immunostaining with CD34 and D2-40 in a hemangioma. The brown-stained structures are CD34-positive blood capillaries, while the blue-stained structures are D2-40-positive lymphatic vessels (×110). (b) Double immunostaining with CD34 and D2-40 in a lymphangioma. The lymphatic vessels are stained blue by D2-40, while the blood capillaries are stained brown by CD34. The basal cell membranes of the squamous epithelium are also positive for D2-40 (×110).

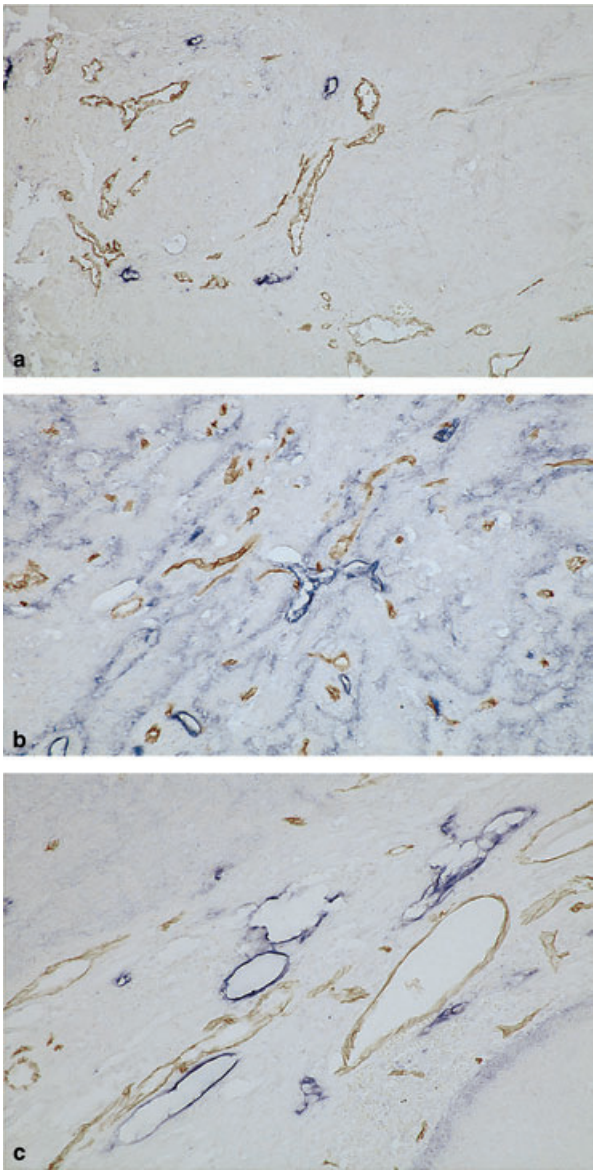

Figure 2 Double immunostaining with CD34 and D2-40 in a well and moderately differentiated oral squamous cell carcinomas. (a) Fewer than five lymphatic vessels, but more than 10 blood vessels were observed in the intratumoral area with well-differentiated oral squamous cell carcinomas. This photograph indicates + for lymphatic vessels and +2 for blood vessels in the intratumoral area (×110). (b) About five lymphatic vessels, but more than 20 blood vessels were observed in the intratumoral area with moderately differentiated oral squamous cell carcinomas. The photograph shows +2 for lymphatic vessels and +3 for blood vessels in the intratumoral area (×110). (c) More than five lymphatic vessels and more than 20 blood vessels were discernible in the peritumoral area with well-differentiated oral squamous cell carcinomas. The scores are +2 for lymphatic vessels and +3 for blood vessels in the peritumoral area (×110).

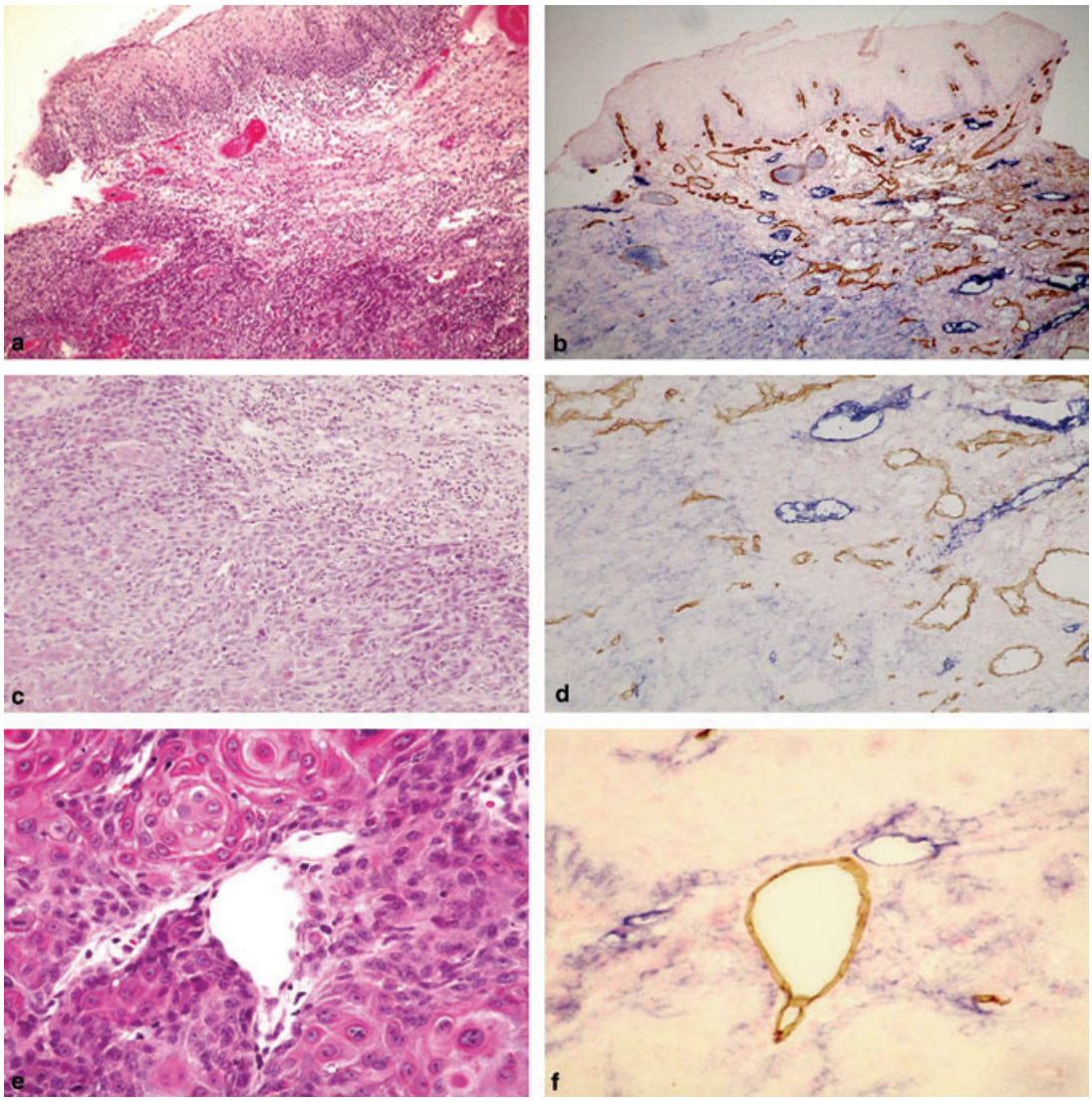

Figure 3 (a) A histopathological section of an oral squamous cell carcinomas, showing the tumoral region in the lower portion and the peritumoral region in the upper portion (hematoxylin and eosin stain, ×70). (b) A serial section of the oral squamous cell carcinomas shown in Fig. 3(a) after double immunostaining with CD34 and D2-40. Lymphatic vessels (blue) are found primarily in the peritumoral area rather than the intratumoral area (×70). (c) The photograph is a high-power view of the section shown in Fig. 3(a). Tumor nests are seen in the lower two thirds (×110). (d) The photograph is a high-power view of the section in Fig. 3(b). Lymphatic vessels (blue) and blood vessels (brown) are observed in the peritumoral area on the right. In the marginal area of the tumor nest, basal-cell like cells are positive (blue) for D2-40 (×110). (e) Histopathological section of an oral squamous cell carcinomas stained with hematoxylin and eosin, showing vessel structures surrounded by nests of atypical cells. Two vessel-like structures can be seen in the middle of the intratumoral region (×300). (f) Serial section of the oral squamous cell carcinomas shown in Fig. 3(d) after double immunostaining with CD34 and D2-40. The section shows intratumoral lymphatic vessels positive for D2-40 (blue) and intratumoral blood vessels positive for CD34 (brown) (×300).

lymphatic vessels (Fig. 2a), moderate (+2): 5 ≤ positive lymphatic vessels ≤ 10 (Fig. 2b and c), and severe (+3): more than 10 positive lymphatic vessels. The degree of CD34 positivity for blood vessels was classified as slight (+): fewer than 10 positive blood vessels, moderate (+2): 10 ≤ positive blood vessels ≤ 20 (Fig. 2a), and severe (+3): more than 20 positive blood vessels (Fig. 2b and c). The number of vessels was counted in a 0.2 mm^2 area under a 20× objective.

Localization of lymphatic and blood vessels

In almost all cases, the endothelial vessels stained by D2-40 showed no overlap with those stained by CD34. However, D2-40 reactivity was detected in the basal cell membranes of squamous epithelium (Fig. 1b). Histopathologically, vessels containing erythrocytes were frequently seen in both well and moderately differentiated OSCC (Fig. 3a, c and e). In these carcinomas, lymphatics were primarily distributed in the peritumoral areas, although a few were found inside the neoplastic tissues (Fig. 3b, d and f). The start of lymphatic invasion by carcinoma cells (Fig. 4a) could be clearly observed, since the lymphatic vessels were stained blue by D2-40 (Fig. 4b). The number of the lymphatic vessels in the peritumoral areas was greater than that inside the cancer tissues (Chi-square-test, $P < 0.01$) (Table 1). The

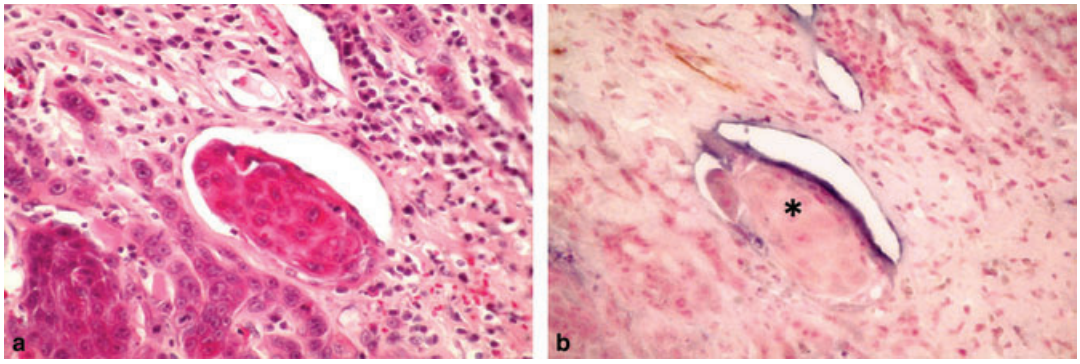

Figure 4 (a) A histopathological section of an oral squamous cell carcinomas showing tumor invasion to lymphatics (hematoxylin and eosin stain, ×300). (b) A serial section of the oral squamous cell carcinomas shown in Fig. 4(a) after double immunostaining with CD34 and D2-40. Carcinoma cells (*) can be seen invading the lymphatic vessels, which are stained blue (×300).

number of blood vessels in the peritumoral areas was also greater tendency than that inside the cancer nests (Table 1).

Discussion

In the differentiation of lymphatic vessels from blood vessels enzymatic histochemical staining techniques have been used for studying these anatomical structures for many years (9). One principle underlying the enzymatic histochemical technique used for identifying lymphatics is that the endothelium of these vessels differs in enzymatic reactivity from that of blood vessels: lymphatic endothelium has been reported to show high reactivity for 5′-nucleotidase, while blood vessel endothelium shows high reactivity for alkaline phosphatase, which is detected only in cryostat sections, and not in paraffin sections. In addition, the specificity of enzymatic histochemical double staining for lymphatics and blood vessels is doubtful, since arterial endothelium has also been confirmed to show reactivity for 5′-nucleotidase (10). Moreover, enzymatic histochemical techniques usually require the use of frozen sections in order to preserve sufficient enzymatic activity. Therefore, enzymatic histochemistry is unsuitable for use on most archival specimens, which are generally fixed with formalin and embedded in paraffin.

Differentiation between lymphatics and blood vessels by light microscopy has long been a problem, since differentiation criteria for these structures are inevitably subjective. Fortunately, several specific antibodies for lymphatics have been found in recent years. These factors include VGFR-3, a tyrosine kinase receptor for vascular endothelial growth factor (VEGF)-C, and VEGF-D. VGFR-3 was the first molecule found to be expressed in the lymphatic endothelium; however, it was later discovered that this factor is also expressed in the fenestrated blood vessels of normal tissues as well as in angiogenic blood vessels in the retina, wounds, and tumors (11, 12). Podoplanin, a membrane protein of glomerular epithelial cells, was found to be expressed in lymphatic endothelium and in vascular tumors. Podoplanin is also present in small lymphatics, but not in large ones that contain smooth muscle (13, 14). Prox1 is a homeobox transcription factor gene product that is involved in the growth and elongation of lymphatic vessel sprouts during development. It is also expressed in non-endothelial cells in the lens, heart, liver, pancreas, and nervous system (15). LYVE-1 is a receptor for extracellular matrix/lymphatic fluid glycosaminoglycan in lymphatic endothelial cells. It is not, however, completely specific for lymphatic endothelial cells since it has also recently been found to be present in normal hepatic blood sinusoidal endothelial cells (15). In addition, D2-40 was detected in the basal cell membrane of the squamous epithelium in the present study. However, D2-40 MoAb has been reported to be specific for lymphatic vessels (16).

Although in a previous study a series of vascular lesions were separately stained with D2-40 or CD34 (17), simultaneous staining with D2-40 and CD34 was not carried out. The results showed that these antibodies stained non-overlapping thin-walled endothelial channels n adjacent sections and that D2-40 also stained lymphatic channels, which were not stained with PAL-E or CD34 (17). These findings support the results of our study employing double immunostaining with CD34 and D2-40 MoAb.

In recent years, many studies have confirmed that the proliferation of lymphatic vessels that occurs during embryogenesis and normal tissue growth is regulated by VEGF-C and VEGF-D and their receptors (18, 19). On the other hand, VEGF-C has been detected in several different types of carcinomas, and in some cases its levels have been shown to correlate with nodal metastasis (20, 21). Therefore, the possibility that lymphangiogenesis induced by malignant cells plays a role in lymph node metastasis has been mentioned in several reports. However, a study of spontaneously arising pancreatic tumors in RIP-Tag/VEGF-C transgenic mice showed that an increase in lymphatic metastasis occurred without intratumoral lymphangiogenesis (22). Moreover, in patients with hepatocellular or metastatic colorectal carcinomas lymphatic vessels have been observed only in the peripheries of the tumors (15). In the present study, lymphatics were

significantly more dense in the perineoplastic area than in the intratumoral areas ($P < 0.01$). Although our study revealed that a few lymphatic vessels were indeed present in the intratumoral region, these lymphatics seem to be pre-existing structures from normal tissue that had become trapped by squamous carcinoma cells, rather than newly proliferating vessels, because they were all located just inside the connective tissue rather than in the carcinoma nests. The number of blood vessels was much more in the peritumoral area than intratumoral area. However, the number of lymphatic vessels were considerably different in the two areas in the well, moderately, and poorly differentiated OSCC. The number of lymphatic vessels was definitely greater in the peritumoral area than in the intratumoral area, regardless of the degree of cancer differentiation. We hope that D2-40 will be helpful in our future attempts to elucidate the mechanisms underlying lymphangiogenesis and the degree to which it facilitates lymph node metastasis.

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