Oral Diseases (2007) 13, 99–104. doi:10.1111/j.1601-0825.2006.01256.x

http://www.blackwellmunksgaard.com

ORIGINAL ARTICLE

Histomorphometrical study in cavernous lymphangioma of the tongue

T Yaita, K Onodera, H Xu, K Ooya

Division of Oral Pathology, Department of Oral Medicine and Surgery, Tohoku University Graduate School of Dentistry, Sendai, Japan

OBJECTIVE: To study the histomorphometrical characteristics of lymphatic vessels in cavernous lymphangiomas of the tongue.
MATERIAL AND METHODS: Immunohistochemical stainings were prepared in the 20 specimens with three antibodies [D2-40, CD31 and proliferating cell nuclear antigen (PCNA)]. Three-dimensional (3D) reconstruction and histometrical analysis of the lymphatic vessels was also examined.
RESULTS: Distinctly positive staining for D2-40 was found in dilated lymphatic vessels located in the lamina propria beneath the thinned covering epithelium. Small blood vessels stained positively for CD31 were present in the lamina propria. PCNA-positive lymphatic endothelial cells were scattered in both control and lymphangioma. The 3D architecture of lymphatic vessels was characterized by a complex network with irregular branches in the lamina propria. Histometrical analysis showed that the number of lymphatic endothelial cells per lymphatic vessel perimeter in cavernous lymphangioma was significantly higher than that in control. There were no significant differences in the lymphatic density and the ratio of PCNA-positive lymphatic endothelial cell nuclei to the total number of lymphatic endothelial cell nuclei between control and lymphangioma.
CONCLUSIONS: These results indicate the absence of excessive endothelial cell proliferation in dilated lymphatic vessels in cavernous lymphangioma. Cavernous lymphangioma may be attributed to the enlargement of lymphatic vessels without the tumorous proliferation of lymphatic endothelial cells.
Oral Diseases (2007) **13**, 99–104

Keywords: cavernous lymphangioma; lymphatic vessel; D2-40

Correspondence: Tomoyuki Yaita, Division of Oral Pathology, Department of Oral Medicine and Surgery, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba, Sendai 980-8575, Japan. Tel: +81 22 717 8303, Fax: +81 22 717 8304, E-mail: onodera@opath.dent.tohoku.ac.jp
Received 21 November 2005; revised 4 January 2006; accepted 30 January 2006

Introduction

Lymphangiomas are uncommon congenital hamartomas of the lymphatic system, usually diagnosed in infancy and early childhood as lobulated masses or cystic lesions arising in the head and neck, axilla, and abdomen (Bill and Sumner, 1965; Enzinger and Weiss, 1995). Oral lymphangiomas occur in the cheek, floor of the mouth, lips, and most commonly the tongue (Brennan *et al*, 1997; Park *et al*, 2002; Hudson and Meszaros, 2003). Tongue lymphangiomas typically show multiple blister-like nodules on the enlarged dorsal surface of the tongue. These lesions may also be diffuse, involving large portions of the tongue, causing macroglossia (Guelmann and Katz, 2003).

Histopathologically, lymphangiomas are classified into three types: simple, cavernous, and cystic (Ikemura *et al*, 1987; Balakrishnan and Bailey, 1991; Enzinger and Weiss, 1995). Simple lymphangiomas consist of a proliferation of thin-walled, endothelium-lined, capillary-sized lymphatic vessels (Enzinger and Weiss, 1995; Shimizu *et al*, 1999). Cavernous lymphangiomas are characterized by the presence of dilated endothelium-lined lymphatic vessels (Enzinger and Weiss, 1995). Cystic lymphangiomas are made up of large macroscopic lymphatic vessels and are formed by multilocular cystic masses of various sizes (Ikemura *et al*, 1987; Enzinger and Weiss, 1995; Shimizu *et al*, 1999). Most tongue lymphangiomas are cavernous, characterized by dilated lymphatic vessels invading surrounding tissues (Enzinger and Weiss, 1995).

Although previous studies have examined lymphatic and blood vessels in lymphangioma, the lack of reliable lymphatic endothelial markers has led to inconsistent results concerning the lymphatic vasculature of lymphangiomas. The architectures of blood and lymphatic vessels share similarities, but have distinct differences (Albertine *et al*, 1982; Azzali, 2003; Rovenská *et al*, 2003). Blood vessels recruit supporting pericytes and smooth muscle cells, which produce extracellular matrix as the vasculature matures (Albertine *et al*, 1982). On the other hand, lymphatic vessels have only a sparse covering of recruited supporting cells and lack a

continuous basement membrane, thus accounting for their permeability (Rovenská *et al*, 2003). Blood vessels show tight junctions, whereas lymphatic vessels have fenestrations, suggesting differences in cell–cell interactions (Rovenská *et al*, 2003).

Despite obvious differences between blood and lymphatic vessels, molecular markers capable of distinguishing these vessels were previously unavailable. During the past decade, however, several molecules unique to lymphatic vessels have been identified (Erovic *et al*, 2003; Streit and Detmar, 2003). The development of new analytical tools has permitted the lymphatic system and its relevance to disease to be studied at the molecular level.

In this study, the histomorphometrical characteristics of lymphatic vessels in cavernous lymphangioma of the tongue were assessed in association with histochemical, immunohistochemical, three-dimensional (3D), and histometrical examinations.

Material and methods

The 20 specimens of cavernous lymphangioma of the tongue examined in this study were gathered from the surgical pathology case files (between 1980 and 2002) retained at Tohoku University Graduate School of Dentistry. The cases were 10 men and 10 women, 2–68 years of age (totally 20 specimens; mean age, 21.7 years). Specimens of oral normal mucosa (control) were taken from the resection margins of cavernous lymphangioma specimens showing no signs of inflammation. Biopsy specimens had been fixed in 10% formalin solution, dehydrated in graded ethanol solutions and xylene, and embedded in paraffin. Paraffin-embedded blocks were sectioned in 3-μm-thick slices and mounted on glass slides.

Immunohistochemical staining

CD31 (1:40, Dako, Kyoto, Japan) and D2-40 (1:40, Tarner, Osaka, Japan), specific markers for panendothelial vessels (CD31) and lymphatic vessels (D2-40), respectively, were used in this study. In addition, proliferating cell nuclear antigen (PCNA, 1:100, DAKO, Kyoto) was used to detect lymphatic endothelial cell proliferation. For CD31 and D2-40 staining, antigen retrieval was done by heating slides for 30 min in citrate buffer (pH 6.0) at 98°C. No antigen retrieval was required for PCNA immunohistochemistry. After antigen retrieval, a cooling off period of 20 min preceded pre-incubation for 15 min in wash buffer (DakoCytomation); after which endogenous peroxidase activity was quenched using peroxidase-blocking solution (DakoCytomation). The slides were incubated with each primary antibody for 16 h at 4°C. Antibody binding was visualized using the EnVision + Dual Link system and diaminobenzidine as chromogen (DakoCytomation). The slides were counterstained and mounted.

Three-dimensional reconstruction

Representative sections were selected for 3D reconstruction, done with the use of a computer graphics analysis system (OZ, Rise Co., Miyagi, Japan) after hematoxylin and eosin (H&E) staining and immunohistochemical staining. Briefly, 100 serial sections 3 μm in thickness were prepared, thereby covering a total thickness of 300 μm. A digital camera (Penguin 600CL; Pixera Co., CA, USA) was used. Serial photographic images of the outlines of the lymphatic vessels and covering epithelium of the cavernous lymphangiomas were traced manually on the computer screen and were converted into computer-generated 3D images suitable for examination from different perspectives.

Histometrical analysis

Microscopic fields were captured at 400× magnification (40 objective and 10 ocular) with a microscope (BH-2; Olympus, Tokyo, Japan) coupled to a digital camera system for microscopy (Penguin 600CL; Pixera Co., Los Angeles, CA, USA). The images were stored in the digital memory and displayed on the monitor. Three microscopic fields were randomly selected on serial histological sections labeled by the primary antibodies, CD31 and D2-40. The same microscopic fields of the serial sections were examined under a light microscope. Immunolabeled lymphatic vessels were manually outlined. The number of lymphatic vessels per square millimeter, the size of lymphatic vessels, and the number of lymphatic endothelial cells per lymphatic vessel perimeter were then examined by image analysis. Moreover, the total number of PCNA-positive lymphatic endothelial cells per lymphatic vessel perimeter was counted. The PCNA index for each lesion was calculated by the following formula: (number of PCNA-positive lymphatic endothelial cell nuclei/total number of lymphatic endothelial cell nuclei) $\times$ 100.

Statistical analysis

All values are expressed as mean $\pm$ standard deviation. The statistical significance of differences between groups was assessed by repeated-measures analysis of variance (ANOVA) or Student's *t*-test, as appropriate. Differences with P values < 0.05 were considered statistically significant.

Results

Histopathologic findings

In the control, there were no dilated vessels or vascular proliferation in the lamina propria. Several vessels showed flat-lined endothelium with a wavy outline. In contrast, the cavernous lymphangioma specimens were composed of proliferating large dilated vessels located in the lamina propria beneath the thinned covering epithelium (Figure 1a). Lymphatic vessels were distinguishable from blood vessels for their very thin vessel wall. Blood vessels had a thicker wall and contained red blood cells (Figure 1b).

Immunohistochemical findings

On immunohistochemical studies, blood vessels stained CD31 positive and D2-40 negative. Blood vessels were recognized by intravascular erythrocytes and smooth

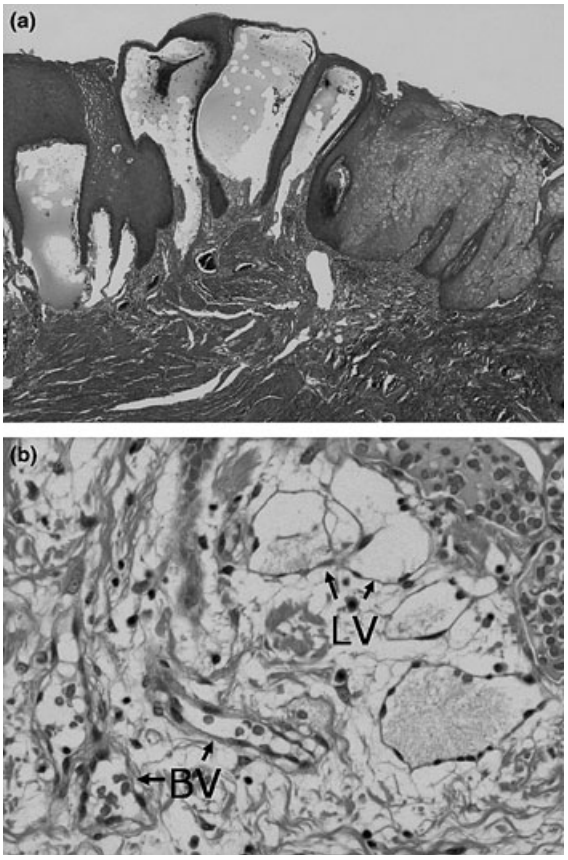

Figure 1 In cavernous lymphangioma, dilated vessels were located in the lamina propria beneath the thinned covering epithelium (a, H&E ×20). Lymphatic vessels were distinguishable from blood vessels for their very thin vessel wall. Blood vessels had a thicker wall and contained red blood cells (b, H&E ×400) (LV: lymphatic vessel, BV: blood vessel)

muscle cells (Figure 2a). Conversely, lymphatic vessels were strongly D2-40 positive and weakly CD31 positive. Lymphatic vessels were confirmed by the presence of flat-lined endothelium with a wavy outline and extremely scant cytoplasm and by the absence of intravascular erythrocytes and a smooth muscle cell layer (Figure 2b). In control, both blood and lymphatic vessels were located in the lamina propria. Lymphatic vessels were comma-shaped small structures with tiny lumens. In the cavernous lymphangiomas, the dilated vessels located in the lamina propria beneath the thinned covering epithelium were stained by D2-40, indicating lymphatic vessels.

Three-dimensional findings (Figure 3)

In control (Figure 3, left side) lymphatic vessels gave rise to progressively smaller branches near the covering epithelium in the lamina propria. An orderly network of lymphatic vessels was visible in the lamina propria.

In cavernous lymphangioma (Figure 3, right side) lymphatic vessels presented a complex network characterized by irregular branching with dilated lymphatic spaces of various sizes. Lymphatic vessels in the lamina propria were larger than those in other regions and

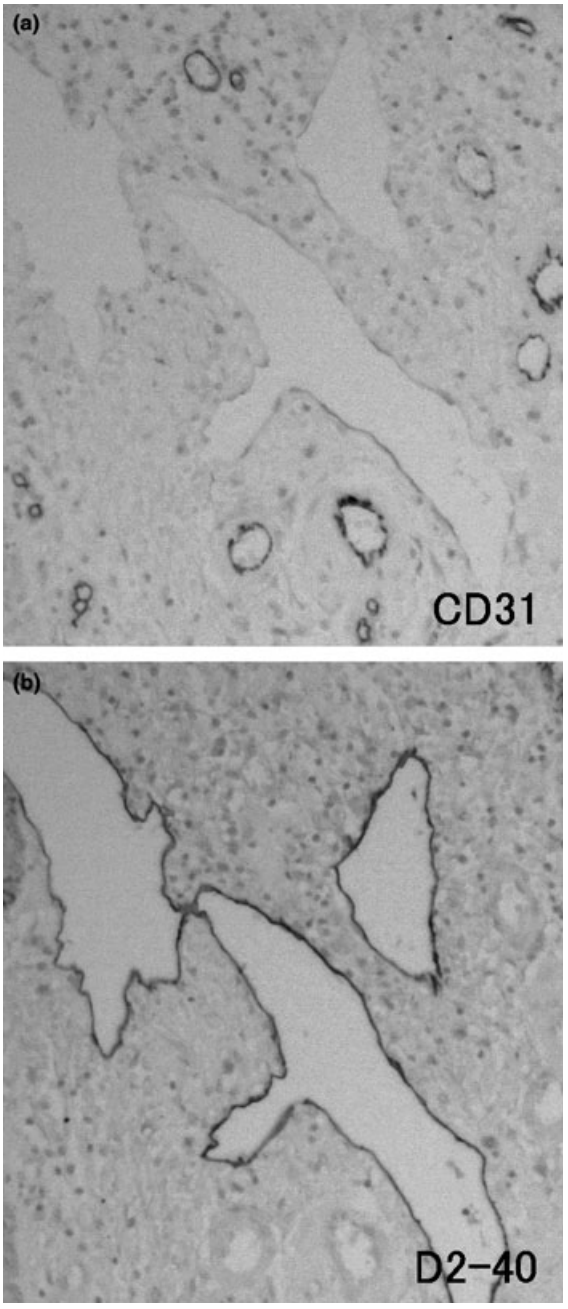

Figure 2 Immunohistochemical staining of consecutive sections of cavernous lymphangioma for CD31 (a, ×100) and D2-40 (b, ×100). Lymphatic vessels stained weakly CD31 positive and strongly D2-40 positive, whereas blood vessels CD31 positive and D2-40 negative

usually presented with island-like dilatations. Island-like lymphatic vessels were connected to or in direct contact with smaller lymphatic vessels.

Histometrical findings

Lymphatic vessel density (number of lymphatic vessels per square millimeter) (Figure 4a)

The mean number (±s.d.) of lymphatic vessels per square millimeter in control and cavernous lymphangioma was 22.5 (±13.7) and 23.9 (±14.0), respectively. This difference was not significant.

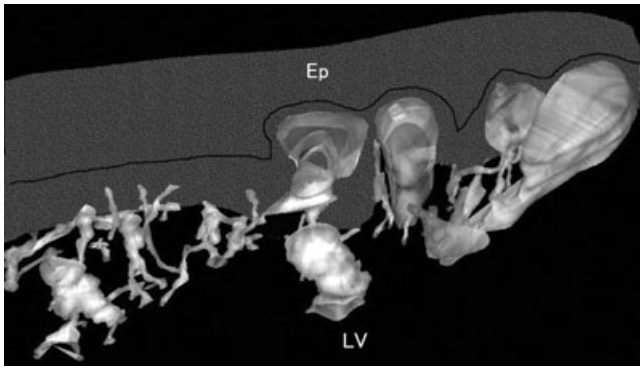

Figure 3 3D architecture of lymphatic vessels in control tissue and cavernous lymphangioma (left side: control, right side: cavernous lymphangioma) (LV: lymphatic vessel, Ep: covering epithelium)

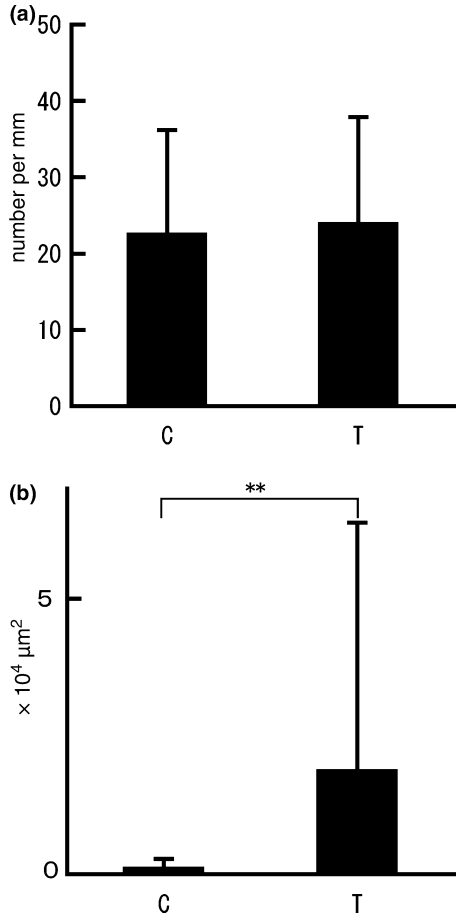

Figure 4 (a) Lymphatic vessel density (number of lymphatic vessels per square millimeter) in control and cavernous lymphangioma. (b) Size of lymphatic vessels in control and cavernous lymphangioma (C: control, T: cavernous lymphangioma, $^{**}P < 0.001$)

Size of lymphatic vessels (Figure 4b)

The mean size (±s.d.) of lymphatic vessels in control and cavernous lymphangioma was 1034.7 (±1770.8) μm^2 and 18663.3 (±45124.8) μm^2, respectively. This difference was significant ($P < 0.001$).

Number of lymphatic endothelial cells per lymphatic vessel perimeter (Figure 5)

The mean number (±s.d.) of lymphatic endothelial cells per lymphatic vessel perimeter in control and cavernous lymphangioma was 27.4 (±13.2) and 55.0 (±22.4), respectively (Figure 5a). This difference was significant ($P < 0.01$). The relations between the numbers of lymphatic endothelial cells and the lymphatic vessel perimeters in control and cavernous lymphangioma are shown in Figure 5b. There were a significant correlation between these variables in both the control and cavernous lymphangioma ($y = 0.023x + 0.52$, $y = 0.033x + 3.97$, respectively).

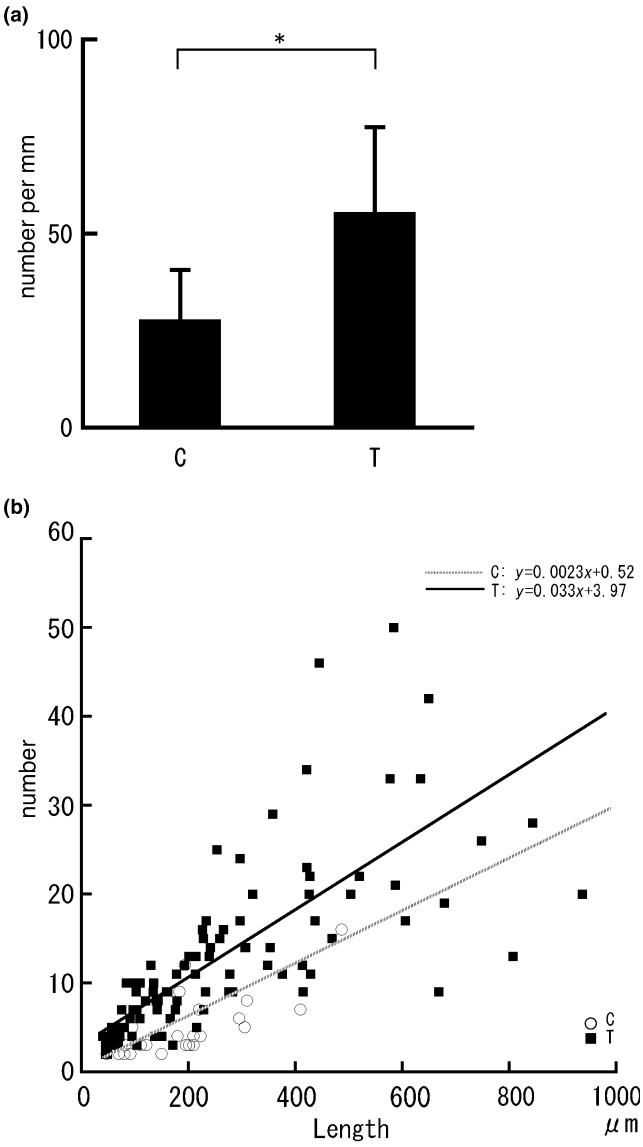

Figure 5 (a) Number of lymphatic endothelial cells per lymphatic vessel perimeter. (b) Relations between the numbers of endothelial cells in lymphatic vessels and the lymphatic vessel perimeters. A statistically significant correlation was present in both the control and cavernous lymphangioma (C: control, T: cavernous lymphangioma, $^{*}P < 0.01$)

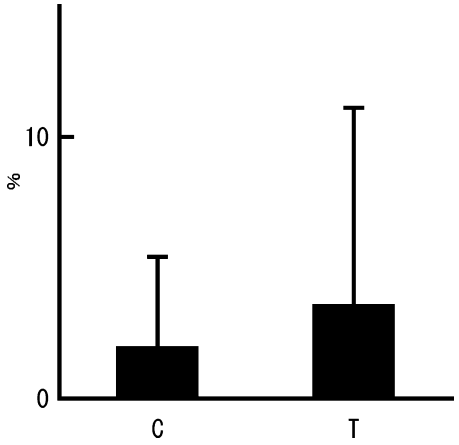

Figure 6 Proliferating cell nuclear antigen index in lymphatic endothelial cells (C: control, T: cavernous lymphangioma)

PCNA index of lymphatic endothelial cells (Figure 6)
The mean PCNA index (±s.d.) of lymphatic endothelial cells in control and cavernous lymphangioma was 1.9% (±3.5) and 3.5% (±7.6), respectively. This difference was not significant.

Discussion

In the past, studies of the lymphatic vasculature were precluded by the lack of specific lymphatic endothelial markers (Sleeman *et al*, 2001). However, the recent identification of various specific lymphatic endothelial markers has greatly facilitated studies of lymphangiogenesis (Kahn *et al*, 2002; Koizumi *et al*, 2002; Jackson, 2003; Streit and Detmar, 2003; Franke *et al*, 2004). These markers have enabled studies of the immunohistochemical characteristics of lymphangiomas. D2-40 is a recently developed, commercially available monoclonal antibody that reacts with a 40-kd antigen in fetal germ cells and germ cell tumors (Marks *et al*, 1999; Ordóñez, 2005). Because the antigen recognized by this antibody is selectively expressed in lymphatic endothelium, it has been shown that it could be very helpful in both the diagnosis of lymphatic derived tumors and determining lymphatic invasion by tumors (Ordóñez, 2005; Renyi-Vamos *et al*, 2005; Van der Auwera *et al*, 2005). The present study showed that D2-40 was excellent immunohistochemical markers of lymphangiomas. This new marker clearly distinguished lymphatic vessels from blood vessels, even when the vessels were in close proximity.

Recently, lymphangiogenesis has been found to play important roles not only in physiologic processes such as embryonic development and tissue regeneration, but also in tumor progression and metastasis. Lymphangiogenesis has central roles in the transition from hyperplasia to neoplasia, in the progression from a low grade to a high grade of neoplasia, and in augmenting metastatic potential (Nisato *et al*, 2003; Pepper *et al*, 2003; He *et al*, 2004; Shields *et al*, 2004). The development and growth of lymphatic vessels is usually quantified on the basis of lymphatic vessel density (Straume *et al*, 2003; Shields *et al*, 2004). This parameter, considered an index of lymphangiogenesis, is regarded to be a useful tool in oncology. In the present study, there was no significant difference in lymphatic density between control and cavernous lymphangioma. This finding may indicate that lymphatic density is not a distinguishing feature of cavernous lymphangioma.

Most regard lymphangiomas as malformations that arise from sequestrations of lymphatic tissue that fail to communicate normally with the lymphatic system (Huang *et al*, 2001). These remnants may have some capacity to proliferate, but more importantly they accumulate vast amounts of fluid, which accounts for their cystic appearance. Most lymphangiomas manifest clinically during childhood and develop in areas where primitive lymph sacs occur. This fact provides presumptive evidence for the hypothesis that lymphangiomas originate from sequestrations of lymphatic tissue. It has also been argued that instead of being a congenital malformation, lymphangioma is a true neoplasm resulting from transformed lymphatic endothelial cells, stromal cells, or both (Enzinger and Weiss, 1995). On the basis of the present findings, this hamartomatous hypothesis seems to be reasonable in cavernous lymphangioma because PCNA indexes of lymphatic vessels were similar in control and cavernous lymphangioma. On the other hand, the present study showed that the number of lymphatic endothelial cells per lymphatic vessel perimeter in cavernous lymphangioma was significantly higher than that in control. This result may indicate that some kind of the stimulation may play an important role in pathogenesis in cavernous lymphangioma.

In the previous study, various histometrical parameters about lymphatic vessels could be manually determined by two-dimensional (2D) analysis. However, 2D analysis could not assess the architecture of lymphatic vessel networks. With the use of a 3D reconstruction program, images showing the 3D structure of lymphatic vessels could be constructed and displayed on a computer screen and repeatedly viewed from different perspectives until various connections and structural interrelationships were discerned. In cavernous lymphangioma, irregular branching of lymphatic vessels as well as lymphatic dilation could be characterized. These results indicate that cavernous lymphangioma may be attributed to the dilated and/or cystic growth of lymphatic vessels into surrounding tissues and not to the tumorous proliferation of lymphatic endothelial cells.

References

Albertine KH, Wiener-Kronish JP, Roos PJ, Staub NC (1982). Structure, blood supply, and lymphatic vessels of the sheep's visceral pleura. *Am J Anat* **165:** 277–294.

Azzali G (2003). Structure, lymphatic vascularization and lymphocyte migration in mucosa-associated lymphoid tissue. *Immunol Rev* **195:** 178–189.

Balakrishnan A, Bailey CM (1991). Lymphangioma of the tongue. A review of pathogenesis, treatment and the use of

surface laser photocoagulation. *J Laryngol Otol* **105:** 924–930.

Bill AH, Sumner DS (1965). A unified concept of lymphangioma and cystic hygroma. *Surg Gynecol Obstet* **120:** 79–86.

Brennan TD, Miller AS, Chen SY (1997). Lymphangiomas of the oral cavity: a clinicopathologic, immunohistochemical, and electron-microscopic study. *J Oral Maxillofac Surg* **55:** 932–935.

Enzinger FM, Weiss SW (1995). Tumors of lymph vessels. In: Enzinger FM, Weiss SW, eds. *Soft tissue tumors*. Mosby-Year Book: St Louis, MO, pp. 679–688.

Erovic BM, Neuchrist C, Kandutsch S, Woegerbauer M, Pammer J (2003). CD9 expression on lymphatic vessels in head and neck mucosa. *Mod Pathol* **16:** 1028–1034.

Franke FE, Steger K, Marks A, Kutzner H, Mentzel T (2004). Hobnail hemangiomas (targetoid hemosiderotic hemangiomas) are true lymphangiomas. *J Cutan Pathol* **31:** 362–367.

Guelmann M, Katz J (2003). Macroglossia combined with lymphangioma: a case report. *J Clin Pediatr Dent* **27:** 167–169.

He Y, Karpanen T, Alitalo K (2004). Role of lymphangiogenic factors in tumor metastasis. *Biochim Biophys Acta* **1654:** 3–12.

Huang HY, Ho CC, Huang PH, Hsu SM (2001). Co-expression of VEGF-C and its receptors, VEGFR-2 and VEGFR-3, in endothelial cells of lymphangioma. Implication in autocrine or paracrine regulation of lymphangioma. *Lab Invest* **81:** 1729–1734.

Hudson JW, Meszaros EJ (2003). Submucosal lymphangioma of the maxillary sinus. *J Oral Maxillofac Surg* **61:** 390–392.

Ikemura K, Hidaka H, Fujiwara T, Ohkuma R, Terashima H (1987). A case of cystic lymphangioma extending from the neck to the tongue. Management of the lesion remaining after surgery. *J Craniomaxillofac Surg* **15:** 369–371.

Jackson DG (2003). The lymphatics revisited: new perspectives from the hyaluronan receptor LYVE-1. *Trends Cardiovasc Med* **13:** 1–7.

Kahn HJ, Bailey D, Marks A (2002). Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. *Mod Pathol* **15:** 434–440.

Koizumi T, Shiraishi T, Hagihara N, Tabuchi K, Hayashi T, Kawano T (2002). Expression of vascular endothelial growth factors and their receptors in and around intracranial arteriovenous malformations. *Neurosurgery* **50:** 117–126.

Marks A, Sutherland DR, Bailey D *et al* (1999). Characterization and distribution of an oncofetal antigen (M2A antigen) expressed on testicular germ cell tumours. *Br J Cancer* **80:** 569–578.

Nisato RE, Tille JC, Pepper MS (2003). Lymphangiogenesis and tumor metastasis. *Thromb Haemost* **90:** 591–597.

Ordóñez NG (2005). Immunohistochemical diagnosis of epithelioid mesothelioma: an update. *Arch Pathol Lab Med* **129:** 1407–1414.

Park YW, Kim SM, Min BG, Park IW, Lee SK (2002). Lymphangioma involving the mandible: immunohistochemical expressions for the lymphatic proliferation. *J Oral Pathol Med* **31:** 280–283.

Pepper MS, Tille JC, Nisato R, Skobe M (2003). Lymphangiogenesis and tumor metastasis. *Cell Tissue Res* **314:** 167–177.

Renyi-Vamos F, Tovari J, Fillinger J *et al* (2005). Lymphangiogenesis correlates with lymph node metastasis, prognosis, and angiogenic phenotype in human non-small cell lung cancer. *Clin Cancer Res* **11:** 7344–7353.

Rovenská E, Rovenská E, Neumüller J (2003). Structure of synovial lymphatic capillaries in rheumatoid arthritis and juvenile idiopathic arthritis. *Int J Tissue React* **25:** 29–39.

Shields JD, Borsetti M, Rigby H *et al* (2004). Lymphatic density and metastatic spread in human malignant melanoma. *Br J Cancer* **90:** 693–700.

Shimizu K, Nishino M, Arita K (1999). Cystic lymphangioma: its orofacial manifestations. *ASDC J Dent Child* **66:** 377–382.

Sleeman JP, Krishnan J, Kirkin V, Baumann P (2001). Markers for the lymphatic endothelium: in search of the holy grail? *Microsc Res Tech* **55:** 61–69.

Straume O, Jackson DG, Akslen LA (2003). Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. *Clin Cancer Res* **9:** 250–256.

Streit M, Detmar M (2003). Angiogenesis, lymphangiogenesis, and melanoma metastasis. *Oncogene* **22:** 3172–3179.

Van der Auwera I, Van den Eynden GG, Colpaert CG *et al* (2005). Tumor lymphangiogenesis in inflammatory breast carcinoma: a histomorphometric study. *Clin Cancer Res* **11:** 7637–7642.