J Oral Pathol Med (2005) 34: 70–6

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Various immunostaining patterns of CD31, CD34 and endoglin and their relationship with lymph node metastasis in oral squamous cell carcinomas

Hitoshi Nagatsuka[1], Kazuteru Hibi[1], Mehmet Gunduz[1], Hidetsugu Tsujigiwa[1], Ryou Tamamura[1], Toshio Sugahara[2], Akira Sasaki[3], Noriyuki Nagai[1]

Departments of [1]Oral Pathology and Medicine, [2]Oral and Maxillofacial Reconstructive Surgery and [3]Oral and Maxillofacial Surgery and Biopathology, Graduate School of Medicine and Dentistry, Okayama University, Shikatacho 2-5-1, Okayama, Japan

BACKGROUND: Intratumoral blood vessels are known to play an important role in cancer growth and metastasis. The discrepancy in previous reports using various endothelial markers individually suggested us to investigate both normal and various tumor areas with a wide panel of vascular markers.
METHODS: Here, we used a panel of three antibodies (CD31, CD34, and endoglin) as blood vessel markers to investigate the distribution and properties of blood vessels in normal oral tissues and squamous cell carcinomas.
RESULTS: Many microvessels with strong remodeling activity as well as undifferentiated tumoral vascular endothelial cells and immature endothelial cells were present in the cancer cell nest and marginal area of cancer infiltration. Our results showed different vascular distribution patterns using various immunostaining markers in normal and tumoral tissues.
CONCLUSION: Vascular distribution and properties of endothelial cells appear to be closely associated with metastasis.
J Oral Pathol Med (2005) **34**: 70–6

Keywords: angiogenesis; CD31; CD34; endoglin; oral cancer

Introduction

Intratumoral blood vessels are known to play an important role in cancer growth by supplying oxygen and nutrients as well as excreting metabolic products, and to be associated with metastasis. In 1945, Algire et al. (1) first reported the phenomenon of active neovascularization by the host to neoplastic tissues. Later Folkman (2) conducted a series of studies on cancer growth and neovascularization, and demonstrated that blood vessels in the host underwent angiogenesis and developed into intratumoral vessels that were closely related to tumor growth. Recent studies of colon cancers have shown differences in gene expression on intratumoral blood vessels and normal vessels (3). Moreover, tumoral blood vessels have been shown to differ from normal blood vessels in that the vascular endothelial cells have high remodeling activities (4).

Previous reports on the identification of tumoral blood vessels generally employed antifactor VIII antibody, anti-CD31 antibody (CD31), or anti-CD34 antibody (CD34) individually as vascular marker (5–7). Using antifactor VIII antibody, Weidner et al. (8, 9) compared the microvascular densities of poorly and well-differentiated adenocarcinomas in prostatic and breast carcinomas. Their report indicated an increased vascular density in poorly differentiated adenocarcinomas and demonstrated a positive correlation between increased vascular density and metastasis. Kitadai et al. (5) used CD34 to study esophageal squamous cell carcinomas, and reported a high frequency of lymph node metastasis in cases with high vascular density.

Several authors have reported tumoral blood vessels in oral squamous cell carcinomas (OSCC). Using antifactor VIII antibody, Williams et al. (7) demonstrated a correlation between recurrence of OSCC and the blood vessel count. Although Moriyama et al. (6) reported a correlation between metastasis and vessel density at the tip of infiltration when measured using CD31, Schor et al. (10) reported no correlation when using antifactor VIII antibody and CD31. The discrepancies in these reports presumably resulted from different antibodies used and lack of standardization of the site of observation.

On the contrary, reports have shown that the immunostaining results using different vascular markers vary depending on the degree of differentiation of the vascular endothelial cells and the degree of maturation

Correspondence: Noriyuki Nagai, Department of Oral Pathology and Medicine, Graduate School of Medicine and Dentistry, Okayama University, 2-5-1 Shikatacho, 700-8525 Okayama, Japan. Tel.: +81-86-235-6650, Fax: +81-86-235-6654. E-mail: nori@md.okayama-u.ac.jp
Accepted for publication January 21, 2004

of the vessels (11, 12). Therefore, when only one antibody is used, some blood vessels or endothelial cells may remain undetected. This raises doubt over whether previous reports indicated the true vascular distribution and density. Moreover, it remains unclear whether normal vessels and tumoral vessels show the same

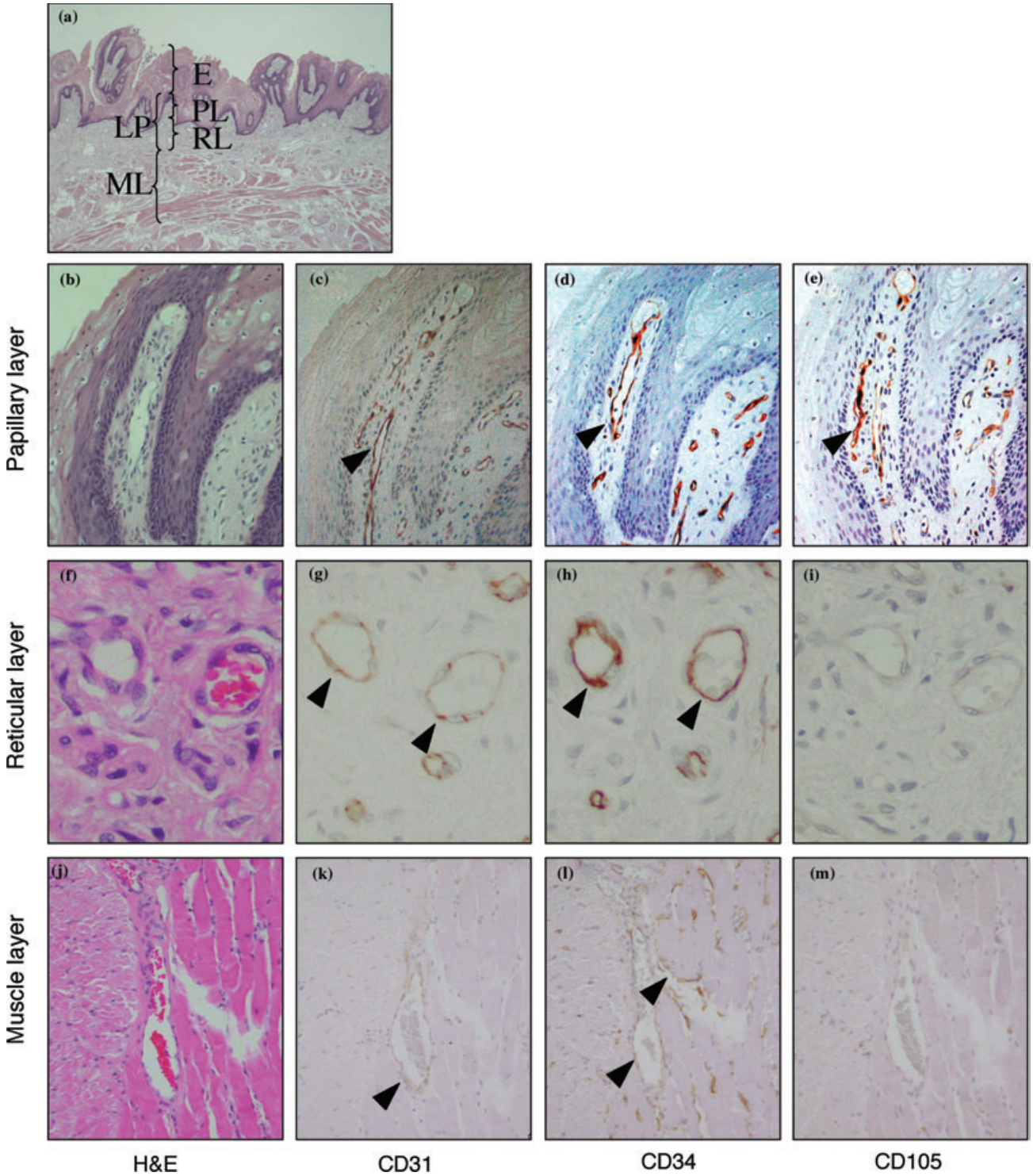


Figure 1 Histological findings and layer identification of normal oral mucosa tissue (a, H&E staining). LP, lamina propria mucosae; ML, muscle layer; PL, papillary layer; RL, reticular layer; E, stratified squamous epithelium. Distribution of blood vessels and properties of endothelial cells in various regions of oral normal mucosa (b–e, ×90; f–i, ×360; j–m, ×180). H&E staining of oral normal mucosa (b, f, j). Vessels in all normal oral mucosa layers were positive for immunostainings of CD31 (c, g, k), CD34 (d, h, l), while CD105 immunostaining (e) was positive only in papillary layer (arrowheads).

immunoreactivity with various antibodies. In this study, we aimed to detect all the blood vessels and endothelial cells at various stages of differentiation as completely as possible using various markers, and examine the correlation between vascular distribution and property with metastasis in OSCC cases.

Recent studies on vascular endothelial cells have identified endoglin, which is a homodimeric glycoprotein of endothelial cells (13). Endoglin has been shown to have high-binding affinity to transforming growth factor (TGF)-β1 and -β3 (14). Anti-CD105 antibody (CD105 hereinafter) is the antibody against endoglin, and has been reported to be useful in identifying specifically neovessels of tumors (15–18).

The purpose of this study was to use multiple antibodies, including new anti-CD105 antibody and other vascular markers, to investigate the distribution of microvessels and the properties of vascular endothelial cells in OSCC, and to examine the correlation with lymph node metastasis.

Materials and methods

Samples

Samples stored at the Department of Oral Pathology, Okayama University Dental hospital were used in the study. Forty cases of OSCC were studied, including 18 cases with lymph node metastasis. For the degree of differentiation in 18 metastatic tumors, eight cases were well-differentiated, three were moderately differentiated, and seven were poorly differentiated. For the 22 cases without metastasis were as follows: 14 cases were well-differentiated, four were moderately differentiated, and four were poorly differentiated. Five samples of normal mucosal tissue were used as control.

All the tissue samples were fixed in 10% neutral buffered formalin and paraffin-embedded according to the conventional methods. From each tissue block, 4-μm serial sections were prepared.

Immunohistochemical staining

Vascular endothelial cell markers were used as the first antibodies, comprising anti-CD31 antibody [JC/70A, 1/50 dilution; Dako Corp., Glostrup, Denmark (19, 20)], anti-CD34 antibody [Nu-4A1, ready-to-use; Nichirei Corp., Tokyo, Japan (21)], anti-CD105 antibody [Sn6h, 1/20 dilution; Dako Corp. (20)]. To identify vascular basement membrane, anti-type IV collagen antibody [H11, 1/200 dilution; Shigei Medical Lab. Inst., Okayama, Japan (22)] was used as the first antibody. After deparaffinization, the serial sections were immersed in 0.03% hydrogen peroxide in methanol. Immunohistochemical staining was performed by the ABC method (ABC Kit, Vector Laboratories, Burlingame, CA, USA). Color was developed with 3,3′-diaminobenzidine tetrahydrochloride. The sections were counterstained with Mayer's hematoxylin and examined under a light microscope. Sections stained by omitting the primary antibodies were used as negative controls. Stainings of normal tissues were considered as positive controls.

Results

Immunostaining pattern in normal oral mucosal tissue

In this study, the vascular distribution and immunohistochemical staining of each antibody in the papillary, reticular and muscle layers of normal mucosa were examined (Fig. 1a).

Papillary layer

The microvessels in the papillary layer were composed of a single layer of endothelial cells. The endothelial cells were flat, spindle-shaped cells poor in cell body. The immunostaining pattern of the vascular endothelial cells was CD31-positive, CD34-positive, and CD105-positive (Fig. 1b–e). The staining pattern of the vascular endothelial cells in the papillary layer was classified as type A.

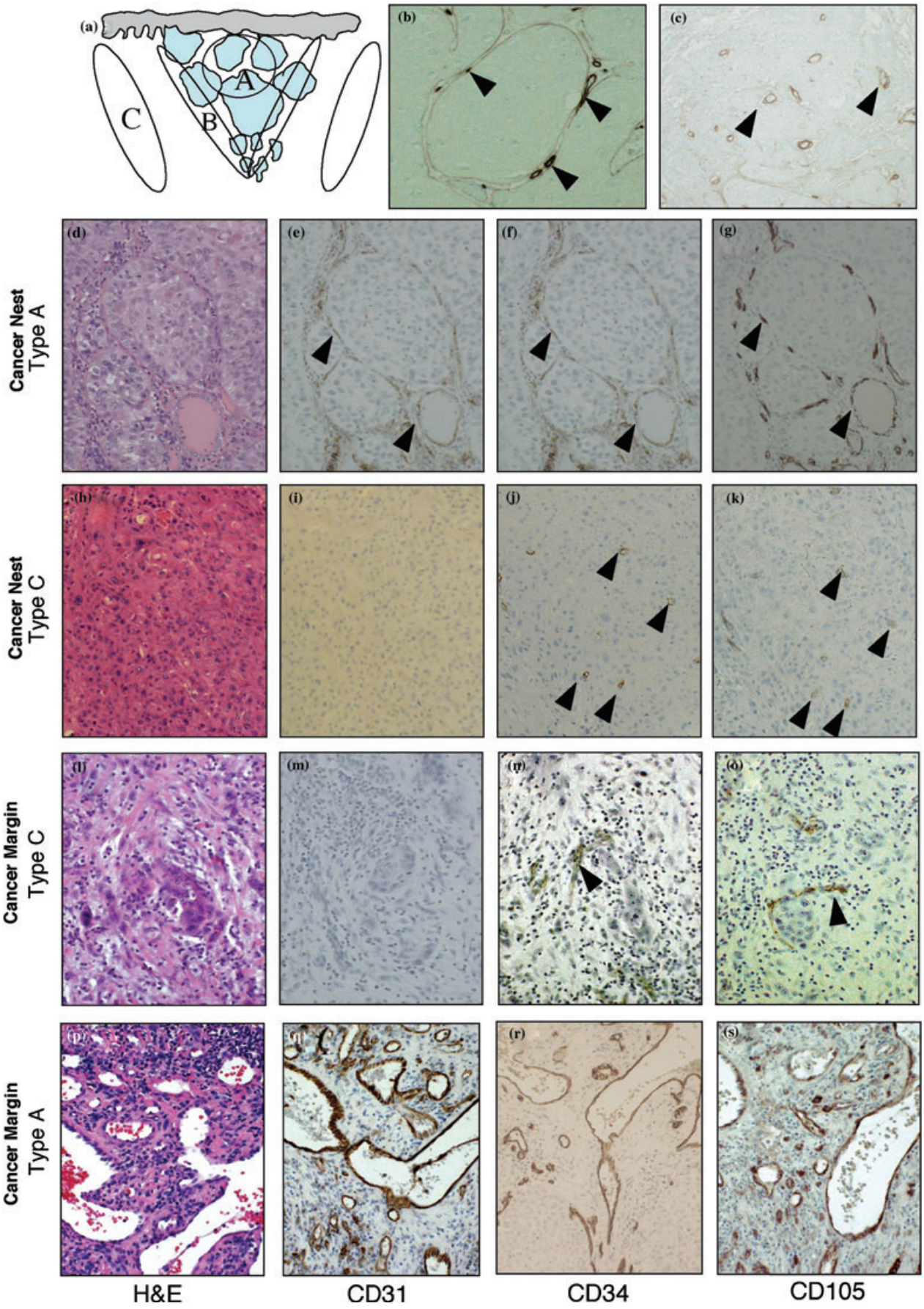
Reticular layer

Morphologically, the blood vessels in the reticular layer were composed of a single layer of endothelial cells and had larger lumen compared with the papillary layer. The immunostaining pattern of the vascular endothelial cells was CD31-positive, CD34-positive, and CD105-negative (Fig. 1f–i).

Muscle layer

Morphologically, arterioles and venules with smooth muscle and capillaries were present in the muscle layer. In all these vessels, the immunostaining pattern of the endothelial cells was CD31-positive, CD34-positive, and CD105-negative (Fig. 1j–m). The staining pattern of the vascular endothelial cells in the reticular and muscle layers was classified as type B.

Figure 2 Schematic representation of tumor nest, margin and stroma (a) (a, tumor nest; b, tumor margin; c, stroma near tumor). Cancer cell nest of oral squamous cell carcinoma (b and c, ×360). Immunohistochemical staining by anti-type IV collagen antibody shows positive staining along the lumen of blood vessel encircling the cancer nest (b, arrowheads, circumscribing type) and in lumens of blood vessels inside the cancer cell nest and surrounding the cancer nest (c, arrowheads, penetrating type). Circumscribing type vessels (type A) around cancer nest in oral squamous cell carcinoma (d–g, ×90). (d) Blood vessel encircles the cancer nest (H&E staining). Positive staining is consistent with vascular endothelial cells by anti-CD31 antibody (e), by anti-CD34 antibody (f), and by anti-CD105 antibody (g, arrowheads). Penetrating type vessels (type C) in cancer nest of oral squamous cell carcinoma (h–k, ×90). (h) Blood vessels penetrate the cancer nest in the form of scattered dots (H&E staining). Immunohistochemical staining of vascular endothelial cells are negative by anti-CD31 antibody (i). Positive staining is consistent with vascular endothelial cells by anti-CD34 antibody (j) and by anti-CD105 antibody (k) (arrowheads). Cancer infiltration marginal area of oral squamous cell carcinoma (type C; l–o, ×90). (l) Cancer cells form small nests and no definite vascular lumen is observed. Endothelial cells are difficult to identify (H&E staining). (m) Immunohistochemical staining of serial section by anti-CD31 antibody showing negative reaction. Immunohistochemical stainings by anti-CD34 antibody (n) and by anti-CD105 antibody (o) show positive reaction in spindle-shaped cells adjacent to cancer nest (arrowheads). Cancer infiltration peripheral area of oral squamous cell carcinoma (type A; p–t, ×90). (p) Many irregularly dilated vascular lumens and an increased number of cuboidal endothelial cells with enlarged nuclei are observed (H&E staining). Positive immunohistochemical stainings of serial sections by anti-CD31 antibody (q), by anti-CD34 antibody (r), and by anti-CD105 antibody (s) are clearly observed in vascular endothelial cells.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Type A
H&E
CD31
CD34
CD105

Table 1 Distribution of blood vessels and properties of endothelial cells in various regions of oral squamous cell carcinoma

Marker				
CD31	*CD34*	*CD105*	*Type*	*Description*
+	+	+	Type A	Commonly seen in circumscribing vessels of cancer nest area and in vessels of cancer infiltration peripheral area Neovessels with strong remodeling activity
+	+	–	Type B	Rarely seen in cancer nest Endothelial cells with weak remodeling activity
–	+	+	Type C	Commonly seen in penetrating type vessels of cancer nest area and cancer infiltration marginal area Immature neovessels

Immunostaining pattern in oral squamous cell carcinoma
For cases of OSCC composed of and infiltrated by large and small cancer cell nests, we divided each specimen into three regions: cancer infiltration area including cancer cell nests (cancer nest area); margin of cancer infiltration, containing cancer cells (cancer infiltration marginal area); and periphery of cancer infiltration, containing no cancer cells (cancer infiltration peripheral area) (Fig. 2a).

Cancer nest area
Anti-type IV collagen antibody immunohistochemically stained the type IV collagen in the vascular basement membrane. Two patterns of vascular distribution were observed in the cancer nest area. For the first pattern, microvessels encircled the cancer cell nest but did not invade the cancer nest. This pattern was designated as circumscribing type (Fig. 2b). For the second pattern, the stroma was narrow, and microvessels invaded the cancer cell nest. This type was designated as penetrating type (Fig. 2c).

Two immunostaining patterns of the vascular endothelial cells were observed in the cancer nest area. The first pattern was type A (positive for CD31, CD34, and CD105) (Fig. 2d–g). Type A blood vessels appeared to encircle individual cancer cell nests, and were commonly observed in the circumscribing type tumors. The second pattern was CD31-negative, CD34-positive, and CD105-positive (Fig. 2h–k). This pattern was classified as type C. Type C blood vessels were commonly found in the penetrating type tumors.

Cancer infiltration marginal area
The immunostaining pattern of vascular endothelial cells in the marginal area of cancer infiltration was type C (CD31-negative, CD34-positive, and CD105-positive) (Fig. 2l–o). These endothelial cells showed irregular orientation and did not form a definite lumen.

Cancer infiltration peripheral area
In the areas peripheral to cancer infiltration, blood vessels were dilated irregularly. The number of cuboidal endothelial cells with enlarged nucleus increased, and aligned along the tubular lumen wall. These blood vessels were all type A (CD31-positive, CD34-positive, and CD105-positive) (Fig. 2p–t).

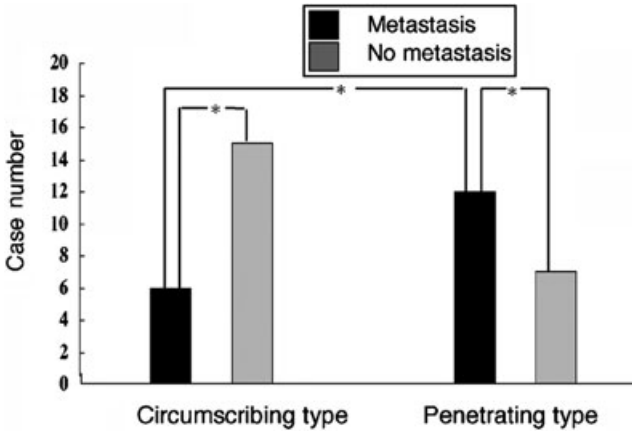

Figure 3 Correlation between blood vessel distribution pattern and metastasis of oral squamous cell carcinoma. Circumscribing type vessels were type A and most penetrating type vessels were type C (*$P < 0.05$).

The distribution and properties of blood vessels in various areas of OSCC are summarized in Table 1.

Vascular distribution and lymph node metastasis
We used chi-squared and Mann–Whitney *U*-tests to analyze the correlation between lymph node metastasis and the two types of vascular distribution (circumscribing type with type A pattern and penetrating type with predominantly type C pattern) in cancer cell nest area. Lymph node metastasis was significantly more frequent in penetrating type cases than in circumscribing type cases (Fig. 3, $P < 0.05$). Six of 21 (28%) cancer nest with circumscribing type vessel showed lymph node metastasis while 12 of 19 (63%) of cancer nest with penetrating type vessels were metastatic.

Discussion

Specific antibodies against vascular endothelial cells are used for histopathological identification of microvessels in tumors. Anti-CD31 antibody is an antibody targeting the platelet-derived cell adhesion factor that is present in endothelial cells. According to Alessandri et al. (23), CD31 is detected in endothelial cells ranging from non-lumen-forming cells to lumen-forming cells. Anti-CD34 antibody is an antibody targeting the transmembranous sialo protein, and according to Asahara et al. (24).

CD-34 is detected in precursors of (undifferentiated) endothelial cells to differentiated endothelial cells. Anti-CD105 antibody targets endoglin, which is known to be expressed throughout the process of vasculogenesis, from immature to mature blood vessels (25).

Among the immunostaining patterns demonstrated in the present study, type A, whether found in the papillary layer of normal mucosa or in tumoral blood cells in OSCC, are considered to represent neovessels with strong remodeling activity. On the contrary, type B found in the reticular layer and muscle layer of normal mucosa was CD105-negative, and probably represent blood vessels with relatively low remodeling activity. This might account for why type B tumoral blood vessels were extremely rare in OSCC.

Type C observed in tumor cell nests were immunostaining patterns not found in normal blood vessels, and these patterns were considered specific characteristics of tumoral blood vessels. Type C was found predominantly in blood vessels infiltrating the tumor cells nests and in the marginal area of cancer infiltration. From the characteristics of the antibodies (23–25) this type probably indicates the presence of actively proliferating endothelial cells or immature neovessels.

Previous study on vascular distribution in squamous cell carcinomas only reported a high vessel count in the tissues surrounding foci of cancer invasion (10). There is so far no report that investigates the vascular distribution and properties in defined regions of the cancer lesion; namely, cancer nest area, cancer infiltration marginal area, and cancer infiltration peripheral area. The results of the present study clearly demonstrated different immunostaining patterns of the endothelial cells in the three regions, suggesting that there are differences in distribution of vessels and properties of endothelial cells in these regions. Therefore, accompanying proliferation of cancer cells, the tumoral blood vessels in the cancer cell nests and marginal area of cancer infiltration are constantly extending endothelial cells and actively undergoing remodeling.

The blood vessel distribution patterns in the cancer nests of OSCC were divided into the circumscribing and penetrating types. The circumscribing blood vessels were all type A, or blood vessels with strong remodeling activity. The penetrating blood vessels were predominantly type C, or immature endothelial cells or blood vessels.

Previous studies on factors affecting the metastatic capability of OSCC indicated a correlation with the degree of differentiation and degree of invasion (26, 27), but a consensus on the correlation with tumoral blood vessels has not been established. While a large number of blood vessels is found in the periphery and margin of the cancer lesion (6, 26), suggesting a correlation with metastasis, Schor et al. (10) reported no correlation between the vessel count and metastasis in OSCC.

The present study revealed the presence of a large number of immature vascular endothelial cells in the cancer cell nest and margin of cancer infiltration, and the blood vessels in the cancer nest could be divided into the circumscribing and penetrating types. Furthermore, this study also demonstrated that cases of the penetrating type containing mostly immature blood vessels had a higher frequency of lymph node metastasis.

Previous reports focused on the correlation between metastasis and vascular density in the periphery of cancer infiltration (8, 9). However, when cancer cells metastasize, invasion into blood vessel generally occurs more readily in leaky blood vessels (4). Our findings showed that while active proliferation of vascular endothelial cells was observed in the peripheral areas of cancer infiltration, relatively mature blood vessels distributed in these areas. Therefore, although blood vessels are abundant in the peripheral area of cancer infiltration, leakage of cancer cells is unlikely considering the degree of maturity of the blood vessels. The finding that the penetrating type correlates with metastasis suggests that metastasis is closely associated with the distribution of blood vessels in the cancer cell nest and marginal area of cancer infiltration, together with the immature property of endothelial cells.

References

1. Algire GH, Chalkey HW. Vascular reactions of normal and malignant tissue in vivo. Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J Natl Cancer Inst* 1945; **6**: 73–85.
2. Folkman J. Tumor angiogenesis: therapeutic implications. *New Engl J Med* 1971; **285**: 1182–6.
3. Croix SB, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. *Science* 2000; **289**: 1197–202.
4. Yoshiji H, Kuriyama S, Fukui H. Analysis of tumoral neovessels using experimental animals. In S Masahi (ed), The Frontline of Vascular Research. Yodosha, Tokyo, 2000; 64–71.
5. Kitadai Y, Haruma K, Tokutomi T, et al. Significance of vessel count and vascular endothelial growth factor in human esophageal carcinomas. *Clin Cancer Res* 1998; **4**: 2195–200.
6. Moriyama M, Kumagai S, Kawashiri S, Kojima K, Kakihara K, Yamamoto E. Immunohistochemical study of tumour angiogenesis in oral squamous cell carcinoma. *Oral Oncol* 1997; **33**: 369–74.
7. Williams JK, Carlson GW, Cohen C, Derose PB, Hunter S, Jurkiewicz MJ. Tumor angiogenesis as a prognostic factor in oral cavity tumors. *Am J Surg* 1994; **168**: 373–80.
8. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis correlation in invasive breast carcinoma. *New Engl J Med* 1991; **324**: 1–8.
9. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993; **143**: 401–9.
10. Schor AM, Pendleton N, Pazouki S, et al. Assessment of vascularity in histological sections: effects of methodology and value as an index of angiogenesis in breast tumours. *Histochem J* 1998; **30**: 849–56.
11. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 1999; **18**: 3964–72.
12. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999; **5**: 434–8.

13. Gougos A, Letarte M. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem* 1990; **265**: 8361–4.
14. Fonsatti E, Del Vecchio L, Altomonte M, et al. Endoglin: an accessory component of the TGF-beta-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. *J Cell Physiol* 2001; **188**: 1–7.
15. Benítez–Bribiesca L, Wong A, Utrera D, Castellanos E. The role of mast cell tryptase in neoangiogenesis of premalignant and malignant lesions of the uterine cervix. *J Histochem Cytochem* 2000; **49**: 1061–2.
16. Kumar S, Ghellal A, Li C, et al. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. *Cancer Res* 1999; **59**: 856–61.
17. Nishijima K, Nishimura M, Hayashi T, et al. Clinical significance of endoglin expression in colonic carcinoma. *Igaku No Ayumi* 1999; **188**: 1043–4 (in Japanese).
18. Tanaka F, Otake Y, Yanagihara K, et al. Evaluation of Angiogenesis in non-small cell lung cancer: comparison between anti-CD34 antibody and anti-CD105 antibody. *Clin Cancer Res* 2001; **7**: 3410–5.
19. Kakolyris S, Giatromanolaki A, Koukourakis M, et al. Assessment of vascular maturation in non-small cell lung cancer using a novel basement membrane component, LH39: correlation with p53 and angiogenic factor expression. *Cancer Res* 1999; **59**: 5602–7.
20. Chang YS, di Tomaso E, McDonald DM, Jones R, Jain RK, Munn LL. Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. *Proc Natl Acad Sci USA* 2000; **97**: 14608–13.
21. Oda T, Takahashi A, Miyao N, et al. Cell proliferation, apoptosis, angiogenesis and growth rate of incidentally found renal cell carcinoma. *Int J Urol* 2003; **10**: 13–8.
22. Borza DB, Bondar O, Ninomiya Y, et al. The NC1 domain of collagen IV encodes a novel network composed of the alpha 1, alpha 2, alpha 5, and alpha 6 chains in smooth muscle basement membranes. *J Biol Chem* 2001; **276**: 28532–40.
23. Alessandri G, Girelli M, Taccagni G, et al. Human vasculogenesis ex vivo: embryonal aorta as a tool for isolation of endothelial cell progenitors. *Lab Invest* 1995; **81**: 875–85.
24. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999; **85**: 221–8.
25. Yamashita J. Vessel formation from ES cells. *Jikken Igaku* 2001; **19**: 830–5 (in Japanese).
26. Yamada K. Quantification of angiogenesis. *Rinsho Kensa* 2000; **44**: 1634–7 (in Japanese).
27. Yamamoto Y, Sanagawa H. Study of disseminated infiltrative oral squamous cell carcinoma. *Nihon Koku Geka Zasshi* 1982; **28**: 1471–9 (in Japanese).

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research (A) (no. 15209060) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.