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Regional difference in intratumoral lymphangiogenesis of oral squamous cell carcinomas evaluated by immunohistochemistry using D2-40 and podoplanin antibody: an analysis in comparison with angiogenesis

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BACKGROUND: Whether tumor cells induce lymphangiogenesis intratumorally or permeate pre-existing lymphatic vessels in the peritumoral area still remains unclear. In this study, we investigated in detail the intratumoral lymphangiogenesis of oral squamous cell carcinomas (SCC) in comparison with tumor angiogenesis.

METHODS: Immunohistochemistry with D2-40, podoplanin antibody, and CD34 antibody were used to evaluate the lymphatic vessel density (LVD) and blood microvessel density (MVD). Vascular endothelial growth factor (VEGF) and VEGF-C expressions of oral SCC were also assessed by immunohistochemistry.

RESULTS: LVD significantly increased in the superficial area of tumor tissue compared with normal mucosa, whereas it decreased in the deep area of intratumoral tissue near the invasion front, in sharp contrast to MVD, which significantly increased throughout tumor tissue. Consistent with the decreased intratumoral LVD and increased intratumoral MVD, VEGF-C expression of tumor cells was down-regulated in the deep area of tumor tissue, while VEGF expression of tumor cells was up-regulated throughout the tumor tissue.

CONCLUSIONS: Lymphangiogenesis in oral SCC varies depending on the region within the tumor tissue. It is not induced in the genuine tumor stroma near the invasion front, probably due to the down-regulation of VEGF-C expression of tumor cells, which is different from VEGF-mediated induction of intratumoral angiogenesis.

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Abbreviations: VEGF, vascular endothelial growth factor; LVD, lymphatic vessel density; MVD, blood microvessel density.

Introduction

Malignant tumor cells in the oral area tend to metastasize to regional lymph nodes from the relatively early stage due to the anatomical features of the oral cavity. The most important lymph node metastasis in patients with oral squamous cell carcinoma (SCC) is cervical lymph node metastasis (1). The treatment of choice for patients with Stage I and II tongue SCC is the surgical resection of the primary tumor without prophylactic neck dissection. However, recurrence in the cervical lymph node after curative operation, so-called subsequent metastasis, is observed at the incidence of 20–40% of SCC patients, accounting for the major prognostic factor (2). Angiogenesis is well known to be essential for tumor growth and hematogenous metastasis (3). Among various angiogenic factors, vascular endothelial growth factor (VEGF) secreted by cancer cells and stromal cells proved to play a major role in tumor angiogenesis (4), and VEGF expression is reportedly an independent prognostic factor in oral cancer (5). Several clinicopathological studies have suggested that lymphangiogenesis is also critically involved in lymphatic metastasis in several malignancies (6, 7). However, it remains unclear whether tumor cells directly induce lymphangiogenesis or not and how they enter the lymphatic vessels in oral SCC. VEGF-C, a ligand for VEGF receptor-3 (VEGFR-3), has been demonstrated to play a crucial role in the growth and differentiation of lymphatic

endothelial cells (8, 9). However, the significance of VEGF-C expression in lymphangiogenesis in oral SCC also remains to be elucidated.

Recently, molecular markers specific to lymphatic endothelial cells, such as LYVE-1, Prox-1, and podoplanin, have been identified (10–13), and the lymphatic endothelial cell-specific antibody, D2-40, has been developed (14, 15). Immunohistochemistry using these antibodies made it possible to distinguish more clearly and readily the lymphatic vessels from the blood vessels than conventional enzyme histochemistry methods, such as 5′-nucleotidase (16). Since then, immunohistochemical studies on tumor lymphangiogenesis using these antibodies have been reported in various malignancies including colon, gastric, prostate, breast cancer, and oral SCC (17–21). One possible problem with these studies is that assessment of intratumoral LVD has been carried out by measuring only the area with the highest vessel density, the so-called hot spot, and not chosen randomly. This poses the risk of over-simplification or generating some selection bias. In the present study, to investigate lymphangiogenesis within tumors more objectively, we therefore immunohistochemically examined lymphatic vessels of oral SCC in more detail, considering locoregional heterogeneity and anatomical features of pre-existing lymphatic vessels of the oral tissue in comparison with tumor angiogenesis. We also examined VEGF-C and VEGF expression of tumor cells to elucidate the reason for the regional difference in lymphangiogenesis and angiogenesis of oral SCC.

Materials and methods

Patients

The study population consists of 50 oral SCC patients including 38 tongue, eight mouth floor, two gingiva and two buccal mucosa carcinomas. These patients underwent operation at the Department of Oral and Maxillofacial Surgery, Aichi-Gakuin University School of Density, and the Department of Head and Neck Surgery, Aichi Cancer Center Hospital, from April 1996 to March 2004. Tumor resection was performed with either modified radical or suprahyoidal neck dissection. The mean age of 37 male and 13 female patients at diagnosis was 62.6 years, ranging from 29 to 86 years. Histologically, 41 (82%) patients were well- to moderately differentiated SCC and nine (18%) poorly differentiated SCC. The population included 10 (20%) patients with lymph node metastases and one (2%) patient with distant metastases. The 50 oral SCC patients include 15 Stage I, 20 Stage II, 12 Stage III and three Stage IV patients. Tumor stage and grade of tongue SCC were classified according to the UICC's criteria.

Immunohistochemistry

Histology and immunohistochemistry were performed on thin sections of 4-μm thickness from formalin-fixed, paraffin-embedded tissues dewaxed through xylene and graded concentrations of ethanol. For the histology, sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were pretreated for antigen retrieval by microwave at 98°C for 10 min (pH 6.0) for D2-40, CD34 and podoplanin or by autoclave at 121°C for 10 min for VEGF-C. No pretreatment was carried out for VEGF. These sections were immersed in methanol with 0.3% hydrogen peroxide for 20 min to inactivate endogenous peroxidase activity, followed by normal horse or goat serum for 30 min to block non-specific reactions. Sections were incubated at 4°C overnight with the following first antibodies diluted with PBS containing 1% BSA; mouse monoclonal antibodies against D2-40 (1:200 dilution, Signet Laboratories, Dedham, MA, USA), CD34 (1:400 dilution, Immunotech, Marseille, France), podoplanin (1:200 dilution, AngioBio Co., Del Mar, CA, USA), MIB-1 (1:100 dilution, Immunotech), VEGF (1:400 dilution, Upstate, Lake Placid, NY, USA) and a rabbit polyclonal antibody against VEGF-C (1:100 dilution, IBL, Gumma, Japan). After washing with PBS, sections were incubated with biotinylated second antibody for 30 min. Sections were washed again with PBS, then incubated with streptavidin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame, CA, USA) for 60 min. Chromogen was developed with 0.01% diaminobenzidine, and sections were counter-stained with hematoxylin.

Double immunostaining

To confirm specificity of D2-40 and podoplanin for lymphatic endothelial cells distinct from vascular endothelial cells, a double immunostaining with D2-40 antibody and anti-CD34 antibody was performed. First, D2-40 monoclonal antibody was applied to the rehydrated paraffin sections after antigen retrieval with microwave for 10 min, followed by avidin/biotin complex system (Vectastain ABC kit) and developed brown color with diaminobenzidine. Then, the first antibody was removed by several washings with 0.1 M glycine–HCl buffer (pH 2.2) for 120 min, and the section was reacted with a paired antibody, and finally stained with alkaline phosphatase-conjugated second antibody (DAKO Cytomation, Kyoto, Japan) using Fast Red as chromogen (red color) to visualize binding of the second antibody. Color resolution of the dual color image of brown and red obtained from a double immunostaining was improved using the Nuance imaging system, a liquid crystal tunable filter-based multispectral imaging platform as reported previously (22).

Lymphatic vessel density and lymphatic vessel invasion

Upon analysis of lymphatic vessels of oral SCC, we first divided the tissue area into three portions normal, peritumoral tissue (means extratumoral tissue), and intratumoral tissue, with the latter two portions further subdivided into superficial area and deep areas, resulting in a total of five portions. The five portions were schematically represented in Fig. 2G. Lymphatic vessel density (LVD) and blood microvessel density (MVD) were measured according to Weidner's method as described previously (23). In brief, the area with the highest vascular density (hot spot) was selected at a low

magnification, and the number of microvessels was counted in each of five microscopic fields with ×20 objective lens. Lymphatic vessel invasion by tumor cells (Ly) was evaluated on slides immunostained with D2-40 antibody.

VEGF and VEGF-C expression of tumor cells
To evaluate VEGF and VEGF-C expression of tumor cells, we used a scoring system based on both parameters: (a) staining intensity (0, negative; 1, weak; 2, intermediate; and 3, strong) and (b) percentage of positive cells (0, 0% positive cells; 1, <25% positive cells; 2, 26–50% positive cells; 3, >50% positive cells), as described previously (24). The sum of (a) + (b) ranged from 0 to maximally 6. A score greater than 3 represented a positive result.

Statistical analysis
The statistical significance of differences in data for LVD and MVD between groups was determined by applying Student's or Welch's two-tailed *t*-tests. Differences in the incidence between the groups were analyzed with Fisher's exact test. A value of $P < 0.05$ was considered statistically significant.

Results

Specificity of D2-40 and podoplanin immunostaining for lymphatic vessels
D2-40 and podoplanin immunohistochemistry showed essentially the same staining pattern and both successfully identified lymphatic vessels much more accurately than conventional histology with HE staining, which is based on morphological criteria, such as lack of erythrocytes in their lumen. Double immunostaining and the resultant dual color images assisted by the Nuance multispectral imaging system clearly demonstrated no overlap between D2-40 (or podoplanin)-positive vessels and CD34-positive vessels in normal and cancer tissue (Fig. 1A and B), confirming the specificity of D2-40 and podoplanin immunohistochemistry for lymphatic vessels.

LVD and MVD in oral SCC
As lymphangiogenesis is influenced by both the anatomical location and depth of tumor invasion, we subdivided intratumoral and peritumoral tissue into 'superficial area' and 'deep area', and compared in detail the LVD to those of normal mucosa as shown in Fig. 2G. Normal tongue mucosa (N) contains dilated lymphatic vessels, most abundantly in the subepithelial layer. LVD significantly increased ($P < 0.01$) in a mucosal area with the hyperplastic or dysplastic epithelium adjacent to tumor tissue (superficial area of peritumoral tissue, PS). In tumor tissue, LVD depends on the location. LVD significantly increased in a superficial area of intratumoral tissue (IS), but decreased ($P < 0.01$) in the deep areas of intratumoral tissue near the invasion front when compared with normal mucosa (Fig. 2A, B, C and H). In the IS portion, lymphatic vessels are dilated and MIB-1-positive lymphatic endothelial cells were observed within vessels (Fig. 2, inset), whereas in the PS portion, only small-sized lymphatic vessels were scantily observed. In the peritumoral tissue surrounding the invasion front of tumor cells (PD), no apparent increase in lymphatic vessels was observed. In contrast, MVD as determined by CD34 immunostaining increased in a homogeneously manner throughout tumor tissue both in the superficial and deep areas (IS and ID) ($P < 0.05$) when compared with normal mucosa (Fig. 2D, E, F and I), indicating that angiogenesis, but not lymphangiogenesis, was induced in the tumor tissues.

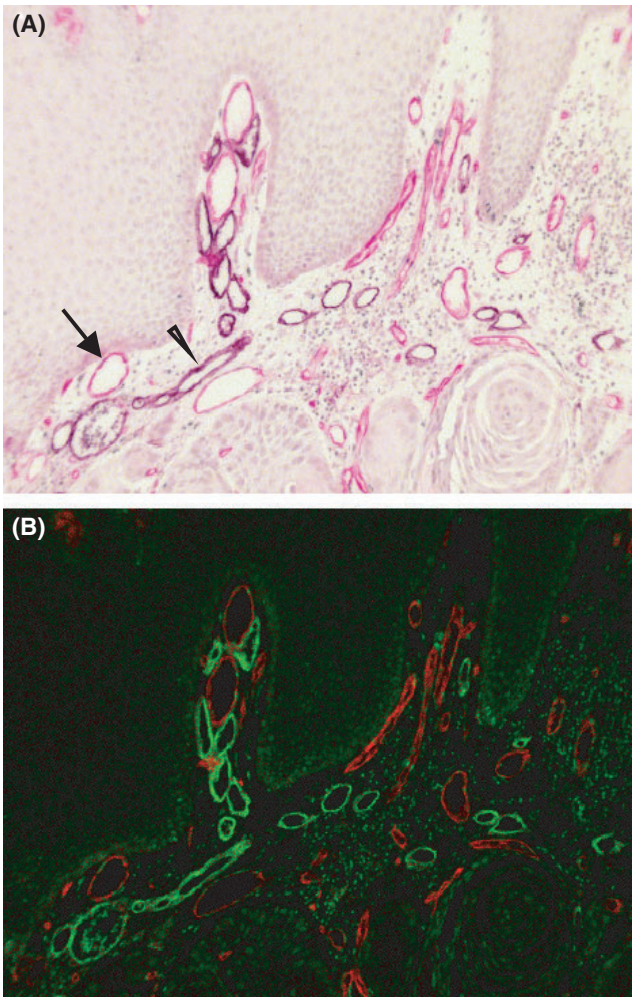

Figure 1 Double immunohistochemical staining of tongue squamous cell carcinoma using lymphatic vessel and blood vessel specific antibodies. (A) Cancer tissue stained for D2-40 (brown, arrowhead) and CD34 (red, arrow). (B) Cancer tissue stained for D2-40 (green fluorescence) and CD34 (red fluorescence) detected by Nuance multispectral imaging system. Lymphatic vessels and blood vessels were more clearly distinguished than conventional double immunostaining image.

VEGF-C expression and lymphangiogenesis
Vascular endothelial growth factor-C stained most strongly in the hyperplastic epithelium adjacent to tumor in the PS portion (Fig. 3A and B). Staining intensity of the tumor cells decreased with depth of

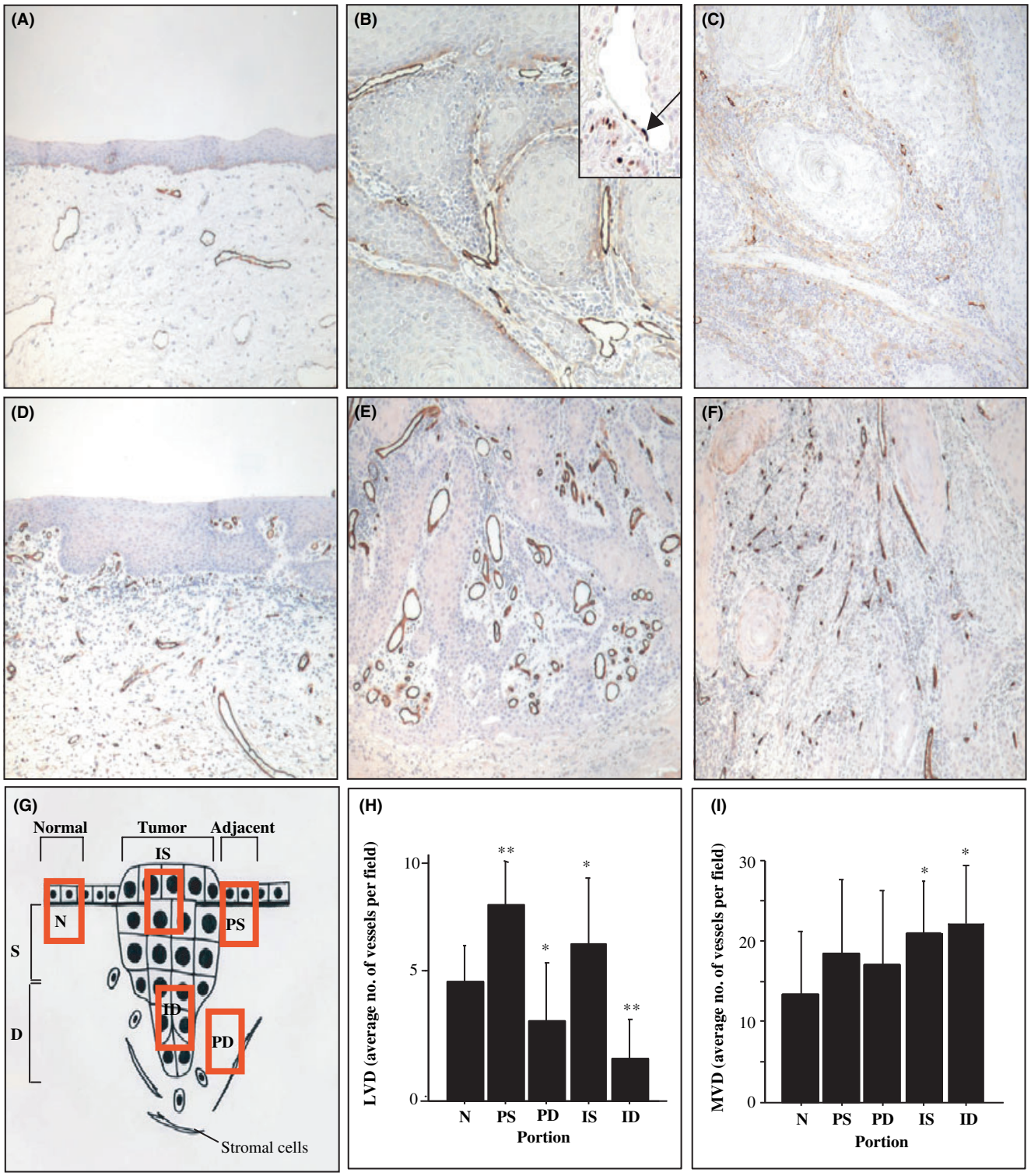

Figure 2 Lymphangiogenesis and angiogenesis in the oral SCC as visualized by D2-40 (A–C) and CD34 immunostaining (D–F). (A, D) Normal mucosa distant from tumor tissue (portion N). (B, E) Superficial area of intratumoral tissue (portion IS). Inset shows MIB-1-positive lymphatic endothelial cells (arrow). (C, F) Deep areas of intratumoral tissue (portion ID). (G) Schematic representation of subdivided intratumoral and peritumoral portions defined in this study (five portions). S, superficial area; D, deep area. (H) Lymphatic vessel density measured on D2-40-stained specimen according to the portions. (I) Blood microvessel density measured on CD34-stained specimen according to the portions. PS, superficial area of peritumoral tissue; PD, deep area of peritumoral tissue. $*P < 0.05$, $**P < 0.01$ (vs. normal mucosa).

tumor invasion from superficial to deep areas (Fig. 3C and D). Accordingly, the positivity rate of tumor cells for VEGF-C significantly increased ($P < 0.01$) in the superficial area of peritumoral tissue (PS portion) and most significantly decreased ($P < 0.05$) in the deep area of intratumoral tissue (ID portion) (Fig. 3E), indicating

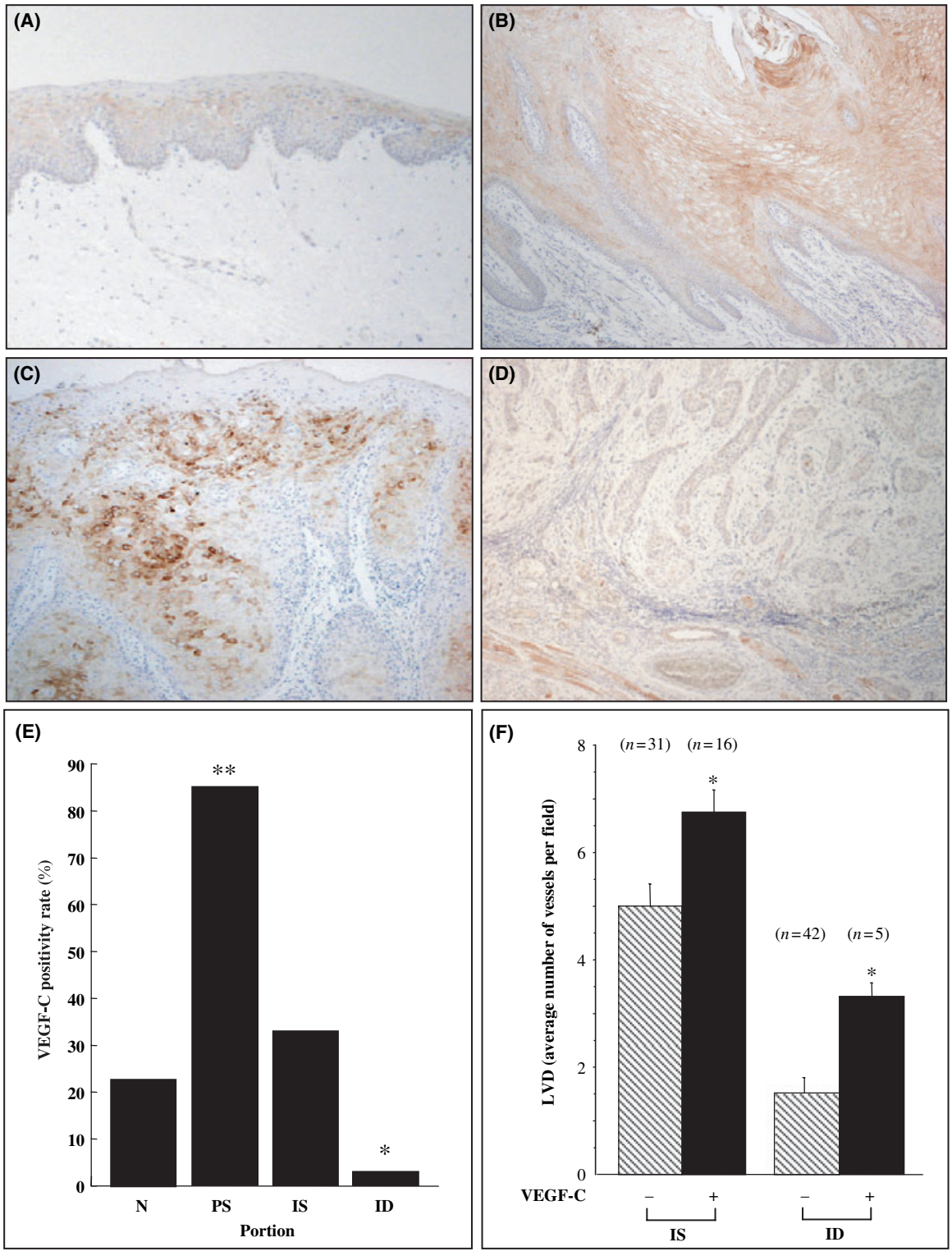

Figure 3 Immunohistochemical analysis of VEGF-C expression of oral squamous cell carcinoma. (A) Normal mucosa distant from tumor tissue (portion N). (B) Superficial area of peritumoral tissue (hyperplastic epithelium, portion PS). (C) Superficial area of intratumoral tissue (portion IS). (D) Deep area of intratumoral tissue (portion ID). (E) VEGF-C positivity rate according to the portions. (F) Relation between VEGF-C expression and LVD according to the portions. $*P < 0.05$, $**P < 0.01$ (vs. negative cases).

down-regulation of VEGF-C expression in the oral SCC tumor cells with depth of invasion. LVD was significantly higher in the VEGF-C-positive tumor than the negative tumor in both superficial and deep areas of tumor tissue, indicating a potential role of VEGF-C in lymphangiogenesis (Fig. 3F).

VEGF expression and angiogenesis

Vascular endothelial growth factor stained more intensely in the hyperplastic epithelium adjacent to tumor in the PS portion than in normal epithelium (Fig. 4A and B). VEGF staining further enhanced in the tumor tissue, especially at their invasion front (Fig. 4C and D). Concomitantly, the positivity rate of VEGF most significantly increased ($P < 0.01$) in the tumor at the invasion front in the ID portion (Fig. 4E), indicating up-regulation of VEGF expression in most oral SCC tumors (77%). MVD was significantly higher in the VEGF-positive tumor than in the VEGF-negative tumor in both superficial and deep areas of tumor tissue (Fig. 4F).

Lymphatic vessel invasion by tumor cells

The start of lymphatic vessel invasion by tumor cells (Ly) could be more clearly observed in D2-40-stained specimen than HE-stained slide (Fig. 5A and B). Ly detected by D2-40 staining was most frequently observed in the dilated lymphatic vessels in the PS portion, followed by IS portion, but Ly was only rarely observed in the deep portion of intratumoral and peritumoral tissues in oral SCC (Fig. 5C).

Discussion

In studies thus far, almost all analyses for tumor lymphangiogenesis were performed simply by comparing intratumoral and peritumoral lymphatic vessels with measurement of LVD using the 'hot spot' method. In the tongue, however, it is well known that lymphatic vessels are abundant in the lamina propria of the mucosa, but relatively scant in the muscle layer (25). Considering this different anatomical distribution of lymphatic vessels in the tongue, it is only reasonable to perform an analysis by subdividing intratumoral tissue and peritumoral tissues into two portions, superficial and deep areas, which correspond to the mucosa and muscle layer respectively. In the present study, we found that LVD was higher in the superficial area of tumor tissue than in normal mucosa, and we also observed the presence of MIB-1-positive lymphatic endothelial cells consisting of dilated lymphatic vessels in the superficial area, suggesting that the increased number of lymphatic vessels in the superficial area of tumor tissue result from hyperplasia or sprouting from pre-existing lymphatic vessels in the normal mucosa. On the other hand, lymphatic vessels in the deep area of the tumor tissue were markedly decreased when compared with peritumoral tissue or were almost lacking in some cases. Indeed, although a decrease in LVD in the deep zone of the tumor tissue reflects the small number of pre-existing lymphatic vessels in the intact muscle layer, the diminished numbers of lymphatic vessels are more than expected anatomically, suggesting that intratumoral lymphangiogenesis in the deep area near the invasion front is impaired or inhibited rather than induced. This is in sharp contrast to the MVD, which is uniformly elevated in both superficial and deep areas of tumor tissue when compared with peritumoral tissue. Some controversy still remains as to whether the intratumor lymphatic vessels are increased or decreased. Beasley *et al*. reported the proliferation of intratumoral lymphatic vessels as discrete hotspots in a subset of human oropharyngeal SCC. On the other hand, Franchi *et al*. reported a significant decrease in the intratumoral lymphatic vessels in oral SCC compared with peritumoral tissue (26, 27). Padera *et al*. further reported the absence of functional intratumoral lymphatics in a murine tumor model (28). Our detailed quantitative analysis in the present study from a locoregional point of view, provides the first evidence that lymphangiogenesis varies depending on the region within a tumor, and is not newly formed in the deep area near the invasion front, where inherent tumor stroma was reconstructed after destruction of muscle layers by the invasion of tumor cells.

Several explanations are possible for the decreased LVD in the deep area of tumor tissues in oral SCC. We found that LVD is significantly higher in VEGF-C-positive tumor than in the VEGF-C-negative tumor, confirming that VEGF-C is a potent mitogen responsible for the proliferation of lymphatic endothelial cells in oral SCC (29). Interestingly, however, VEGF-C expression was down-regulated in the intratumoral tissue, especially in the deep area near the invasion front in the most cases (95%) when compared with normal adjacent epithelium. Onogawa *et al*. previously reported down-regulation of VEGF-C expression in colon cancer tissue when compared with paired non-cancerous tissues and also showed that the VEGF-C-positive cancer cell lines is less than VEGF-positive colonic cancer cell lines by RT-PCR analysis (30). Therefore, poor lymphangiogenesis in the tumor tissue may be at least partly due to the low VEGF-C expression of oral SCC. As VEGF-C is reportedly up-regulated by inflammatory cytokines produced by leukocytes, poor inflammatory cell infiltration occasionally observed in the fibrous tumor stroma may be related to the down-regulation of VEGF-C in ID portion. Alternatively, it can also be speculated that a natural inhibitor of lymphangiogenesis may be present in the tumor stroma. In contrast, VEGF expression was found to be up-regulated in tumor tissue, consistent with the elevated MVD throughout the tumor tissue of oral SCC. These results suggest that lymphangiogenesis and angiogenesis in the tumor tissue are reciprocally regulated mainly by the mediation of VEGF-C and VEGF respectively.

Another question to be resolved is how and where tumor cells enter the lymphatics to disseminate to the lymph node despite the decrease in the lymphatic vessels within the tumor (31). We found that tumor cells utilize pre-existing dilated lymphatic vessels in the superficial zone of the intra- and peritumoral tissue, yet do not seem to enter into lymphatic circulation from the invasion front in the deep area. In the superficial area, proliferating tumor cells seem to contact lymphatic vessels in clumps directly and break through the thin-walled lymphatic vessel to enter the lumen. These findings suggest that pre-existing lymphatic channel in

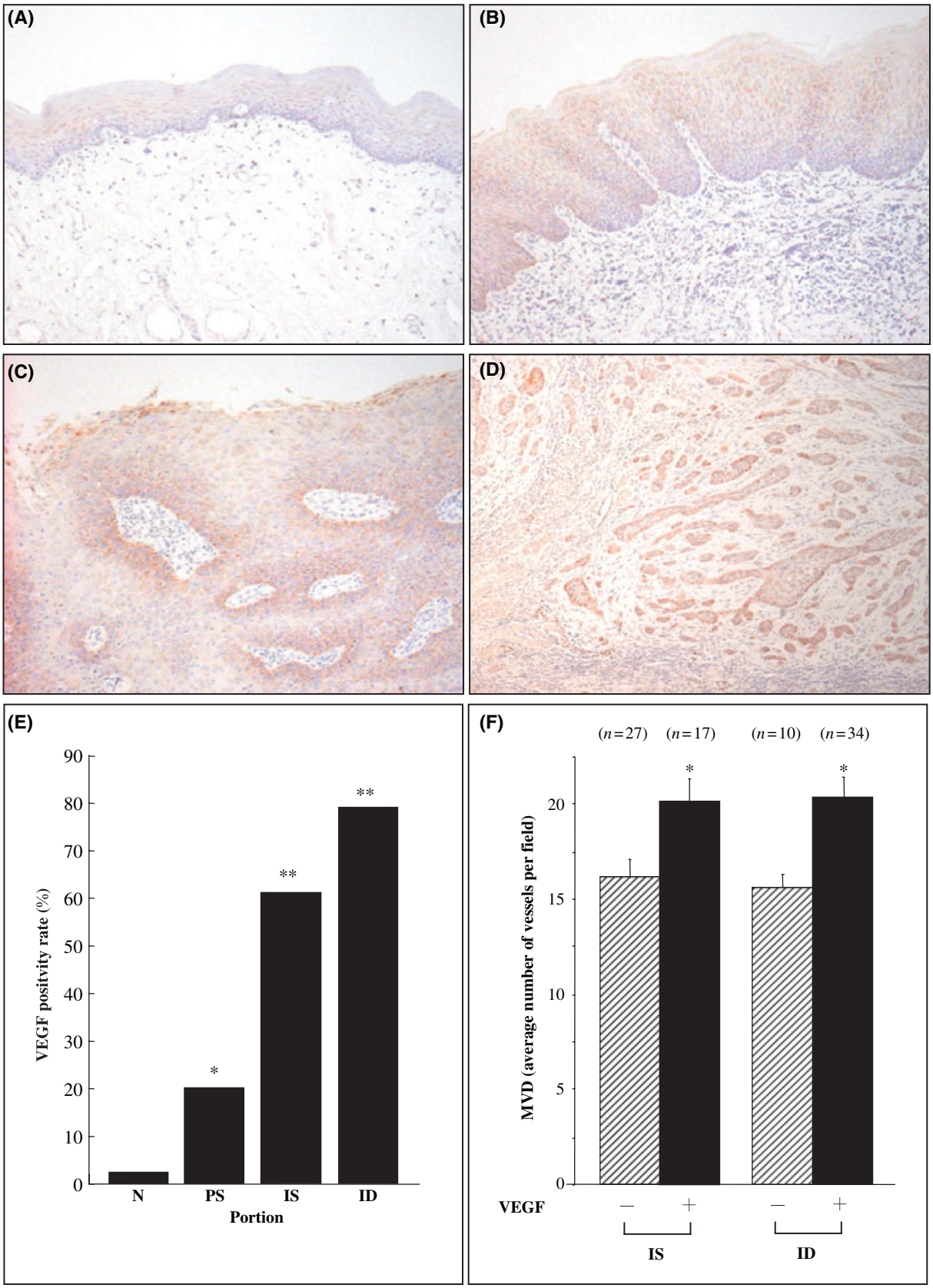

Figure 4 Immunohistochemical analysis of VEGF expression of oral squamous cell carcinoma. (A) Normal mucosa distant from tumor tissue (portion N). (B) Superficial area of peritumoral tissue (hyperplastic epithelium, portion PS). (C) Superficial area of intratumoral tissue (portion IS). (D) Deep area of intratumoral tissue (portion ID). (E) VEGF positivity rate according to the portions. (F) Relation between VEGF expression and blood microvessel density according to the portions. $*P < 0.05$, $**P < 0.01$ (vs. normal epithelium).

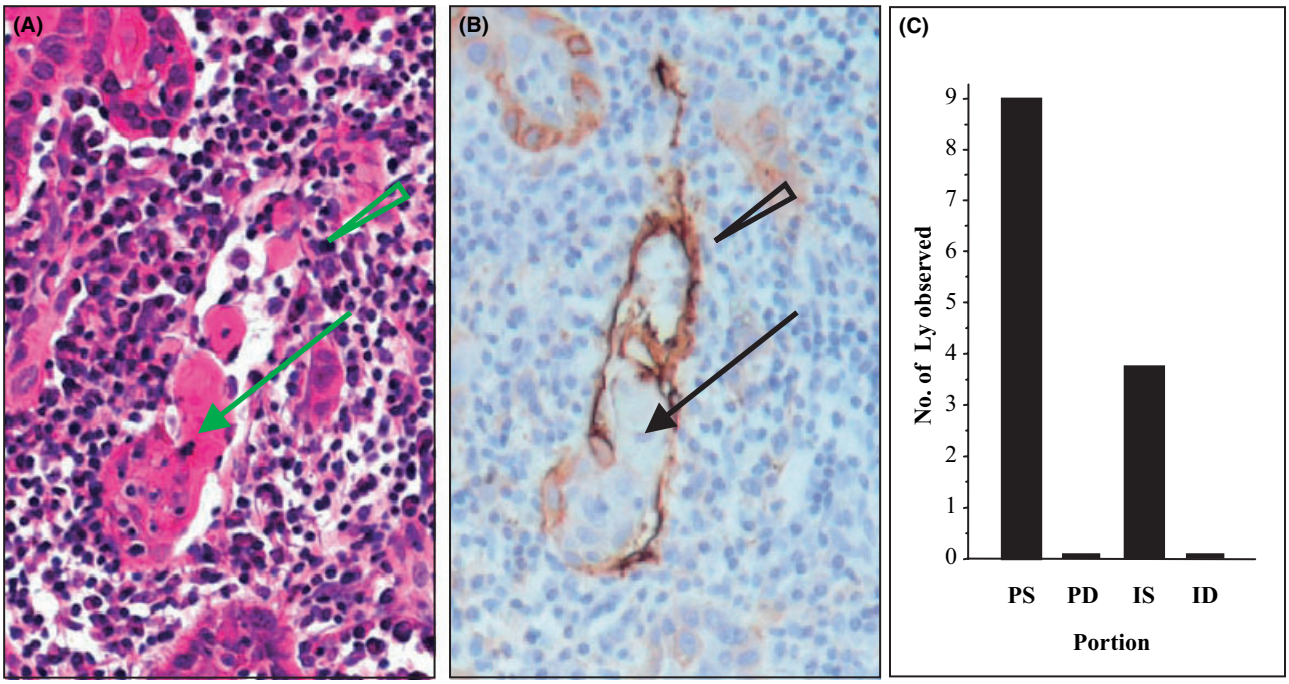

Figure 5 Detection of lymphatic vessel invasion by tumor cells (Ly) using D2-40 immunohistochemistry. (A) Ly observed on HE-stained slide. (B) Ly observed on D2-40 immunostained slide. Arrow, tumor cells; arrowhead, lymphatic endothelial cells. (C) Number of Ly observed according to the portions.

the superficial area (lamina propria), as opposed to poor lymphatic vessels in the deep area near the invasion front, is most likely involved in the process of lymphatic dissemination. In fact, we found that Ly factor, but not LVD, was significantly correlated with the lymph node metastasis (data not shown).

In conclusion, we provided evidence for the first time that intratumoral lymphangiogenesis varies depending on the area and is not induced in the deep intratumoral tissue in OSCC, probably due to down-regulation of VEGF-C expression of tumor cells, in sharp contrast to VEGF-mediated induction of intratumoral angiogenesis, suggesting the presence of reciprocal regulatory mechanism between lymphangiogenesis and angiogenesis. D2-40 and podoplanin are new selective immunohistochemical markers for lymphatic endothelium and proved to be useful for sensitive detection of lymphatic vessels as well as lymphatic invasion by tumor cells. Thus, it is an important tool for investigating tumor lymphangiogenesis in clinical samples.

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