Abstract: Neovascularization in the stroma of a tumor plays an important role in tumor growth and the establishment of metastases. The present study examined the immunohistochemical expression of vascular endothelial growth factor receptor 2 (VEGFR2) in 34 cases of oral squamous cell carcinoma (OSCC). Moreover, the relationships between parameters of tumor neovascularization (count of VEGFR2-positive [+] cells and total size of vessel lumen [TSVL]) and those of histology (differentiation type and mode of invasion) were analyzed statistically. Immunohistochemical expression of VEGFR2 was localized in stromal cells at the tumor invasive front. The VEGFR2+ cell count around poorly differentiated tumors was significantly higher than that around well differentiated tumors \( (P = 0.032, \text{one-way ANOVA}) \). The TSVL around the well differentiated type was found to be significantly larger than that around the poorly or moderately differentiated type \( (P < 0.001, \text{respectively; one-way ANOVA}) \). With regard to the mode of invasion, the TSVL was significantly larger for lower-grade (Grades 1+2) than for higher-grade (Grades 3+4) tumors \( (P < 0.001, \text{unpaired t-test}) \). On the basis of our results, we suggest that vascular development at the invasive front of OSCC is governed by the following factors: the tumor cells themselves may induce hemangiogenesis in the adjoining stromal tissue; hemangiogenic activity is higher when parenchymal intercellular adhesion is looser and when the parenchymal area exposed to the stroma is greater; and the rate of blood flow is higher when parenchymal intercellular adhesion is tighter and parenchymal nests are larger. (J Oral Sci 51, 551-557, 2009)

Keywords: tumor neovascularization; oral squamous cell carcinoma; vascular endothelium; VEGF; VEGFR2.

Introduction

Since oxygen can diffuse for a distance of only 100-200 \( \mu \text{m} \) from vessels permeating tissue, tumor cells beyond this distance cannot survive without new vessel formation (1). Therefore, in addition to the pre-existing vasculature, a supply of new vessels is necessary in order for solid tumors to continue growing (1). Such newly formed vessels are also closely related to the establishment of metastases in other organs (2,3). Tumor neovascularization develops through two different mechanisms: vasculogenesis from circulating endothelial precursors (angioblasts) (4,5) and angiogenesis through sprouting or splitting from the pre-existing vasculature (6,7).

Vascular endothelial growth factor (VEGF), first isolated in the 1980s, is a 45-kDa multifunctional glycoprotein cytokine (8). Binding to endothelial receptors, VEGF acts as both a potential vascular permeability-inducing factor (9,10) and a selective endothelial mitogen (8,11,12). Besides the importance of physiological vessel formation in the endometrium (13,14) and ovary (15,16) in adult females during the estrous cycle, VEGF is an essential regulator of tumor neovascularization (1,5), and its expression has been reported in many types of human
solid cancer (17,18). Endothelial cell surface receptor tyrosine kinases for VEGF, constituting the VEGFR family, have been identified (19,20); VEGFR2 is known to be the most essential receptor for the actions of VEGF on permeability and growth (21,22). VEGFR2 is also known to be a marker of hemangiogenesis (hematopoiesis and vasculogenesis) (6) and is reported to play an important role in the differentiation of angioblasts to endothelial cells during vasculogenesis (23). VEGF and VEGFR2 are therefore considered to be important targets for anti-tumor therapy (22,24). However, the relationship between tumor neovascularization and the histologic characteristics of oral carcinomas is still unclear (25).

The aim of the present study was to investigate immunohistochemically the expression of VEGFR2 and to statistically analyze the relationships between parameters of tumor neovascularization (count of VEGFR2-positive [+] cells, and total size of vessel lumen [TSVL]) and those of histology (differentiation type and mode of invasion) in oral squamous cell carcinoma (OSCC).

Materials and Methods

Patients and tumor specimens

This study included 34 cases of untreated OSCC (11 well, 11 moderately, and 12 poorly differentiated types) (Table 1). All cases were diagnosed in our department between 1989 and 2005 on the basis of the World Health Organization criteria (26), taking into account the degree of differentiation, including the formation of keratin, the presence of intercellular bridges, cellular and nuclear pleomorphism, and mitotic activity. The study cases were divided into four modes of invasion using the criteria of Jacobsson et al. (27,28), based on the relationship between tumor cells and adjacent connective tissue (tumor-host relationship): 4 cases of Grade 1 (well defined borderline), 3 cases of Grade 2 (cords, less marked borderline), 15 cases of Grade 3 (groups of cells, no distinct borderline), and 12 cases of Grade 4 (diffuse invasion). Grades 1 and 2 were considered to constitute a lower-grade group, and Grades 3 and 4 a higher-grade group. Sections 4 µm thick were obtained from formalin-fixed and paraffin-embedded blocks, and subjected to further study.

Immunohistochemical staining

The sections were deparaffinized and then treated with 10 mM sodium citrate buffer, pH 6.0, for 5 min at 95°C for antigenic retrieval. After blocking the endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol, immunohistochemistry was performed using a polymeric enzyme-labeled antibody method (Histofine

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### Table 1 Clinicopathological findings of oral squamous cell carcinomas

<table>
<thead>
<tr>
<th></th>
<th>WD* (11)</th>
<th>MD† (11)</th>
<th>PD‡ (12)</th>
<th>Total (34)</th>
</tr>
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<tr>
<td>Age distribution, year (mean, year)</td>
<td>56-78 (68.6)</td>
<td>49-88 (64.2)</td>
<td>39-97 (63.9)</td>
<td>39-97 (65.5)</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>3/8</td>
<td>9/2</td>
<td>10/2</td>
<td>22/12</td>
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<tr>
<td>Anatomical sites</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>17</td>
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<tr>
<td>Lower gingiva</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Upper gingiva</td>
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<td>0</td>
<td>1</td>
<td>2</td>
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<td>Palate</td>
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<td>2</td>
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</tr>
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<td>Mouth floor</td>
<td>1</td>
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<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mode of invasion</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
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<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Grade 2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Grades 1+2 (lower grade group)</td>
<td>6</td>
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<td>7</td>
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<td>Grade 3</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Grade 4</td>
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<td>3</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Grades 3+4 (higher grade group)</td>
<td>5</td>
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<td>11</td>
<td>27</td>
</tr>
</tbody>
</table>

*: well differentiated type, †: moderately differentiated type, ‡: poorly differentiated type
Simple Stain Max PO(M) kit, Nichirei, Tokyo, Japan) at room temperature in humidified chambers. Mouse anti-VEGFR2 (FLk-1) monoclonal antibody (Santa Cruz Biotechnology, CA, USA) at 1:200 dilution, and mouse anti-CD34 monoclonal antibody (Nichirei) at 1:100 dilution were used as the primary antibodies. 3,3'-diaminobenzidine tetrahydrochloride (DAB; Wako, Osaka, Japan) was used as the substrate. The sections were counterstained with hematoxylin or methyl green. Normal murine immunoglobulin G (IgG) diluted to an equivalent protein concentration served as a negative control in place of the primary antibody.

**VEGFR2+ cell count and TSVL**

VEGFR2+ cell count and TSVL were measured using an ocular graticule (H1221, 20 × 20 squares, MeCan Imaging, Inc., Saitama, Japan) with 420 vertical- and 420 horizontal segments within a total area of 62,500 µm² (250 × 250 µm) at ×400 magnification. Five random dots (0.05 mm in diameter) were marked on the cover glasses on the stromal tissue on each slide stained for VEGFR2 or CD34. The center line of the graticule coincided with a perpendicular line extending from each random dot to the nearest tumor, and then the baseline of the graticule was aligned with the intersection point between the perpendicular line and the tumor-host borderline (Fig. 1A). The incidence of VEGFR2+ cells within the graticule was counted at ×400 magnification (Fig. 1B), whereas TSVL within the graticule was estimated by counting the number of vertical/horizontal segments falling within CD34-positive microvascular lumina at ×400 magnification (Fig. 1C). The mean of these five counts was obtained as the value for each case. Every quantitation was performed by two trained observers.

**Statistical analysis**

Differences between mean values (VEGFR2+ cell count and TSVL, respectively) related to the differentiation type or mode of invasion were assessed by one-way analysis of variance (ANOVA). The significance of individual differences was evaluated using Scheffe’s F post-hoc comparison test if ANOVA was significant. Differences between mean values (VEGFR2+ cell count and TSVL, respectively) related to the mode of invasion, lower-grade group (Grades 1+2) and higher-grade group (Grades 3+4), were assessed using unpaired t-test. All statistical analyses were performed using the Stat View software package, version 4.5 (Abacus Concepts Inc., Berkeley, CA), and differences were deemed significant at a 95% confidence interval.

**Fig. 1** Illustration of the method used to obtain the VEGFR2+ cell count and TSVL at the tumor invasive front: A) The baseline of the ocular graticule was aligned with the intersection point between the perpendicular line and the tumor-host borderline. B) The number of VEGFR2 cells within the graticule was counted. C) The TSVL within the graticule was estimated by counting the number of segments (red bars) falling within microvascular lumina fringed with CD34-positive endothelial cells. Scale: 100 µm. T: tumor.
Immunohistochemistry

A number of VEGFR2+ cells were observed in the stroma adjacent to the parenchyma at the tumor invasive front (Fig. 2A). VEGFR2 was detected on the surfaces of cells showing scant cytoplasm and rounded nuclei (Fig. 2B); however, it was not present on either carcinoma cells or mature capillary endothelial cells. Distinct CD34 expression was found on the membrane surface of endothelial cells lining the microvascular lumina at the tumor invasive front (Fig. 2C).

VEGFR2+ cell count and TSVL

The count of VEGFR2+ cells was significantly larger in poorly differentiated (5.7 ± 6.0; mean ± SD) than in well differentiated (P = 0.032; Fig. 3A) cases, and tended to gradually increase from well differentiated to poorly differentiated tumors. Regarding the mode of invasion, the VEGFR2+ cell count was significantly higher in Grade 3 cases (5.9 ± 7.6; mean ± SD) than in Grade 4 cases (P = 0.039; Fig. 3B). No significant difference in the VEGFR2+ cell count was found between the lower grades (1+2) and higher grades (3+4) (P = 0.562; Fig. 3C). TSVL was significantly larger in well differentiated (64.3 ± 37.6; mean ± SD) than in either moderately or poorly differentiated (P < 0.001, each; Fig. 3D) tumors. TSVL tended to increase gradually from poorly differentiated to well differentiated tumors. With regard to parameters related to the mode of invasion, TSVL was significantly larger in Grade 2 (80.1 ± 47.0; mean ± SD) than in either Grade 3 or Grade 4 (P < 0.001, each; Fig. 3E) tumors. TSVL was significantly larger in the lower-grade (1+2) (66.5 ± 38.3; mean ± SD) than in higher-grade (3+4) tumors (P < 0.001; Fig. 3F).

Discussion

OSCC cells themselves may induce hemangiogenic cells (hemangioblasts, hematopoietic stem cells, or angioblasts) in adjoining stromal tissue. In this immunohistochemical study, expression of VEGFR2 was detected on the surface of round stromal cells adjoining the tumor invasive front (Figs. 2A and B). Since VEGFR2 is known to be a marker of hemangiogenesis (hematopoiesis and vasculogenesis) (6), VEGFR2+ cells located around the tumor may be regarded as hemangiogenic cells. However, the origin of such VEGFR2+ cells still remains unclear (29).

In OSCC, hemangiogenic activity at the tumor invasive front may be greater when parenchymal intercellular adhesion is looser and the parenchymal area exposed to the stroma is wider. In this study, the count of VEGFR2+ cells was significantly larger around poorly differentiated...
than around well differentiated tumors (Fig. 3A, \(P = 0.032\)). The proportion of VEGFR2+ cells at the tumor invasive front may represent hemangiogenic activity there (6). In poorly differentiated or higher-grade (3+4) tumors, intercellular bridges are scarce (26) and the cells tend to be separate from one another, so that the parenchymal area exposed to the stroma becomes wide. Thus, the VEGFR2+ cell induction signal passing from tumor cells to the stroma in poorly differentiated or higher-grade (3+4) tumors should be stronger and have a greater induction capacity than that in well differentiated or lower-grade (1+2) tumors. Since the mode of invasion, in contrast to the type of differentiation, is restricted to the tumor periphery (27,28), this study appears to indicate that there is no significant difference in the count of VEGFR2+ cells between grades 1+2 tumors and grades 3+4 tumors.

In OSCC, the blood flow rate at the tumor invasive front may be greater when parenchymal intercellular adhesion is tighter, and when parenchymal nests are larger. In this study, the TSVL around well differentiated tumors was found to be significantly larger than around poorly or moderately differentiated tumors (\(P < 0.001\)). Moreover, in terms of the mode of tumor invasion, the TSVL was significantly higher in Grades 1+2 than in Grades 3+4 tumors (\(P < 0.001\)). WD: well differentiated type, MD: moderately differentiated type, PD: poorly differentiated type.
well differentiated or lower-grade (1+2) tumors should be larger than the others.

This study has demonstrated the relationship between tumor neovascularization and histologic characteristics using immunohistochemical and statistical methods. We can conclude that vascular development at the invasive front of OSCC is governed by the following factors: the tumor cells themselves may induce hemangiogenic cells in the adjoining stromal tissue; the hemangiogenic activity is greater when the parenchymal intercellular adhesion is looser and the parenchymal area exposed to the stroma is wider; and the rate of blood flow may be greater when the parenchymal intercellular adhesion is tighter and the parenchymal nests are larger.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research for High-Tech Research Project (2005-2009) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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