Vascular Endothelial Growth Factor and Dendritic Cells in Human Squamous Cell Carcinoma of the Oral Cavity

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Abstract. Dendritic cells (DCs) play an important role in the host immune defense against tumors, and there is an inverse correlation between DC density and the expression of vascular endothelial growth factor (VEGF). However, the relationship between VEGF expression in tumors and infiltration of CD1a+ or CD83+ DCs, which express the VEGF receptor (VEGFR), remains unclear. Therefore, in vivo and in vitro studies were conducted to investigate the relationship between VEGF expression and DC subsets in oral squamous cell carcinomas (OSCCs). Strong VEGF expression was detected in cancer tissues from patients with regional lymph node metastasis (PN+ cases). In these tissues, the VEGF expression correlated inversely with the number of CD1a+ DCs, but positively with the number of CD83+ DCs. Large amounts of VEGF were secreted by OSCC cell lines, and their culture supernatants significantly inhibited the production of differentiated CD1a+ DCs from peripheral blood mononuclear cells (PBMCs), whereas differentiated CD83+ DCs were increased. VEGFR-1 and -2 were detected in a few PBMCs and CD1a+ DCs. Furthermore, CD1a mRNA disappeared when recombinant human VEGF165 (rhVEGF165) was added to CD1a+ DCs, while CD83 mRNA increased. These results suggest that, in OSCCs, secreted VEGF might promote escape from tumor immunity by inhibiting the differentiation of CD1a+ DCs from progenitor cells and increasing the levels of dysfunctional CD83+ DCs.

Dendritic cells (DCs) are the most potent antigen-presenting cells in the immune system, playing an important role in tumor immunity (1, 2). Infiltration of DCs into primary tumors has been reported to correlate with the prognosis of various human malignant neoplasms, such as cancer of the oral cavity (3-5), larynx (6), esophagus (7), stomach (8), colon (9), lung (10), thyroid gland (11), uterine cervix (12) and breast (13). However, it remains unclear whether a decrease in the number of DCs correlates with a poor prognosis in patients with oral squamous cell carcinoma (OSCC). Moreover, in recent years, the suitability of DCs as a basis for immunotherapy for various malignant neoplasms has been challenged, as it has been shown that cancer cells can produce and secrete immunosuppressive cytokines which induce a defective immune cell function and a defective host immune response.

In a recent study, Gabrilovich et al. found that the production of vascular endothelial growth factor (VEGF) by human cancer cells inhibited the functional maturation of DCs from CD34+ cells (14). Furthermore, Saito et al. reported an inverse correlation between the infiltration of S100+ DCs and VEGF expression in gastric carcinoma, and that these factors had an impact on prognosis (15). Similarly, Takahashi et al. showed that the expression of VEGF-C was inversely-related to the density of S100+ DCs, and that a low S100+ DC density and strong VEGF-C expression were related to a poor prognosis in gastric cancer patients (16). Moreover, Homer et al. reported that serum VEGF was raised in patients with head and neck tumors (17), while Kumar et al. reported significantly higher serum VEGF levels in patients with node-positive colorectal cancers than in healthy subjects and patients with node-negative disease (18). Yamamoto et al. reported that the circulating level of VEGF was aberrantly increased in patients with breast, gastrointestinal, hepatobiliary and lung cancers (19).

Regarding oral cancer, we have previously shown that the distribution of various DC subsets (S100+, CD1a+ and CD83+ DCs) was related to regional lymph node (RLN)
metastasis. Fewer S100+ and CD1a+ DCs but more CD83+ DCs were exhibited in PN+ cases compared with PN- cases (5). However, the effect of these factors in patients with OSCCs remains unknown. We hypothesized that, in patients with PN+ OSCCs, high serum VEGF concentrations might inhibit the differentiation of progenitor cells to DCs, and that, for some unknown reason, the levels of CD83+ DCs would be increased near to the tumor nests. To our knowledge, the effects of VEGF expression and DC subsets, including those expressing the VEGF receptor (VEGFR), on OSCCs have not been described to date.

In this study, the relationship between VEGF expression in OSCCs and VEGFR expression by DCs, and the effects of VEGF on the generation of CD1a+ and CD83+ DCs, in vivo and in vitro, were investigated. VEGF expression was shown to be related to RLN metastasis and the distribution of DC subsets. Furthermore, VEGFRs were detected in CD1a+DCs and peripheral blood mononuclear cells (PBMCs), and the production of differentiated DCs from PBMCs was inhibited by VEGF secreted by human OSCCs. These results suggest that VEGF, secreted by tumor cells, can induce a defective immune cell function and a defective host immune response in patients with OSCCs.

**Materials and Methods**

**Collection of samples.** Fifty-two patients with primary oral cancer were selected, taking care to avoid any influence of radio- or chemotherapy. The case series consisted of 22 patients with OSCCs that had metastasized to the RLNs and 30 with no metastasis. Thirty-one patients were male and 21 were female. The median age was 61.3 years (range, 29-86 years). The tumors were located in the tongue (n=18), lower gingiva (n=17), upper gingiva (n=8), buccal mucosa (n=4), oral floor (n=3) and lip (n=2).

**TNM staging.** Tumor stage was initially classified according to the tumor, node, metastasis (TNM) staging system of the International Union Against Cancer, TNM Atlas – Illustrated Guide to the Classification of Malignant Tumors (20). The patients were categorized as follows: T1 (n=5), T2 (n=21), T3 (n=4), T4 (n=22), N0 (n=26), N1 (n=15), N2 (n=10) and N3 (n=1). All patients were graded M0. Accordingly, they were classified as stage I (n=4), stage II (n=13), stage III (n=11) and stage IV (n=24).

The staging categories for the level of involvement of the cervical lymph nodes were determined according to the criteria set out in the International Union Against Cancer, TNM Atlas – Illustrated Guide to the Classification of Malignant Tumors (21). A total of 439 affected RLNs were identified, and the level of involvement was classified as follows: level 1 (n=130 nodes); level 2 (n=80); level 3 (n=123); and level 4 (n=106).

Samples were fixed in 10% buffered formalin and embedded in paraffin wax, and the tissue sections were prepared for the immunohistochemical procedures as described below.

**Immunohistochemical procedures.** Deparaffinized sections were immersed in absolute methanol containing 0.5% H2O2 for 20 min at room temperature to block endogenous peroxidase activity, then treated with 2% bovine serum albumin for 20 min to block non-specific reactions. The sections were subsequently incubated with rabbit anti-human VEGF polyclonal antibody (1:200, Santa Cruz Biotechnology, Inc., USA), rabbit anti-S100 protein polyclonal antibody (1:500, DAKO, Glostrup, Denmark), anti-CD1a monoclonal antibody (1:2, Immunotech, Marseille, France), or anti-CD83 monoclonal antibody (1:100, Immunotech) for 60 min at room temperature. After washing with phosphate-buffered saline (PBS, pH 7.4), biotinylated goat anti-rabbit IgG (H+L) (1:200, Vector Labs, Burlingame, CA, USA) or biotinylated horse anti-mouse IgG (H+L) (1:200, Vector Labs) was applied to each section for 30 min. An appropriate dilution of streptavidin-peroxidase (GIBCO-BRL, Life Technologies Inc., MD, USA) was applied to the tissue sections for 30 min, after which they were immersed in 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% H2O2 (DAB solution) for 10 min and counterstained with Mayer’s hematoxylin.

Assessment of VEGF expression. VEGF immunoreactivity was evaluated semi-quantitatively by two of the authors, who remained blind to the origin of each tissue section. All specimens were classified into one of four subgroups: 0, 0-<5% positive cells; 1+, 5-25% positive cells; 2+, 25-<50% positive cells; 3+, >50% positive cells (23).

**Assessment of DCs.** The numbers of S100+, CD1a+ and CD83+ DCs in the primary tumor, adjacent tissue including the peritumoral epithelium and the paracortex of the RLNs were counted in the five most densely infiltrated areas using a 1/400 mm2 square field. The counts were then averaged to determine the population density (8).

**Tumor cell lines.** Various OSCC cell lines (HSC-2, -3 and -4, Ca9-22 and KB) and a uterine cervical cell line (HeLa) were obtained from the Japanese Cancer Research Resources Bank. The cells were cultured in complete medium (CM) consisting of RPMI 1640 medium supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 10% heat-inactivated fetal bovine serum (FBS; GIBCO-BRL) at 37°C in a humidified 5% CO2/air mixture. The cultured cells or culture supernatants were subjected to immunocytochemical and Western blot analyses, reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) to detect VEGF.

**Generation of DCs from human blood.** Peripheral blood was obtained from a healthy 32-year-old male donor (first author). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lympho Separation Medium (ICN Biomedicals, OH, USA) and depleted of lymphocytes by washing with PBS after incubation with CM for 2 h. Granulocyte/macrophage colony-stimulating factor (GM-CSF; Genzyme-Techne, Minneapolis, MN, USA) and Interleukin-4 (IL-4; Genzyme-Techne) were then added to final concentrations of 800 and 500 U/ml, respectively (24). The medium was changed on day 4, and the presence of non-adherent cells on day 8 was confirmed by an immunocytochemical method using cytospin sections. To determine whether VEGF suppressed the differentiation of DCs, recombinant human VEGF165 (rhVEGF165, Genzyme-Techne) or culture supernatant from the cancer cell lines listed above was added to this system. In other
tests, CD1a+ DCs and CD83+DCs were used in this system to detect the expression of VEGFR-1 and -2. As a positive control for VEGFR-1 and -2, human umbilical vein endothelial cells (HUVEC; Bio Whittaker, Inc., Walkersville, USA) were used. The HUVECs were cultured using the EGM*-2 BulletKit® (Bio Whittaker, Inc.), which contains EGM-2 medium supplemented with 0.4% hFGF-B, 0.1% hEGF, 0.1% VEGF, 0.1% ascorbic acid, 0.1% R-IGF-1, 0.1% heparin, 0.1% GA-1000, 0.04% hydrocortisone and 2% FBS. The culture was performed at 37 °C in a humidified 5% CO₂/air mixture.

Immunochemical and immunofluorescence procedures. The cultured cells were fixed in 95% ethanol and 5% acetic acid or acetone for 10 min at 4 °C, then stained for CD14 (anti-human CD14 monoclonal antibody, 1:100; DAKO), S100 protein, CD1a, CD14 monoclonal antibody, 1:10, DAKO), S100 protein, CD1a, acetone for 10 min at 4 °C, then stained for CD14 (anti-human CD14 monoclonal antibody, 1:100; DAKO) and VEGF, using the method described above. To block endogenous peroxidase activity, DAB solution containing 0.05% H₂O₂ solution and 3% H₂O₂ solution containing 0.05% H₂O₂ solution was used.

For immunofluorescence studies, incubation with a primary antibody against VEGFR-2 (anti-human VEGFR-2/Fk-1 monoclonal antibody, 1:200; Santa Cruz Biotechnology, Inc., USA) was followed by the addition of biotinylated horse anti-mouse IgG (H+L) (Vector Labs) and the products were visualized using streptavidin-Texas Red (Vector Labs). In a subsequent secondary step, incubation with a primary antibody against VEGFR-1 (anti-human VEGFR-1/Fit-1 monoclonal antibody, 1:200; Santa Cruz Biotechnology, Inc.) was followed by the addition of biotinylated goat anti-rabbit IgG (H+L) (Vector Labs) and the products were visualized using streptavidin-FITC (Vector Labs).

In PBMCs, incubation with primary antibodies against CD14 was followed by the addition of biotinylated horse anti-mouse IgG (H+L) and the products were visualized using streptavidin-FITC (Vector Labs). In a subsequent secondary step, VEGFR-1 or VEGFR-2 antibody was followed by biotinylated goat anti-rabbit IgG (H+L) or horse anti-mouse IgG (H+L), and the products were visualized using streptavidin-Texas Red.

Western blotting VEGF: Cultures of each type of tumor cells were grown to 70-80% confluence, then lysed by adding lysis buffer (consisting of 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% NaN₃, 100 µg/ml phenylmethyl sulfonyl fluoride, 1 µg/ml aprotinin and 1% TritonX-100) for 30 min on ice. The lysate was centrifuged at 12,000 rpm and the supernatant was collected. The total protein concentration of the soluble cell extract was determined using the DC Protein Assay Reagent Kit (Bio-Rad Laboratories). Thirty µg of the protein was dissolved in sample buffer (0.5 M Tris-HCl [pH 6.8], 2 ml, 10%[W/V] SDS 4 ml, β-mercaptoethanol 1.2 ml, glycerol 2 ml, distilled water 0.8 ml and 1%[W/V] bromophenol blue 0.2 ml) and separated using 12% (VEGF, HLA-DR and CD83), 6% (VEGFR-1/Fit-1) or 5% (VEGFR-2/Fit-2) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The immunoblots were transferred onto pure nitrocellulose membranes (Bio-Rad Laboratories), probed with the anti-VEGF polyclonal antibody (diluted 1:500), anti-HLA-DRt monoclonal antibody (diluted 1:300), anti-CD83 monoclonal antibody (diluted 1:300), anti-VEGFR-1 polyclonal antibody (diluted 1:500) and anti-VEGFR-2 monoclonal antibody (diluted 1:500), and visualized as described above.

ELISA for human VEGF secretion. The tumor cells were seeded at a density of 1×10⁶ cells/ml in CM in culture flasks. After incubation at 37 °C for 24, 48, 72, 96 and 120 h, the supernatants were collected, rendered cell-free by centrifugation at 12,000 rpm for 5 min, and stored at –80 °C. The culture supernatants from each cell line were assayed for VEGF using a quantitative solid-phase ELISA (Human Vascular Endothelial Growth Factor Sandwich ELISA Kit, Chemicon International, Inc.). All samples were assayed in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR). RNA was isolated from each cell culture and the control HUVECs using the RNeasy kit (Qiagen, Tokyo, Japan). cDNA was synthesized from 1 µg of total RNA by using the RNA PCR Kit (Takara, Tokyo, Japan) with random hexamers as primers and AMV reverse transcriptase, according to the manufacturer’s protocol. To exclude the possible effects of DNA contamination, the reactions were also performed without AMV reverse transcriptase (DNA control).

The RNA samples were reverse-transcribed in reverse transcriptase (RT) solution for 10 min at 30 °C and 30 min at 42 °C to produce first-strand cDNA. The cDNA samples were incubated at 99 °C for 5 min to inactivate the reverse transcriptase, then chilled to –80 °C. The samples were amplified by PCR under the following conditions: initial denaturation at 94 °C for 2 min; annealing temperatures 56 °C for VEGF (VEGF121, VEGF 165, VEGF189, VEGF206), 55 °C for VEGFR-1 and -2, 53 °C for CD1a, 55 °C for CD83 and 55 °C for GAPDH; 30 PCR cycles for VEGF (VEGF121, VEGF165, VEGF189, VEGF206), 40 for VEGFR-1 and -2, 35 for CD1a and CD83 and 30 for GAPDH; final extension at 72 °C for 2 min. The primers were prepared according to published data (25-28) and were:

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<th>Gene</th>
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<th>Reverse Primer</th>
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</tr>
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</tr>
<tr>
<td>CD83</td>
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<td>antisense 5'-GGACATGCGTGGCCTG-GGTTTAC-3'</td>
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<tr>
<td>GAPDH</td>
<td>sense 5'-CCAAAGCGCGAAGACATCACA-3'</td>
<td>antisense 5'-GGCCGCGCTTCCG-3'</td>
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Ten-µl aliquots of the PCR products were analyzed by agarose gel (2%) electrophoresis and visualized by ethidium bromide staining.

Statistical analysis. The significance of differences between the means was determined using the Mann-Whitney test (for comparing two categories), the Kruskal-Wallis and Scheffe’ tests.
Figure 1. VEGF expression and DC density in PN− and PN+ cases. (A) Immunohistochemical detection of VEGF in the cytoplasm of SCC cells: poorly-differentiated (1) and well-differentiated (2) SCC cells in the cases with metastasis to the RLNs (PN+ cases). (B) Strong reaction for VEGF was observed in the PN+ cases (PN+ cases versus PN− cases, p<0.01). RLNs, regional lymph nodes; PN−, without metastasis to RLNs; PN+, with metastasis to RLNs. (C) Infiltration of S100+, CD1a+ and CD83+ DCs in the primary tumor area. The numbers of S100+ and CD1a+ DCs were greater in the adjacent tissue of PN− cases. The number of CD83+ DC was greater in the tumor region of PN+ cases. *p<0.05; **p<0.005. (D) Infiltration of S100+, CD1a+ and CD83+ DCs in the RLNs. The numbers of S100+ DCs in both metastatic and non-metastatic RLNs of PN+ cases were fewer compared with those of PN− cases. The numbers of CD1a+ DCs in metastatic RLNs of PN+ cases were few compared with those of PN− cases. In contrast to S100+ and CD1a+ DCs, the number of CD83+ DCs in metastatic RLNs was greater in PN+ cases. *p<0.05; **p<0.01; ***p<0.001.
Figure 1C.

Figure 1D.
(for comparing three or more categories) and the Bonferroni/Dunn test (for comparing controls to all other categories). The correlation between the expression of VEGF and the density of the DC subsets was analyzed using the Spearman rank correlation coefficient. The accepted level of significance was $p<0.05$.

**Results**

**In vivo**

*VEGF expression and infiltration of DC subsets in cancer tissue:* In the primary cancer tissue, VEGF expression was detected in the cytoplasm of the tumor cells. Strong VEGF immunoreactivity was observed in the patients with metastasis to the RLNs (Figure 1A). Comparison of the VEGF expressions ($p<0.01$) between PN- cases and PN+ cases is given in Figure 1B. VEGF expression in the primary cancer tissue and metastasis into the RLNs was correlated.

Although there were more S100+ and CD1a+ DCs in the tissues adjacent to the tumor in PN- cases, there were more CD83+ DCs in the primary tumor in PN+ cases (Figure 1C). With regard to the RLN tissue, there were significantly fewer S100+ and CD1a+ DCs in the metastatic RLNs in PN+ cases than in PN- cases (Figure 1D). Conversely, there were more CD83+ DCs in the metastatic RLNs in PN+ cases (Figure 1D).

Inverse correlations were found between the numbers of S100+ and CD1a+ DCs and the degree of VEGF expression (Figure 2A and B), while the degree of CD83+ DC infiltration correlated positively with that of VEGF expression (Figure 2C).

**In vitro**

*VEGF expression and secretion in the tumor cell lines:* Positive reactivity for VEGF was observed in the cytoplasm of the HSC-2, -3 and -4, Ca9-22, HeLa and KB cells (Figure 3A). Furthermore, all OSCC cell lines expressed VEGF mRNA isoforms (VEGF121, VEGF165, VEGF189, VEGF206) on RT-PCR (Figure 3B). Western blot analysis also revealed a signal for VEGF in all the tumor cell lines examined, the signals agreeing with the molecular weight of rhVEGF165 (Figure 3C). VEGF was also detected in the culture supernatants from the tumor cell lines, and was significantly increased in a time-dependent manner (HSC-2, $p<0.05$; HSC-3, $p<0.01$; HSC-4, $p<0.05$; Ca9-22, $p<0.01$; KB, $p<0.01$; HeLa, $p<0.01$) (Figure 3D).

**VEGFR(-1, -2 and -3) expression in DCs grown from PBMCs:** After 8 days, DCs had developed in cultures of PBMCs supplemented with GM-CSF and IL-4, as observed under an inverted phase contrast microscope (Figure 4A). HLA-DR was overexpressed when the PBMCs differentiated into DCs, as observed by Western blot analysis (Figure 4B). Proliferating DC aggregates from PBMCs consisted largely of CD1a- and HLA-DR-positive cells (Figure 4C and D).
In protein lysates of these cells obtained at various time-points (0, 24, 48, 96 and 192 h of culture), the antibody against human VEGFR-1 reacted with a 110-kDa band, and the antibody against human VEGFR-2 reacted with a 195-kDa band (Figure 5A). VEGFR-3 was not detectable in this system.

In the double-staining experiments, the PBMCs and DCs were positive for both VEGFR-1 and VEGFR-2, as indicated by the green fluorescence produced by FITC (CD14) and the red fluorescence produced by Texas-red (VEGFR-1 or VEGFR-2) on the surface of the PBMCs, and the green fluorescence produced by FITC (VEGFR-1) and red fluorescence produced by Texas-red (VEGFR-2) on the surface of the DCs (Figure 5B).

VEGFR-1 and -2 mRNA expressions by PBMCs and DCs were also analyzed using RT-PCR. As shown in Figure 5C, VEGFR-1 and VEGFR-2 mRNA were initially evident in both cell types; however, after treatment with rhVEGF165 over the last 24 h of culture, no signal could be observed in the DCs. The RT-PCR analysis was not designed to detect VEGFR-3.

Effect of VEGF on DC differentiation: When rhVEGF165 or the culture supernatant from the Ca9-22 cell line was added to the PBMC culture system, the differentiation of DCs from PBMCs was significantly inhibited (Figure 6A). After this treatment, the numbers of HLA-DR+ (data not shown) and CD1a + DCs was markedly decreased, whereas the expression of CD68 in the cultured PBMCs was not affected by treatment with either rhVEGF165 or the culture supernatant from the Ca9-22 cells (data not shown).

The expressions of CD1a mRNA and CD83 mRNA were also investigated after adding rhVEGF165 to the PBMCs and DCs. The rhVEGF165 induced an obvious and significant decrease in CD1a mRNA in the DCs; however, CD83 mRNA levels increased markedly after the addition of rhVEGF165 (Figure 6C). The PBMCs did not express CD1a or CD83 mRNAs, irrespective of whether or not they had been treated with rhVEGF165 (Figure 6C).

The increasing CD83 protein level in DCs after rhVEGF165 treatment was confirmed by Western blot analysis (Figure 6D).

Discussion

In this study, it was demonstrated that the expression and secretion of VEGF by OSCCs had a considerable influence on the total number of immature DCs (CD1a+ DCs) from progenitor cells, the distribution of nature DCs (CD83+ DCs) and the metastasis to RLNs. VEGFRs were detectable in both the PBMCs and DCs obtained from a PBMC culture system, and the differentiation of DCs from PBMCs was significantly inhibited by VEGF. In addition to the fact that increased numbers of CD83+ DCs were located near to the tumor nests in PN+ cases, CD83 expression by DCs was increased by adding rhVEGF165 to CD1a+ DCs.

Previous work has suggested that DCs play a key role in antineoplastic immunity. Clinical studies on malignant tumors of various organs have shown that the presence of a dense DC infiltrate in the primary tumor is associated with improved patient survival (3-13). With regard to oral cancers, Wei et al. (4) and Goldman et al. (3) reported that a decreased number of S-100 or CD1a+ DCs was associated with a poor prognosis and lymph node metastasis in OSCCs. We therefore examined the DC subsets present in the primary tumors, the adjacent tissue and the RLNs (with or without metastasis) in patients with OSCC using immunohistochemistry. PN+ cases exhibited a significantly reduced density of S-100+* and CD1a+ DCs in both the primary tumor area and the RLNs, suggesting that DCs are associated with the metastasis of oral cancer to the RLNs (5). This concurs with the results that were described for gastric cancer by Tsujitani et al. (29). Lymph node metastasis is a factor with a critical influence on prognosis, and patients with PN+ have a less favorable clinical course than those with PN− (30).

Lucas and Halliday reported that progressive skin tumors in mice produce a factor that inhibits Langerhans cell migration from the epidermis to local lymph nodes (31). It is thought that the decreased number of DCs in PN+ cases may be attributable to the production of soluble factors (immunosuppressive cytokines) by the tumor. It has also been reported that IL-10, tumor growth factor (TGF)-β and VEGF influence the differentiation, maturation and function of DCs (1, 14, 32, 33).

We found that VEGF was expressed more strongly in primary tumors in patients with PN+ than in those with PN−, thus demonstrating that VEGF is associated with metastasis to RLNs in OSCCs. VEGF protein is expressed by a wide variety of cancer tissues (34-38) and is also considered to be a marker of tumor invasion, metastasis and a prognostic factor in cancer patients. Saito et al. reported that the expression of VEGF was inversely related to the density of S-100+* DCs in gastric adenocarcinoma tissue (15). Similarly, we also showed that the expression of VEGF is inversely-related to the density of S-100+ and CD1a+ DCs, although it is positively correlated with the density of CD83+ DCs in OSCC tissues.

The serum concentrations of VEGF have been reported to be abnormally elevated in patients with various cancers, including head and neck, compared with healthy subjects (17-19, 39). Takano et al. described time-dependent increases in the VEGF concentrations in the culture supernatants of various glioma cells (40). We also found that VEGF was expressed in the cytoplasm of human OSCC cell lines, and that the VEGF concentrations in their culture
Figure 3. VEGF expression and secretion in human SCC cell lines. (A) VEGF expression could be detected in the cytoplasm of the human SCC cell lines, HSC-2, HSC-3, HSC-4, Ca9-22, HeLa and KB. (B) In RT-PCR showing the expression of VEGF in the human SCC cell lines, HSC-2, HSC-3, HSC-4, Ca9-22, KB and HeLa, four bands were detected, as shown, corresponding to VEGF_206, VEGF_189, VEGF_165 and VEGF_121. (C) Western blot analysis showed the expression of VEGF in the human SCC cell lines, HSC-2, HSC-3, HSC-4, Ca9-22, HeLa and KB. Bands were detected with 30 ng of recombinant human VEGF_165 protein (rhVEGF) under the same conditions. (D) The VEGF concentration in the culture supernatants time-dependently increased in the various human SCC cells (HSC-2, p<0.05; HSC-3, p<0.01; HSC-4, p<0.05; Ca9-22, p<0.01; HeLa, p<0.01; KB, p<0.01).
supernatants increased in a time-dependent manner in vitro, suggesting that VEGF is secreted from tumor cells into the serum of patients with various cancers including OSCCs, consequently producing abnormally high circulating concentrations. Conversely, it has been reported that the circulating density of DCs was reduced in the peripheral blood of various cancer patients (41). In particular, the antigen-presenting function of DCs toward T cells in the peripheral blood and lymph nodes of patients with head and neck cancer was decreased, this phenomenon being caused by the accumulation of immature hematopoietic cells in the peripheral blood secondary to elevated VEGF levels (41). Furthermore, the number of immature hematopoietic cells in the peripheral blood of patients with lung cancer was usually restored to normal by VEGF-neutralizing antibodies, but not by IL-10- or TGF-β-neutralizing antibodies (41-43).

Gabrilovich et al. demonstrated that VEGF inhibited the differentiation and functional maturation of DCs from CD34+ precursors (14), thus indicating that the VEGF produced and secreted by various tumor cells is a significant immunosuppressive factor. We also indicated that when rhVEGF165 or the culture supernatant from the OSCC cell lines was added to a PBMC culture system, the differentiation of DCs from PBMCs was significantly inhibited. Strangely, the number of CD83+ cells tended to increase even though the population of DCs markedly decreased after the addition of rhVEGF165 and the culture supernatant of OSCC. The increase of CD83+ cells by rhVEGF165 and the OSCC culture supernatant was difficult to understand. However, it is consistent with the results of the immunohistochemical tests performed on samples taken from patients with OSCCs. Schwaab et al. reported that the expression of VEGF in colon cancer tissue was associated with an increase in the density of mature DCs (CD83+DC) in colon cancer (44).

The glycoprotein CD83 is a member of the immunoglobulin superfamily. It is expressed by cells of the DC lineage, including skin Langerhans cells and by DCs in the T cell zones of lymphoid organs (45). Bell et al. reported the presence of immature CD1a+ DCs within carcinoma nests, whereas...
mature CD83+ DCs were located in the circumferential carcinoma nests (46). Similarly, in our study, CD83+ DCs were detected in circumferential tumor tissue. To elucidate whether the expression of CD83 in DCs is indeed increased by VEGF, we stimulated CD1a+ DCs with rhVEGF165 for 24 h. RT-PCR and Western blot analysis subsequently revealed an increase in the expression of CD83 mRNA and protein. These results suggest that rhVEGF165 may also induce CD83 expression.

It has also been reported that the differentiation of DCs from CD34+ hematopoietic progenitor cells is induced by signal transduction between TNF-α/TNF-α receptors on the cell surface and the nuclear factor (NF)-κB/inhibitory (I)κB complex in the nucleus, whereas VEGF inhibits the activation of NF-κB in hematopoietic progenitor cells, the dominant negative effect of IκB subsequently preventing DC differentiation (42, 43, 47).

VEGF and its receptors have profound effects on the early development and differentiation of both vascular and hematopoietic progenitors (48). VEGFR-1 and -2 were detected in a few PBMCs and CD1a+ DCs. VEGF secreted by OSCCs may also induce unusual angioid cells and incomplete CD83 expressed cells via PBMCs and CD1a+ DCs carrying VEGFRs. By way of explanation, Pujol et al. reported that VEGFR-1, -2 and -3 were detected in human CD14-positive monocytes and DCs derived from peripheral monocytes and, furthermore, that these cells converted into endothelial-like cells with the formation of a tubular structure by VEGF (26, 27).

In summary, high concentrations of VEGF were detected in the culture supernatants of OSCC cell lines. The differentiation of DCs from PBMCs was significantly inhibited after treatment with the culture supernatants or rhVEGF165 in vitro. This effect was significantly more prominent with the culture supernatants of OSCC cell lines than with rhVEGF165, implying the existence of immunosuppressive cytokines other than VEGF in the culture.

Figure 4. Differentiated DC originating from normal human PBMCs. (A) On day 0, adherent mononuclear cells (MCs) were depleted of lymphocytes by washing with PBS after incubation with CM for 2 h; on day 8, DCs grown from normal human PBMC with GM-CSF and IL-4 were observed as large non-adherent cells with some protruding cells. (B) Western blot analysis showed expression of the HLA-DR band detected in the DCs grown from PBMC. (C) Cytospin preparation; DCs grown from normal PBMC with GM-CSF and IL-4. Immunocytochemical analysis revealed a strong reaction for CD1a (left and right upper panels) and HLA-DRα (right lower panel) in non-adherent cells. The left lower panel shows hematoxylin and eosin staining (H.E.). (D) The phenotype and characteristics of DCs generated from PBMC with GM-CSF and IL-4. Most non-adherent cells were HLA-DR- and CD1a-positive. A small number of CD83-positive cells was also observed. There were only a few CD68-positive cells.
**Figure 4C, D.**

C

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<th>CD1a+ DC</th>
<th>CD1a+ DC</th>
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<tbody>
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<td><img src="image2.png" alt="Image of CD1a+ DC" /></td>
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</tbody>
</table>

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<th>HLA-DR+ DC</th>
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<tbody>
<tr>
<td><img src="image3.png" alt="Image of DC (H.E.)" /></td>
<td><img src="image4.png" alt="Image of HLA-DR+ DC" /></td>
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D

**Control**

<table>
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<tr>
<th>HLA-DR</th>
<th>CD1a</th>
<th>CD83</th>
<th>CD68</th>
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<tbody>
<tr>
<td><img src="chart1.png" alt="Bar chart of HLA-DR" /></td>
<td><img src="chart2.png" alt="Bar chart of CD1a" /></td>
<td><img src="chart3.png" alt="Bar chart of CD83" /></td>
<td><img src="chart4.png" alt="Bar chart of CD68" /></td>
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</table>

*Figure 4C, D.*
Figure 5. VEGFR expression in differentiated DC originating from normal human PBMC with GM-CSF and IL-4. (A) Western blot analysis showing VEGFR-1 and VEGFR-2 protein in differentiated DCs originating from the PBMC culture system. VEGFR-1 and -2 bands were detected in HUVECs under the same conditions. VEGFR-1 was detected in the 110-kDa band and VEGFR-2 was detected in the 195-kDa band, as in HUVECs. (B) Expression of VEGFR-1 and VEGFR-2 protein was observed in CD14 positive cells and in CD1a+ DCs by fluorescence microscopy. (C) RT-PCR showing VEGFR-1 and VEGFR-2 in the differentiated DCs from the PBMC culture system with HUVECs under the same conditions. VEGFR-1 was detected in 449 bp and VEGFR-2 was detected in 619 bp, as in HUVECs.
supernatants of these OSCC cell lines. In vitro studies demonstrated that VEGF, IL-10 and TGF-β can inhibit the function and maturation of DCs (14, 32, 33, 49). These results suggest that immunosuppressive cytokines, including VEGF, secreted by tumor cells, may interfere with the dedifferentiation and function of DCs, and this phenomenon may partially explain why tumor immunity is not effectively induced in patients with SCC of the oral cavity. The efficient
elimination of cancer cells via immunodefense mechanisms remains the ideal goal of therapy. Therefore, in order to enable an immunotherapeutic challenge, it is necessary to restore the increased levels of immunosuppressive factors, including VEGF, in the peripheral blood of OSCC patients to normal levels.

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References


Figure 6. Inhibition of the production of differentiated DCs from PBMCs by VEGF. (A) When rhVEGF_{165} or the culture supernatants from SCC cell lines (Ca9-22) were added to the PBMC culture system, the production of differentiated DCs from PBMCs was significantly inhibited in comparison to the control. *p<0.0001. (B) The population of immature CD1a⁺ DCs tended to decrease even though the number of CD83⁺ cells was markedly increased after adding the culture supernatants (Ca9-22) and rhVEGF_{165}. Inhibition of the production of differentiated CD1a⁺ DCs from PBMCs by the culture supernatant from the SCC cell line was significantly higher than that effected by rhVEGF_{165}. *p<0.05, **p<0.0001. (C) RT-PCR analysis revealed an increase in the expression of CD83 mRNA and a decrease in the expression of CD1a mRNA, if rhVEGF_{165} was added to the CD1a⁺ DCs. (D) Western blot analysis revealed an increase in the expression of the CD83 protein, if rhVEGF_{165} was added to the CD1a⁺ DCs.


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