

Clinicopathological implications of vascular endothelial growth factor 165b expression in oral squamous cell carcinoma stroma

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Abstract. Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors. VEGF165b was recently isolated as the anti-angiogenic VEGF splice variant. In the present study, we examined the association between VEGF165b expression and clinicopathological characteristics in order to determine how VEGF165b produced from oral squamous cell carcinoma (OSCC) affects the stromal cell biological activity. We examined the relationships between the expressions of both VEGF isoforms in normal human dermal fibroblasts (NHDFs) and OSCC cell lines (HSC2, 3, 4 and SAS). Our analyses indicated that both the mRNA and protein expression levels of VEGF165b in the HSC2 and SAS cells were higher than those in the NHDFs. VEGF165b did not promote cell growth or invasive capabilities, but it induced the cell adhesive capabilities to ECM. Although strong expression of the VEGF165 isoforms in tumor cells of OSCC tissues was observed, there was no significant difference in the VEGF165b expression level among the various degrees of malignancy. OSCC cells secrete VEGF165b into the stroma, and this factor

may contribute to the process of anti-angiogenesis by inhibiting gelatinase-expressing cells and activating cell adhesive capabilities to ECM, such as that of fibroblasts surrounding tumor cells.

Introduction

In the cancer microenvironment, complex interactions between tumor cells and the surrounding tissues have important roles in tumor growth and metastasis. In addition to their direct biological activities mediating invasive processes by degrading the extracellular matrix (ECM) via the production of cytokines and proteolytic enzymes, cancer cells alter their adjacent stroma in order to form a supportive cancer microenvironment (1). Fibroblasts within the tumor stroma, known as carcinoma-associated fibroblasts (CAFs), were reported to accelerate tumor progression and metastasis (2). CAFs express alpha-smooth muscle actin (α -SMA) and produce ECM proteins such as fibronectin and collagen. CAFs also produce a number of important factors that directly promote growth in the adjacent epithelium.

Oral squamous cell carcinoma (OSCC) is one of the leading causes of cancer-related death. Although OSCC accounts for approximately 3% of all common cancers, the relative survival rates of OSCC patients are poor because of high local metastasis and recurrence rates, despite the development of various treatment methods (3). Kawashiri *et al* (4) reported that the presence of CAFs in OSCC patients was correlated with the tumor stage, metastasis and a poor prognosis. CAFs thus, appear to play a critical role in many of the biological functions of the cancer-stroma interplay at the tumor-host borderline, called the invasive front, via cancer cell-cell adhesion, cancer cell-ECM interactions, ECM degradation and the expression of cytokines.

Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors. In OSCC patients, the overexpression of VEGF has been identified as an independent prognostic indicator for survival and recurrence (4). In VEGF gene expression, alternate splice site selection in the terminal exon of mRNA yields two families of VEGF isoforms: the pro-angiogenic family and the anti-angiogenic family of VEGF. Proximal splice site selection in exon 8 yields the

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Abbreviations: bFGF, basic fibroblast growth factor; CAF, carcinoma-associated fibroblast; ECM, extracellular matrix; FAK, focal adhesion kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; HUVEC, human umbilical vein endothelial cell; IP-10, interferon gamma-induced protein 10; NHDF, normal human dermal fibroblast; OSCC, oral squamous cell carcinoma; qPCR, quantitative real-time polymerase chain reaction; TGF- β , transforming growth factor-beta; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; α -SMA, alpha-smooth muscle actin

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pro-angiogenic VEGF_{xxx} isoform (xxx is the number of amino acids such as 121, 145, 165, 183, 189 and 206), whereas distal splice site selection generates the anti-angiogenic VEGF_{xxx} isoform (5).

Among the pro-angiogenic isoforms, VEGF165 is known to play critical roles in angiogenesis (6). VEGF165b has been identified as an alternative isoform of VEGF165 via differential splice acceptor site selection in the 3'-untranslated region (3'-UTR) within exon 8. VEGF165b antagonizes VEGF165-induced endothelial cell proliferation and competitively binds to vascular endothelial growth factor receptor 2 (VEGFR2), resulting in the inhibition of downstream signal transduction pathways (7-10). However, it has been unknown whether VEGF165b affects OSCC stroma cell biological activity, particularly in fibroblasts.

In the present study, we investigated whether VEGF165b produced from OSCC cells affects the biological activity of stromal cells and whether VEGF165b acts on specific molecular targets associated with the biology of OSCC patients.

Materials and methods

Materials. For these *in vitro* experiments, recombinant human VEGF165 (rVEGF165) and VEGF165b (rVEGF165b) were purchased commercially from R&D Systems (Minneapolis, MN, USA) and dissolved in sterile phosphate-buffered saline (PBS) containing at least 0.1% bovine serum albumin (BSA) at a concentration of 100 µg/ml, and stored at -20°C prior to use.

Patients and cell lines. Seventy-one consecutive patients with OSCC (44 men, 27 women; median age, 70 years; range, 32-93 years) who underwent surgical resection with curative intent between January 2006 and December 2013 at the Showa University Dental Hospital were enrolled in this study. Four OSCC cell lines (HSC2, 3, 4 and SAS) derived from human oral squamous cell carcinoma were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Normal human dermal fibroblasts (NHDFs) derived from human dermals were purchased from Lonza Biologics (Basel, Switzerland) and used between passages 2 and 10. These cells were also cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS.

Human umbilical vein endothelial cells (HUVECs) derived from human umbilical cords were purchased from Lonza Biologics and used between passages 6 and 10. These cells were cultured in endothelial cell basal medium (EBM)-2 complete medium (Lonza Biologics). Prior to their use in experiments, the HUVECs were maintained in EBM-2 medium without hydrocortisone for ≥24 h. The removal of hydrocortisone was necessary, since it inhibits metalloproteinase production. Once passed and plated, the HUVECs grew normally even in the absence of hydrocortisone. Hypoxia experiments were performed for the indicated times in a humidified multigas incubator (model BL-43MD; Bio-Labo, Tokyo, Japan) calibrated to deliver 5% CO₂, 2% O₂ and 93% N₂ at 37°C.

Bead-based assays. Bio-Plex Pro™ assays with protein profiles using an immunobead-based system were performed following the Bio-Rad systems protocol (Bio-Rad Laboratories,

Hercules, CA, USA). Bio-Plex Pro™ Human Cytokine Standard Group I 24-Plex panels of capture antibody-coated beads and labeled detection antibodies were used with the cell lysate samples. Initially, the 96-well filter-bottom plates were pre-wet with phosphoprotein wash buffer, and 50 µl of the sample was added to each well in duplicate. The plate was then incubated for 1 h and washed three times with phosphoprotein wash buffer. Next, 50 µl of diluted biotin antibody was added to each well and incubated for 30 min. The plate was then washed, and 50 µl of diluted Streptavidin-PE (a component of the Bio-Plex reagent kit) was added to each well and incubated for 10 min. All incubations were performed at room temperature on a shaker set at 300 rpm. Finally, the plate was washed again with 100 µl of phosphoprotein wash buffer, and 125 µl of bead resuspension buffer was added. The median relative fluorescence units were measured using a Bio-Plex Assay Reader (Bio-Rad Laboratories).

RNA extraction and quantitative real-time polymerase chain reaction (qPCR). For the detection of *Vegf165b* mRNA on various OSCC cell lines, total RNA was extracted from growing OSCC and NHDF cells at the logarithmic phase in a 3.5-cm dish using TRIzol® reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's directions.

For the detection of *gelatinase* mRNA, subconfluent NHDF cells in a 3.5-cm dish were cultured for 24 h in DMEM containing 10% FBS. The cells were replenished with fresh medium without FBS and further cultured for 6 h. One of various concentrations of rVEGF165 or rVEGF165b was then added, and the culture was continued for 12 h. The complementary DNA (cDNA) mixture was generated by reverse transcription using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Tokyo, Japan) and then used as a template for the subsequent real-time PCR analysis using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) and the MyiQ™ 2 Two-Color Real-time PCR detection system (Bio-Rad Laboratories). The primers used were 5'-tggtgtacaagatccgcagacgtg-3' and 5'-tca ccgctcggtgtcacaatctgcaagtacgtt-3' for *vegfl65* and 5'-tggtgt tacaagatccgcagacgtg-3' and 5'-gtctgtatcagcttctctggtgagagatc tga-3' for *vegfl65b* (11). For *vegfl65*, an initial denaturation at 96°C for 5 min was followed by 30 cycles of denaturation at 96°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 60 sec.

For *vegfl65b*, an initial denaturation at 96°C for 5 min was followed by 45 cycles of denaturation at 96°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 60 sec. The other primers were: for MMP-2, 5'-ccacgtgagaagccatgg ggcccc-3' and 5'-gcagctagccagcagctcggtatgatg-3'; for MMP-9, 5'-gccccaccgagctgactc-3' and 5'-ttcaggcgaggaccatagagg-3'; and for 18S ribosomal RNA, 5'-tctgcccagtagcatatgctg-3' and 5'-agaggagcggagcggaccaaagg-3'. All results were normalized to 18S. The threshold cycle (Ct) values for 18S and the genes of interest were measured for each sample, and the relative transcript levels were calculated by the ΔΔCT method.

Western blot analysis. A western blot analysis was carried out as previously described (12). For the detection of VEGF165b expression on the various OSCC cell lines, total cellular proteins were harvested from growing OSCC and NHDF cells at the logarithmic phase in a 3.5-cm dish.

For the detection of α -SMA and the determination of the focal adhesion kinase (FAK) expression, subconfluent NHDF cells in a 3.5-cm dish were cultured for 24 h in DMEM containing 10% FBS. The cells were replenished with fresh medium without FBS and further cultured for 6 h, and then the cells were exposed to one of various concentrations of rVEGF165 or rVEGF165b and harvested after 12 h or at the indicated time for α -SMA expression or FAK expression, respectively.

For the hypoxic experiments, subconfluent NHDFs and the OSCC cell lines in a 3.5-cm dish were cultured for 24 h in 2 ml of DMEM containing 10% FBS. The cells were replenished with 2 ml of fresh medium and left under normoxic or hypoxic conditions for the desired times. The primary antibodies human VEGF165 (catalog no. AF-293-NA) and VEGF165b (cat. no. MAB3045) were obtained from R&D Systems. Anti-actin antibodies, anti-FAK, anti-phospho-FAK antibodies, and peroxide-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal mouse IgG anti- α -SMA antibody was obtained from Abcam (Cambridge, MA, USA).

Cell proliferation assays. Cell proliferation assays were performed as previously described (13). The monolayer cell proliferation was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay kit (Roche Diagnostics, Indianapolis, IN, USA) that measures a purple formazan compound produced by viable cells. The cells (5×10^3 /well) were seeded with DMEM in 96-well plates (Falcon Laboratories, McLean, VA, USA). After 24 h, the cells were treated with various concentrations (1, 10, 20, 50 and 100 ng/ml) of rVEGF165 or rVEGF165b and then further cultured for 3 days.

ECM adhesion assay. Adhesion assays were performed using the CytoSelect™ 48-Well Cell Adhesion Assay (Cell Biolabs, Inc., San Diego, CA, USA). NHDF cells (1×10^5) were seeded onto the wells in serum-free DMEM containing 100 ng/ml of rVEGF165, rVEGF165b or both isoforms and then incubated at 37°C for 6 h in a tissue culture incubator prior to washing and staining. Gelatin zymography was performed using a Gelatin Zymography kit (Cosmo Bio Co., Ltd., Tokyo, Japan).

Subconfluent NHDF cells in a 3.5-cm dish were cultured for 24 h in 2 ml of DMEM containing 10% FBS. The cells were replenished with 2 ml of fresh medium without FBS containing one of various concentrations of rVEGF165, rVEGF165b or both isoforms and further cultured for 18 h. Next, 2 ml of the supernatant was harvested and then concentrated to 50 μ l by the Amicon Ultra-0.5 centrifugal filter device (Merck Millipore, Billerica, MA, USA).

Immunohistochemical assay. For immunohistochemical staining, paraffin-embedded tissues were cut at 4 μ m. Slides were deparaffinized in xylene for 5 min three times, in 100% ethanol for 5 min twice, in 90% ethanol for 5 min, in 80% ethanol for 5 min, in 70% ethanol for 5 min, in distilled water for 5 min, and finally in Tris-buffered saline (TBS) for 5 min three times. After being deparaffinized and rehydrated, the sections were heated in 10 mM sodium citrate buffer for 20 min in an autoclave at 120°C. The sections were incubated

in 3% H₂O₂-methanol for 10 min to inactivate endogenous peroxidase. Non-specific binding was blocked with Protein Block Serum-Free (Dako Japan, Tokyo, Japan) for 30 min at room temperature.

The tissue sections were then incubated in a 1:50 dilution of monoclonal mouse IgG anti-human VEGF165b antibody (R&D Systems) and with a ready-to-use monoclonal mouse antibody IgG CD34 (Monosan, Uden, The Netherlands) placed on the sections for 2 h in humidified boxes at room temperature. A 1:50 dilution of antigen affinity-purified polyclonal goat IgG anti-human VEGF165 antibody (R&D Systems) and polyclonal mouse IgG anti- α -SMA actin antibody (Abcam) were placed on the sections in humidified boxes and left at 4°C overnight. After being rinsed with TBS for 3 min three times, the sections were incubated with a biotinylated secondary antibody, EnVision+ mouse/HRP (Dako) or polyclonal rabbit anti-goat immunoglobulins/HRP (Dako) for 30 min at room temperature. The sections were stained using the Liquid DAB⁺ Substrate chromogen system (Dako). The slides were mounted prior to observation under a conventional light microscope. The clinicopathological findings were assessed based on the International Union Against Cancer TNM staging system (14). The histological mode of invasion was classified according to the method of Anneroth *et al* (15).

Statistical analyses. The statistical analyses for the qPCR and adhesion assays were carried out using Student's t-test. The resulting data are shown as the means \pm standard deviations. P-values <0.05 were considered significant. We used Pearson's Chi-square test to analyze the correlation between the clinicopathological features and the expressions of VEGF165 and VEGF165b.

Results

The protein expression profiles of the NHDFs and the OSCC lines. We used immunobead-based systems to compare the expression protein profiles of a variety of cytokines in NHDFs and four OSCC lines. A detailed comparison of the concentrations from the cell layer led to the identification of interleukin (IL)-1 β , IL-8, basic fibroblast growth factor (bFGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ -induced protein 10 (IP-10) and VEGF, which showed significant differences between the NHDFs and the OSCC lines. Of these identified cytokines, the expression of one angiogenic factor, VEGF, was markedly increased on several OSCC lines compared to the NHDFs (Table I). We therefore focused on this potent angiogenic factor of interest as a clinicopathological characteristic of OSCC.

The RNA and protein levels of both VEGF165 and VEGF165b in the NHDFs and OSCC lines. It is well known that various isoforms of VEGF are produced from a single gene by means of alternative splicing. Of these isoforms, VEGF165b was isolated as the anti-angiogenic VEGF splice variant (16). In the present study, we first evaluated the mRNA and protein levels of both VEGF165 and VEGF165b expressions in the NHDFs and the four OSCC lines. As shown in Fig. 1A, the NHDFs and the HSC3 and HSC4 cells faintly expressed *vegfl65* mRNA, whereas the HSC2 and SAS cells more strongly expressed

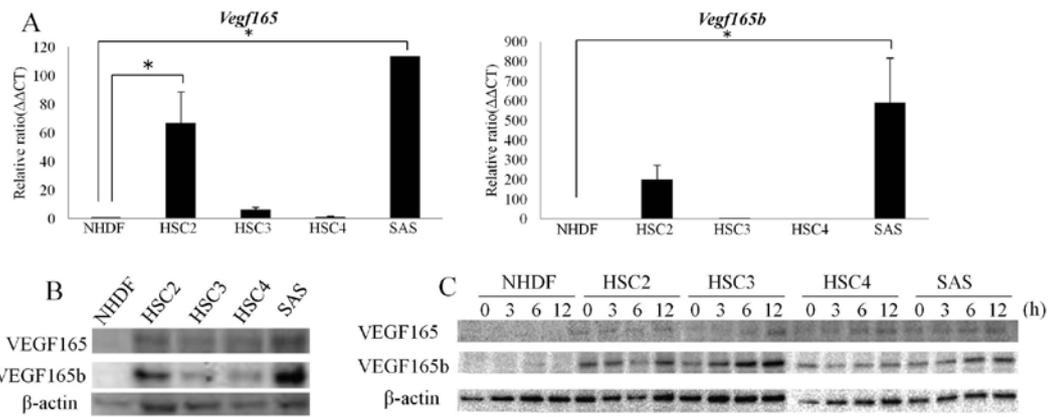


Figure 1. The RNA and protein levels of VEGF165 and VEGF165b in the NHDFs and the OSCC lines. (A) The *vegfl65* and *vegfl65b* mRNA expressions were analyzed by real-time PCR in the NHDFs and OSCC lines. The *vegfl65b* mRNA levels displayed a pattern of expression similar to those of the *vegfl65* mRNA level in the NHDFs and all four OSCC lines (HSC2, 3, 4 and SAS). (B) The expression of VEGF165 and VEGF165b protein was analyzed by an immunoblot assay. (C) The production of the hypoxia-increased VEGF isoforms was analyzed by an immunoblot assay. The hypoxic treatment (2% O₂) of these cells along a 12-h time course resulted in significant VEGF165b protein enhancement in the HSC2, 3 and SAS cells in a time-dependent manner. *P<0.05 vs. the control.

Table I. Protein expression profiles of the NHDFs and the OSCC lines.

Gene name	Functional class	Fibroblast	HSC2	HSC3	HSC4
IL-1β	Cytokine	OOOR<	152	16.8	22.9
IL-1ra	Cytokine	19.4	36.9	72.4	32.6
IL-2	Cytokine	OOOR<	0.4	OOOR<	OOOR<
IL-4	Cytokine	0.5	1.7	1.3	0.7
IL-5	Cytokine	OOOR<	OOOR<	OOOR<	OOOR<
IL-6	Cytokine	1.4	14.3	35.5	25.2
IL-7	Cytokine	5.9	18.4	21.1	14.1
IL-8	Cytokine	1.9	162.9	214.4	7.2
IL-9	Cytokine	OOOR<	3	4.3	2
IL-10	Cytokine	OOOR<	17.7	10.2	11.9
IL-12	Cytokine	0.6	9.4	3.9	7.6
IL-13	Cytokine	0.2	0.7	0.9	0.6
IL-15	Cytokine	OOOR<	OOOR<	OOOR<	OOOR<
IL-17	Cytokine	OOOR<	0.6	2.4	0.8
bFGF	Cytokine	2777.3	945.1	2820	576.7
G-CSF	Cytokine	145.6	243.6	571	248.4
GM-CSF	Cytokine	OOOR<	41.9	55.3	34.4
IFN-γ	Cytokine	9.5	20.3	17.8	9.8
IP-10	Chemokine	OOOR<	152	16.8	22.9
MCP-1	Chemokine	0.9	1	81.2	0.4
MIP-1	Chemokine	1	0.4	0.2	0.2
RANTES	Chemokine receptor	2	1	0.4	0.2
TNF-α	Chemokine	44.8	116.5	95.7	53.8
VEGF	Growth factor	OOOR<	278.2	78.9	194.3

Bio-Plex panels were hybridized with cell layer protein prepared from NHDFs and the OSCC lines HSC2, 3, 4 and SAS cultivated for 12 h. The protein expression profiles of a variety of cytokines (pg/ml) are given together with their functional classes. NHDFs, normal human dermal fibroblast; OSCC, oral squamous cell carcinoma; OOR<, undetectable.

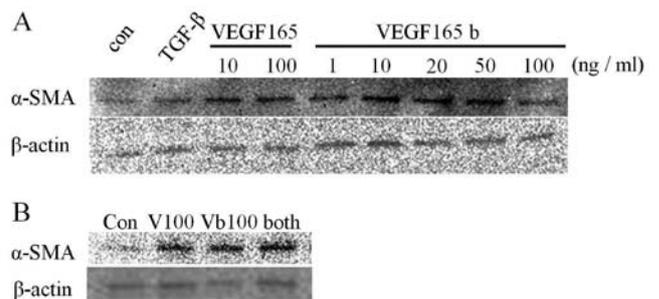


Figure 2. VEGF165b stimulates the transdifferentiation of fibroblasts into myofibroblasts. (A) Subconfluent NHDFs in a 3.5-cm dish were cultured for 24 h in 2 ml of DMEM containing 10% FBS. The cells were replenished with 2 ml of fresh medium without FBS containing a various concentration of rVEGF165, rVEGF165b or both isoforms and further cultured for 18 h. TGFβ (10 ng/ml) as positive control is known to promote the transdifferentiation of fibroblast to myofibroblasts. (B) VEGF165 or VEGF165b (100 ng/ml) modulated the transdifferentiation of fibroblasts into myofibroblasts. Addition of both isoforms also induced α-SMA expression.

vegfl65 mRNA compared to the NHDFs and HSC3 and HSC4 cells.

Notably, the *vegfl65b* mRNA levels displayed a pattern of expression similar to that observed for the *vegfl65* mRNA levels in the NHDF, HSC2, HSC3, HSC4 and SAS cells (Fig. 1A). At the protein level, the HSC2 and SAS cells highly expressed both VEGF165 and VEGF165b, whereas the NHDF, HSC3 and HSC4 cells expressed VEGF165 and VEGF165b only weakly, suggesting that the protein expression patterns of both VEGF165 and VEGF165b are correlated with the patterns observed for the mRNA levels.

Since hypoxia could be a contributing factor in the stimulation of the switch of VEGF165b to VEGF165 during angiogenesis (17), we proceeded to determine whether or not the VEGF165b expression could be upregulated in OSCC cells in response to hypoxic culture conditions. As shown in Fig. 1C, the hypoxia treatment of the OSCC lines along a 12-h time course resulted in significant VEGF165b protein enhancement until 12 h of hypoxic exposure in HSC2, HSC3 and SAS cells, whereas the VEGF165 protein level showed a

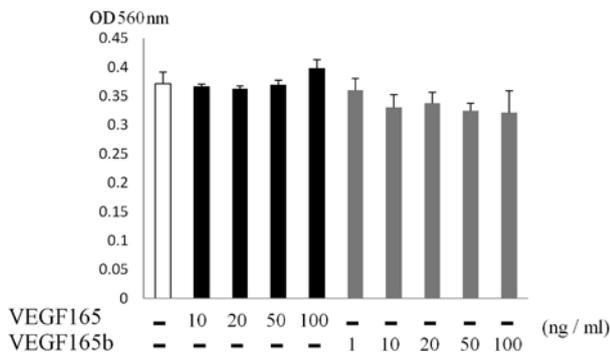


Figure 3. VEGF165b did not stimulate the proliferation of fibroblasts. NHDFs were inoculated at a density of 5×10^3 /well in 96-well plates and cultured in DMEM containing 10% FBS. After 24 h, the cells were replenished with fresh medium without FBS containing the indicated concentration of rVEGF165 or rVEGF165b and then further cultured for 3 days.

faint but similar tendency to increase until the 12-h time point of hypoxic exposure in all four OSCC lines.

VEGF165b modulates the fibroblast transdifferentiation to myofibroblasts. Transforming growth factor-beta (TGF- β) was identified as the main factor in the promotion of the transdifferentiation of fibroblast to myofibroblasts (18). Myofibroblasts are differentiated fibroblasts that express α -SMA. Here we examined whether VEGF165b regulates the transdifferentiation of fibroblasts into myofibroblasts. As shown in Fig. 2A, not only VEGF165 but also VEGF165b induced α -SMA expression, indicating that the normal fibroblasts transdifferentiated into myofibroblasts, i.e., the so-called CAFs, promoted by VEGF165 and VEGF165b secreted from the tumor cells.

Notably, the addition of both VEGF isoforms also induced α -SMA expression despite the isoforms' competitive binding to the same receptor (VEGFR2).

VEGF165b did not stimulate the proliferation of fibroblasts. Since CAFs promote tumor invasion and migration through paracrine stimulation (2), we examined the ability of VEGF165b to induce the proliferation of fibroblasts at various concentrations of VEGF165b (1-100 ng/ml). As shown in Fig. 3, VEGF165 or VEGF165b had no effect on the proliferation of fibroblasts at any of the concentrations used. In the HUVECs, although VEGF165 stimulated the direct proliferation of HUVECs, no proliferation activity of VEGF165b was observed even with the dose of 100 ng/ml (data not shown).

The effect of VEGF165b on the ECM adhesion in fibroblasts. Since it is well known that CAFs produce ECM proteins such as fibronectin and collagen, we examined the ECM adhesion in fibroblasts incubated with 100 ng/ml of VEGF165, VEGF165b, or both isoforms in a cell adhesion assay. As shown in Fig. 4A, VEGF165b more strongly increased adhesion to collagen II, collagen IV, and fibrinogen compared to VEGF165. Interestingly, the addition of both isoforms resulted in less adhesion than the use of either isoform alone.

It was reported that for the VEGF signal transduction related to adhesion of ECM, the activation of FAK and the protein paxillin was essential, leading to the recruitment of actin-anchoring proteins to organize the focal adhesion plaque (19). We therefore examined the phosphorylation of FAK when VEGF165b was added to NHDFs, and the results demonstrated that both VEGF165 and VEGF165b alone at 100 ng/ml induced the phosphorylation of FAK as early as at 15 min (Fig. 4B).

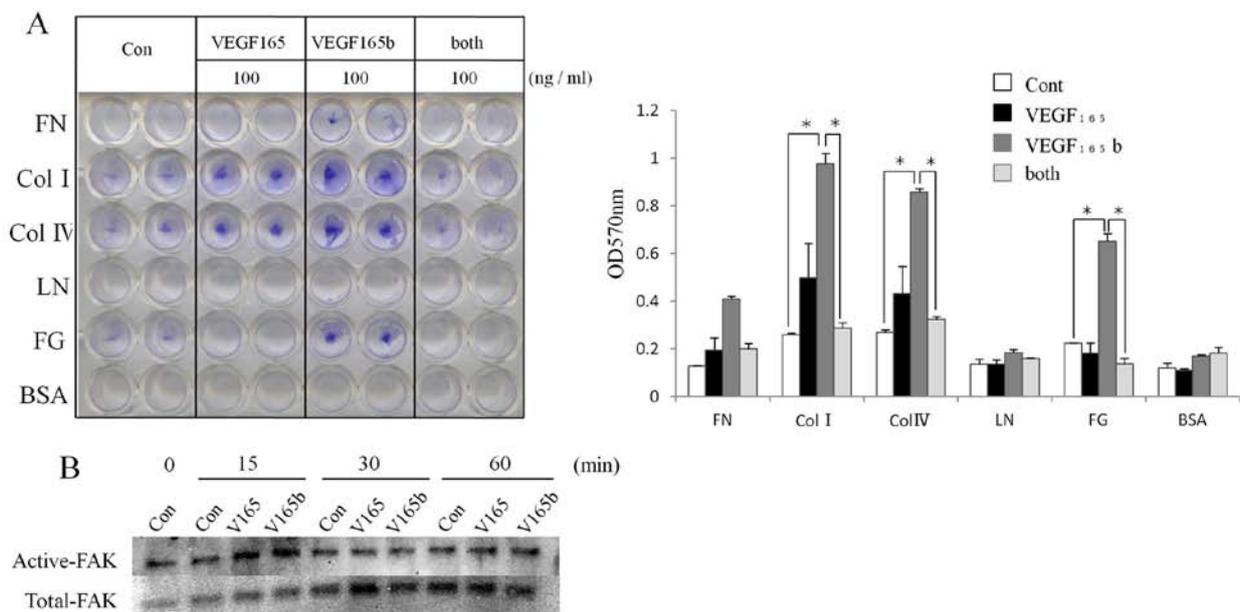


Figure 4. The effects of VEGF165b on ECM adhesion. (A) The cell adhesion to ECM after the addition of VEGF165b (100 μ g/ml) on NHDFs. NHDFs (1×10^5) were plated in wells in serum-free DMEM containing 100 ng/ml of rVEGF165, rVEGF165b or both isoforms and incubated for 6 h. VEGF165b strongly stimulated adhesion to collagen II, IV (Col IV) and fibrinogen (FG). FN, fibronectin; Col I, collagen I; LN, laminin I; BSA, bovine serum albumin. (B) Immunoblotting analysis of the expression of FAK, a cell adhesion-related protein. NHDFs were cultured after stimulation by rVEGF165, rVEGF165b or both isoforms (100 μ g/ml) for the indicated times, after which phosphorylated or total FAK. Phosphorylated cells were analyzed by a western blot analysis. * $P < 0.05$.

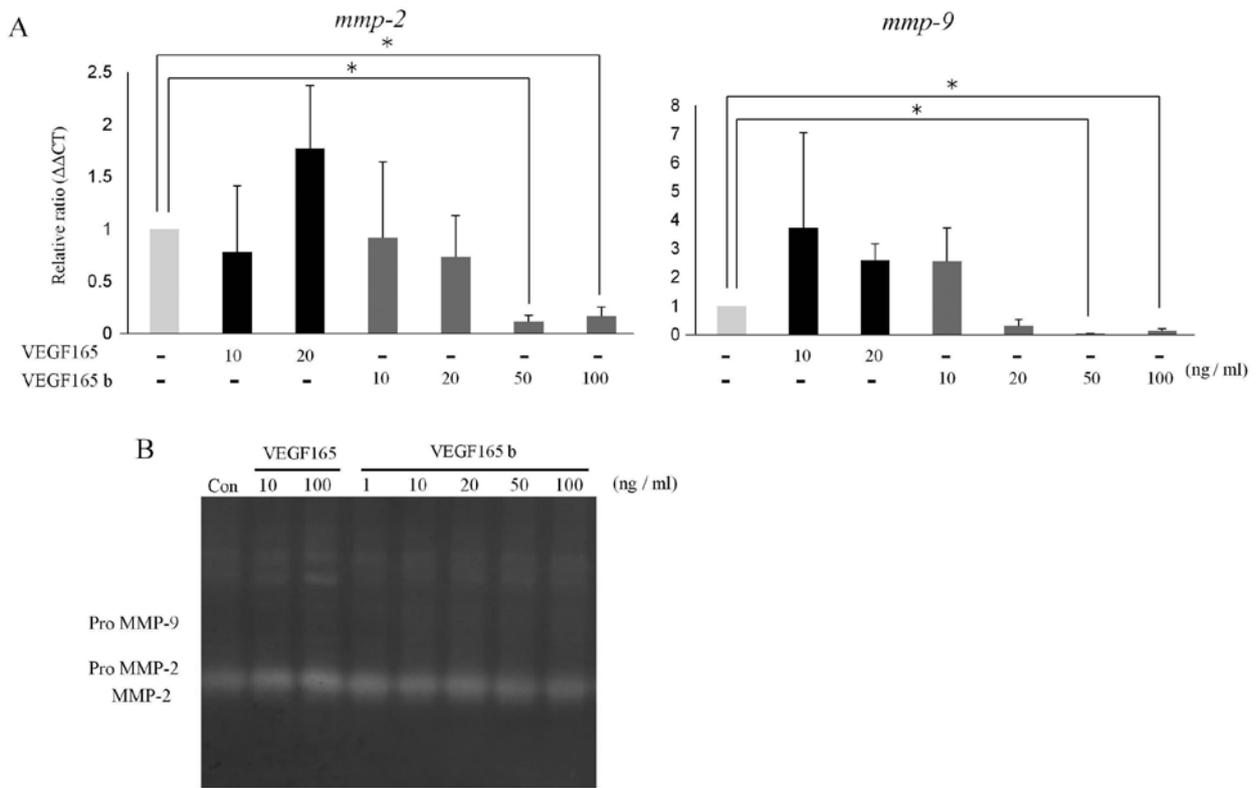


Figure 5. The effect of VEGF165b on the proteolytic activity in fibroblasts. (A) Subconfluent NHDFs in a 3.5-cm dish were cultured with DMEM containing 10% FBS. The cells were replenished with fresh medium without FBS and further cultured for 6 h. One of the indicated concentrations of rVEGF165 or rVEGF165b was then added. After 12 h, total RNA was extracted and the levels of gelatinase mRNAs were determined by qPCR with human-specific primers. The relative transcript levels were normalized using 18S mRNA levels by the $\Delta\Delta CT$ method. (B) Subconfluent NHDFs in a 3.5-cm dish were cultured in 2 ml of DMEM containing 10% FBS, and then replenished with 2 ml of fresh medium without FBS containing one of the indicated concentrations of rVEGF165 or rVEGF165b and further cultured for 18 h. Then, 2 ml of the supernatant was harvested and concentrated to 50 μ l by a centrifugal filter device. We examined the levels of the proteolytic activities and those of MMP-2 and -9 with the indicated concentrations of rVEGF165 or rVEGF165b. Although the MMP-2 induction was increased with 100 ng/ml of VEGF165, VEGF165b incubation at the various concentrations did not result in a significant increase in the proteolytic activities. *P<0.05.

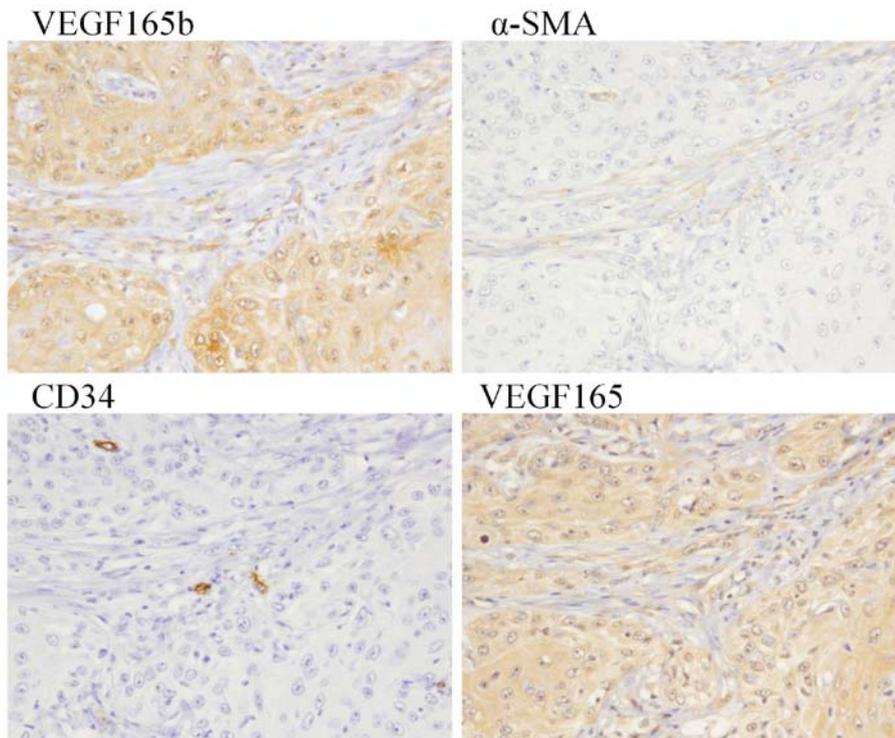


Figure 6. A representative histological pattern of the expression of both VEGF isoforms, α -SMA and CD34 observed in a patient with OSCC in the tongue region.

Table II. Correlations between the clinicopathological features and the expressions of VEGF165 and VEGF165b.

	VEGF165 aberrant expression			VEGF165b aberrant expression		
	Positive (n=28)	Negative (n=43)	P-value	Positive (n=69)	Negative (n=2)	P-value
Age (years)						
≥0	21	38	0.141738	55	2	0.477108
<60	7	5		14	0	
Gender						
Male	20	24	0.185323	42	2	0.261113
Female	8	19		27	0	
T classification						
T1-T2	16	28	0.498811	43	1	0.723506
T3-T4	12	15		26	1	
N classification						
≥1	6	8	0.770075	14	0	0.477108
<0	22	35		55	2	
Distant metastasis						
Yes	0	2	0.0455	2	0	0.807048
No	2	0		67	2	
YK-classification						
1/2/3	23	38	0.46087	59	2	0.56135
4C/4D	5	5		10	0	
Differentiation						
Well/moderate	24	40	0.312676	62	2	0.635188
Poor	4	3		7	0	

The correlations were analyzed using Pearson's Chi-square test.

The effect of VEGF165b on the proteolytic activities in fibroblasts. Matrix metalloproteinases (MMPs) secreted from cancer cells are critical for tumor invasion by the degradation of the ECM (20), but it was recently observed that MMPs produced from surrounding normal cells (such as fibroblasts and endothelial cells) may be important in the tumor microenvironment. We next examined the expression of MMPs and the proteolytic activities in fibroblasts incubated with VEGF165 and VEGF165b, using qPCR and gelatin zymography. As shown in Fig. 5A, VEGF165b did not affect the levels of the gelatinase (*mmp-2* and *mmp-9*) mRNA significantly, but VEGF165 induced the expression of gelatinases. Likewise, predominant proteolytic bands migrating at molecular weights indicating MMP-2 species were increased with 100 ng/ml of VEGF165, but VEGF165b incubation at the various concentrations did not result in a significant increase in the proteolytic activities.

The relationships between VEGF165b expression and clinicopathological factors. We evaluated the correlations between the VEGF165b expression and clinicopathological criteria (i.e., the T, N and M categories, the mode of invasion, and the degree of differentiation). As shown in Table II, VEGF165b-positive staining was seen in almost all categories regardless of the T

or N category. Distant metastasis related to the difference of VEGF isoforms was not evaluated, because we observed only two cases of metastasis.

We also observed that the VEGF165b-positive staining in OSCC cells was not related to the mode of invasion or the degree of differentiation. Fig. 6 provides a representative histological pattern of both VEGF isoforms and the α -SMA and CD34 expression observed in a patient with OSCC in the tongue region. Strong expression of VEGF165 and VEGF165b in both the nucleus and cytoplasm of tumor cells was observed. However, there was a difference in the level of VEGF165b expression compared to that of VEGF165 expression; i.e., an increasing tendency of VEGF165b expression was observed in the boundary between tumor cells and stroma cells that express α -SMA. No correlation was observed between the VEGF165b expression or the VEGF165 expression in tumor cells and the CD34 expression in endothelial cells.

Discussion

Cancer development and progression are controlled by cellular interactions derived from a complex relationship between stromal, epithelial and ECM components. The stromal microenvironment surrounding cancer cells is known as 'reactive

stroma' that is characterized by modified ECM composition, increased microvessel density, inflammatory cells, and fibroblasts with an activated phenotype, termed CAFs (21,22). These CAFs are thought to play a central role in the complex process of tumor development and progression.

The VEGF_{xxx}b family of isoforms is generated by C-terminal distal splice site selection. VEGF165b was identified as an alternative isoform which was an alternative splice site in the terminal exon 8 of the *veg*f mRNA. The precise mechanism of the biological action of VEGF_{xxx}b has not been fully elucidated, but it was reported that VEGF165b binds to both VEGFR1 (23) and VEGFR2 (5), competing with VEGF165, and it initiated only weak signaling of the receptor to induce tyrosine phosphorylation, leading to the inhibition of endothelial cell proliferation, migration and vasodilation as well as *in vitro* experimental angiogenesis and tumor growth (23,24).

In the present study, both the mRNA and protein expression levels of VEGF165b in some of the OSCC lines were higher than those in the NHDFs. We also observed that VEGF165b did not promote the cell growth or invasive capabilities on the NHDFs. One aspect of the molecular mechanisms that was elucidated is that VEGF165b did not affect the level of gelatinase on NHDFs, whereas it induced the cell adhesive capabilities to ECM through activated FAK signaling pathways. In addition, VEGF165b modulates fibroblasts transdifferentiation to myofibroblasts through α -SMA expression. We also observed an increasing tendency of VEGF165b expression in the boundary between cancer cells and stroma cells.

In light of our present findings, it is plausible that the reactive stroma of OSCC indirectly acts in the process of anti-angiogenesis via these VEGF165b-activated fibroblasts in a paracrine manner, although OSCC cells themselves secrete an anti-angiogenic factor, VEGF165b. On the other hand, Chen *et al* (25) reported that VEGF165b could inhibit the migration and invasion of cancer cells in an autocrine-dependent manner through the inhibition of the expression of VEGF165 as well as the competitive binding of VEGF165b to VEGFR. Since the switching of the expression of VEGF from VEGF165 to VEGF165b is related to a reduction in tumor growth rates (8,17) and to microenvironmental conditions such as hypoxia (17), it is possible that OSCC cells themselves enable a finely tuned modulation of the expression ratio among VEGF isoforms under hypoxia, and these cells may promote their own progression by controlling the surrounding stroma via cellular interactions such as CAFs.

Kawashiri *et al* (4) reported that in the invasive front of OSCC tissue, myofibroblasts increased gradually with the mode of invasion and degree of differentiation. They also observed that patients with α -SMA-positive staining had increased locoregional lymph node metastasis and myofibroblast appearance, leading to a prolonged disease-specific 5-year survival rate. Of note, our present results confirmed that VEGF165b secreted from OSCC cell lines may modulate the fibroblast transdifferentiation into myofibroblasts through α -SMA expression. However, our data (Table II) do not permit us to conclude that OSCC tissue with VEGF165b-positive staining is related to the mode of invasion or degree of differentiation. Since VEGF165-positive staining was not

seen most of our OSCC samples despite the degree of invasion, VEGF165b-positive staining may be more useful for the detection of malignant cells, especially in the invasive front of 4C/4D tissue.

Our present findings showed that VEGF165b secreted from OSCC cells may contribute to the process of anti-angiogenesis by stopping the activity of MMPs and by activating cell adhesive capabilities to ECM in CAFs surrounding tumor cells. Further studies are necessary for the elucidation of the biological activity of VEGF165b in OSCC cells.

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