Abstract.

To study the role of TNF-α in tongue cancer metastasis, we made highly metastatic cells from a human oral squamous cell carcinoma cell line (SAS) by repeating the passage in which the cells were injected into a nude mouse tongue and harvested from metastasized cervical lymph nodes. Cancer cells after 5 passages (GSAS/N5) increased invasive activity 7-fold in a TNF-α receptor 1 (TNFR1)-dependent manner and enhanced mRNA expression of TNF-α and TNFR1. In the highly metastatic cells, NF-κB activation was upregulated via elevated phosphorylation of Akt and Iκκα/β in the signaling pathway and secretion of TNF-α, active MMP-2 and MMP-9 increased. Suppression of increase of TNF-α mRNA expression and MMP secretion by NF-κB inhibitor NBD peptide suggested a positive feedback loop in GSAS/N5 cells; TNF-α activates NF-κB and activated NF-κB induces further TNF-α secretion, leading to increase of active MMP release and promotion of invasion and metastasis of the cells. GSAS/N5 cells that had been injected into the nude mouse tongue and harvested from metastasized lungs multiplied angiopoietin-like 4 (Angptl4) expression with enhanced migration activity, which indicated a possible involvement of Angptl4 in lung metastasis of the cells. These results suggest that TNF-α and Angptl4 promote metastasis of the oral cancer cells, thus, these molecules may be therapeutic targets for patients with tongue cancer.

Introduction

The American Cancer Society estimated 45,780 new cases of oral cavity and pharyngeal cancer, and 8,650 deaths from these tumors, in 2015 in the United States (1). By advances in surgery and radiation therapy, the 5-year survival rate for oropharyngeal cancer has increased to 66%, but the rate is still unsatisfactory in comparison with some other site cancers including prostate, thyroid and breast; the rates of these types of cancer are >90% (1). A primary cause for the unfavorable prognosis is patient death from the cancer metastasized at regional and distant sites (2-5). Squamous cell carcinoma (SCC) accounts for approximately 90% of oral and oropharyngeal malignancies in the United States and tongue is a common site of the malignant diseases (6,7). The rate of nodal metastasis is higher in tongue cancer patients than oral cavity cancer patients whose rate is 30% on their initial evaluation (8,9). Several studies have shown a high rate of occult nodal metastasis (20-40%) in tongue SCC patients with no evidence of regional spread on clinical or radiographic evaluation (8,10-15). There is a tendency that tongue cancer increases in young females in recent 2-3 decades (16-18). Thus, metastasis suppression is a main and urgent subject in the treatments of patients with tongue SCC.

The process of metastasis is complex and involves tumor growth, the extra-cellular matrix breakdown, invasion to the vessels, escape from immune surveillance, transport to other sites with adhesion to the vessel, subsequent invasion into an organ where tumor cells proliferate, grow and spread. Various molecules participate in the process but their roles remain incompletely understood. Tumor necrosis factor (TNF)-α is one of major regulators of inflammation, playing...
in the cytokine network. TNF-α is synthesized primarily by immune cells as a 34-kDa type II transmembrane protein (19) and a soluble form of the C-terminal 17 kDa portion is released from cells through cleavage by TNF-α-converting enzyme (20). TNF-α forms a homotrimer that activates cells via two distinct receptors (21). TNFR1 (p55, CD120a) binds the soluble ligand and is expressed on most cells, and TNFR2 (p75, CD120b) primarily binds the transmembrane form and is found on hematopoietic cells (22). TNF-α required high doses or injection to the tumor tissue for induction of hemorrhagic tumor necrosis (19). In contrast, TNF-α at low doses exerted angiogenic activity in both the rabbit cornea and chick chorioallantoic membrane models (23,24). By treating tumor cells or mice TNF-α augmented the metastatic activity of transplanted tumor cells (25,26). These reports suggest TNF-α activity for cancer progression. Thus, whether TNF-α is an anticancer molecule or therapeutic target is an important issue in the treatment of cancer including tongue SCC.

To study a relationship between TNF-α and tongue cancer metastasis, we made highly metastatic oral squamous cell carcinoma (OSCC) cells by repeated implantation of lymph node-metastasized OSCC cells into a nude mouse tongue (27) and investigated secretion of TNF-α. In contrast, TNF-α at low doses exerted angiogenic activity in both the rabbit cornea and chick chorioallantoic membrane models (23,24). By treating tumor cells or mice TNF-α augmented the metastatic activity of transplanted tumor cells (25,26). These reports suggest TNF-α activity for cancer progression. Thus, whether TNF-α is an anticancer molecule or therapeutic target is an important issue in the treatment of cancer including tongue SCC.

Table I. RT-PCR conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Annealing temperature (˚C)</th>
<th>Cycles</th>
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<tr>
<td>TNF-α</td>
<td>Forward: CCGGACGTGCAGCTGGCCGAGGAG</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTGCAGTGTGTATTACCGTGCTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFR-1</td>
<td>Forward: TACATTGCAGCTCTCTGCCCTC</td>
<td>50</td>
<td>35</td>
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<tr>
<td></td>
<td>Reverse: AGAGCTTGACTCTCCACCCGT</td>
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</tr>
<tr>
<td>TNFR-2</td>
<td>Forward: ACATCAGACGTGGTGTGCAA</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCACTGGAAGAGCAGAAGTC</td>
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<td></td>
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<tr>
<td>Angptl4</td>
<td>Forward: CCTCAAGGGGTCTCCGCCCAATT</td>
<td>60</td>
<td>30</td>
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<tr>
<td></td>
<td>Reverse: GGGCCGTTGAAATCCACTGA</td>
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<td></td>
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<tr>
<td>β-actin</td>
<td>Forward: CCAAGGCAACCCGGCGAGAAGATTGAC</td>
<td>57</td>
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<tr>
<td></td>
<td>Reverse: AGGGTACATGGTGTTGCCAGCAG</td>
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</table>

Materials and methods

Preparation of highly metastatic cell lines from a GFP-expressing OSCC cell line. The human OSCC cell line SAS cells were transfected with the pAcGFP1-C1 vector (Clontech Laboratories, Mountain View, CA, USA) using FuGENE 6 (Roche Diagnostics, Indianapolis, IN, USA), then GFP-expressing OSCC cell line. Highly metastatic cell lines were prepared according to the method previously described (27). Briefly, GSAS cells (5.0x10⁵ in 50 µl serum-free DMEM medium were injected into the tongue of a nude mouse and after 21 days were harvested from metastasized cervical lymph nodes. These GSAS cells were cultured in DMEM medium supplemented with 10% FBS. GSAS/N5 was injected into the tongue of another nude mouse. The cells that metastasized to the lungs (GSAS/L) were cultured for use.

TNF-α assay. TNF-α in the GSAS culture medium was assessed by enzyme-linked immunosorbent assay (ELISA) using Human TNF-α Quatikaine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). Cells (2.0x10⁵) were cultured in serum-free DMEM medium for 24 h and 25 µl of the supernatant was incubated in an anti-TNF-α antibody-adsorbed well for 4 h at room temperature, followed by washing. Biotin-labeled anti-TNF-α antibody was poured into the well, and after washing streptavidin-labeled peroxidase was incubated for 10 min in the well. After washing, 100 µl of TMB solution was added and incubated for 10 min, followed by 50 µl of 2 M HCl. The absorbance of the solution was measured at 450 nm.

Total RNA extraction and reverse transcription-PCR. The cells were incubated in serum-free DMEM in the presence or absence of 100 µM NBD peptide (Imgenex Corp., San Diego, CA, USA). After 24 h, total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The RNA quantity, purity, and integrity were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Complementary DNA was synthesized from total RNA using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan). Gene-specific primer sets were designed using the Custom Primers software program (Invitrogen, Carlsbad, CA, USA). The primer sequences and the amplification conditions are shown in Table I. The expression of β-actin was used as an internal control.

Flow cytometry. GSA/L cells were treated for 30 min with a rabbit anti-Angptl4 antibody (Abgent, Inc., San Diego, CA, USA) or a control rabbit IgG (Dako Corp., Carpinteria, CA,
Western blotting. GSAS, GSAS/N3 and GSAS/N5 cells (2.0x10^6) were cultured in serum-free DMEM medium for 24 h. Nuclear extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction reagents (Pierce Chemical Co., TX, USA). The DNA binding reaction was performed with a biotin end-labeled NF-κB oligonucleotide (5'-AGCTTGGGGACTTTCCGAG-3') using a LightShift™ Chemiluminescent EMSA kit (Pierce Chemical). A total of 20 µl of the extracts was electrophoresed in 6% polyacrylamide gels and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). Biotin-labeled DNA was detected with the Chemiluminescent Nucleic Acid Detection Module (Pierce Chemical). For supershift reactions, extracts were preincubated with an anti-p65 antibody (Santa Cruz Biotechnology). Bands were visualized using ECL (GE Healthcare, Buckinghamshire, UK).

Electrophoretic mobility shift assay (EMSA). GSAS, GSAS/N3 and GSAS/N5 cells (2.0x10^6) were cultured in serum-free DMEM medium for 24 h. Nuclear extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction reagents (Pierce Chemical Co., TX, USA). The DNA binding reaction was performed with a biotin end-labeled NF-κB oligonucleotide (5'-AGCTTGGGGACTTTCCGAG-3') using a LightShift™ Chemiluminescent EMSA kit (Pierce Chemical). A total of 20 µl of the extracts was electrophoresed in 6% polyacrylamide gels and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). Biotin-labeled DNA was detected with the Chemiluminescent Nucleic Acid Detection Module (Pierce Chemical). For supershift reactions, extracts were preincubated with an anti-p65 antibody (Santa Cruz Biotechnology) for 20 min. All of the above procedures were done according to the manufacturer's protocols.

Gelatin zymography. Cells were seeded in a well of a 24-well plate to form a confluent sheet, then cultured in serum-free DMEM medium containing 0.5% BSA for 24 h, followed by a 24-h incubation in 10 ng/ml TNF-α in the presence or absence of 100 µM NBD peptide. The supernatant was obtained by centrifugation. To adjust the sample conditions, cells in a well were treated with 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and protein amount was measured using DC protein assay (Bio-Rad Laboratories). Supernatant samples were diluted with 50 mM Tris-HCl, pH 6.8, 10% SDS, 50% glycerol to adjust to a same concentration per the protein amount of the cells in a well and 40 µl of the sample was electrophoresed in a 10% SDS polyacrylamide gel containing 1 mg/ml gelatin. After washing twice in 10 mM Tris-HCl, pH 8.0, 2.5% Triton X-100, the gel was incubated in 50 mM Tris-HCl, pH 8.0, 0.5 mM CaCl_2, 1 µM ZnCl_2 at 37°C for 16 h. Then, the gel was stained in Coomassie brilliant blue solution. To determine the MMPs, standard pro-MMP-2, active MMP-2 and pro-MMP-9 (Cosmo Bio Co., Ltd., Tokyo, Japan) were electrophoresed in the same gel together with the samples.

Invasion assay. CytoSelect™ 24-well Cell Invasion Assay (Cell Biolabs, Inc., San Diego, CA, USA) was used to measure cancer cell invasion activity according to the manufacturer's instructions. Cells (2.0x10^5) were suspended in serum-free DMEM, and then seeded into the upper chamber in the presence or absence of 100 µM NBD peptide. Serum-free DMEM was placed in the lower chamber. After incubation for 24 h, cells that invaded through Matrigel (BD Biosciences) layer to the lower surface of the 8 µm-pored membrane partitioning the chambers were fixed in 100% methanol, stained with 1% toluidine blue and counted in 4 microscopic fields (x400). The average cell number per a field in triplicate assay is shown.

Gene expression microarrays. Total RNA of GSAS, GSAS/N3, GSAS/N5 or GSAS/L cells was isolated using the RNeasy Mini kit (Qiagen). The RNA quantity, purity, and integrity were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The cRNA was amplified, labelled, and hybridised by an Agilent Human GE 4x44K v2 Microarray (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Signals of probes in all hybridised microarrays were scanned by an Agilent scanner and calculated using Feature Extraction Software version 9.5.1.1 (Agilent Technologies).

Wound healing assay. GSAS, GSAS/N3 and GSAS/N5 cells (5.0x10^5) were seeded in 6-well plates and were cultured in DMEM containing 10% of FBS for 24 h. A line was scratched with a yellow pipette tip in the 70-80% confluent monolayer, and the cellular debris were removed by washing with serum-free DMEM. Then, the cell sheets were incubated in serum-free DMEM and the cell migration into the wound area was photographed with a phase-contrast microscope for 24 h. The distance between the cell migration fronts of both sides was measured vertically against the initial scratch border at three points at random after 24 h from scratching and the percentage of the average distance vs. the distance between the initial scratch borders was calculated. All the assays were performed as a set of three independent experiments.

Statistical analysis. Statistical analysis was performed using the unpaired Student's t-test. Values are expressed as means ± SD.

Results

Enhancement of invasive activity of GSAS/N5 cells. High invasiveness facilitates spread and metastasis of cancer cells, thus, invasive activity was measured for GSAS cells and GSAS/N5 cells and compared between them. Invasive activity of GSAS/N5 cells was 7-fold higher than that of GSAS cells (Fig. 1). The invasive activity of GSAS/N5 cells was mostly inhibited in the presence of soluble TNF-R1 and the inhibitory effect of the receptor was decreased by TNF-α addition (Fig. 1B). The result indicated that invasive activity was enhanced in GSAS/N5 cells and the enhanced invasive activity of the highly metastatic cancer cells was mainly dependent on TNF-R1.

Elevation of TNF-R1-NF-κB pathway and TNF-α secretion in GSAS/N cells. TNFR1-dependent enhancement of
GSAS/N5 cell invasive activity suggested involvement of TNF-α-TNF-R1 system and elevation of the TNF-R1-initiated signaling pathway in the highly metastatic cancer cells. Hence, this system of the cancer cells was investigated. TNF-α and the receptor TNF-R1 mRNA expression increased in GSAS/N3 and GSAS/N5 cells in comparison with GSAS cells, but TNF-R2 mRNA expression did not change among the three GSAS cells (Fig. 2A). Suppression of TNF-α mRNA expression by NBD peptide (Fig. 2B) that inhibits NF-κB activation (28) indicated that increase of TNF-α mRNA expression was caused by NF-κB activation. The signaling pathway triggered by binding of TNF-α to TNFR1 mediates NF-κB activation in human head and neck squamous cell carcinoma (29). Thus, molecules participating in the signaling pathway from TNFR1 to NF-κB (30) were examined. Phospho-Akt protein increased in GSAS/N3 and GSAS/N5 cells, but Akt expression did not change (Fig. 3A). Phospho-Iκkα/β was significantly increased in the highly metastatic cells, whereas it was negligible in GSAS cells (Fig. 3A). Expression of p50/p65, components of NF-κB (29), was enhanced in GSAS/N5 cells (Fig. 3B). These...
results indicated elevation of the signaling pathway to NF-κB and resultant increase of NF-κB activation in the highly metastatic oral SCC cells. NF-κB activation leads to TNF-α production in cancer cells (31,32), thus, TNF-α secretion from the cancer cells was measured. In agreement with increased TNF-α mRNA expression (Fig. 2A) GSAS/N5 secreted significantly more TNF-α into the culture medium than GSAS and GSAS/N3 (Fig. 4).

**Enhancement of active MMP release from GSAS/N5 cells by TNF-α and its inhibition by NBD peptide.** Elevation of TNF-R1-NF-κB pathway in GSAS/N5 cells suggested that TNF-α stimulation was associated with enhanced invasive activity of the cells. We previously showed that elevated mRNA expression of MMP-2 and MMP-9 in GSAS/N5 cells (27). We examined whether the cancer cells secreted active forms of these MMPs by TNF-α. By stimulation with TNF-α, cancer cells secreted active MMPs that were converted from latent forms; GSAS/N5 cells released more active MMPs than other cells (Fig. 5). NBD peptide induced pronounced inhibition of TNF-α-elicited active MMP-2 and MMP-9 secretion from the cancer cells (Fig. 5), which indicated a close association of TNF-α-triggered NF-κB activation to upregulation of active MMP release from the cancer cells.

**Elevated angiopoietin-like 4 (Angptl4) expression and mobility of GSAS/L cells.** To study tongue cancer metastasis further, we made another highly metastatic GSAS cell line (GSAS/L), which were GSAS/N5 cells that metastasized to the lung by implantation into a nude mouse tongue. GSAS, GSAS/N5 and GSAS/L cells were examined for exhaustive mRNA expression by the microarray assay and mRNAs that increased in comparison with GSAS cell mRNAs were sought. Table II shows the ten highest increase ratio mRNAs of GSAS/L cell and corresponding mRNAs of GSAS/N5 cells. This assay revealed mRNA elevation of several genes in the highly metastatic cells and Angptl4 increased most among the mRNAs. Angptl4 mRNA expression of GSAS/L increased 141-fold for GSAS and 25-fold for GSAS/N5. Indeed, high expression of Angptl4 mRNA and the protein were confirmed in GSAS/L cells but GSAS cell Angptl4 mRNA expression was faint (Fig. 6). To study GSAS/L cells further, migration activity of the cells was investigated. In the scratch assay GSAS/L cells migrated more rapidly than GSAS/N5 and GSAS cells (Fig. 7). These results may suggest that the lung metastatic activity of GSAS/L is associated with enhancement of Angptl4 expression and cell mobility.

**Discussion**

We revealed enhancement of invasive activity in the highly metastatic OSCC cells (Fig. 1) accompanying elevated TNF-α signaling via TNFR1 (Fig. 2A), leading to NF-κB activation through phosphorylation of Akt and IKKα/β (Fig. 3). The increase of TNF-α mRNA expression (Fig. 2A) and protein secretion (Fig. 4) in relation to elevated NF-κB activation (Fig. 3B) agrees with that NF-κB activation leading to TNF-α production in tumor cells (31,32). NF-κB is a key regulator of MMP-2 and MMP-9 expression (33,34), even in SAS cells (35).
used in the present study, which suggests that the high NF-κB level of GSAS/N5 cells implicated secretion enhancement of these proteases from the cells (Fig. 5). Upregulation of MMP-2 and MMP-9 expression is associated with tumor invasion and metastasis (36,37). Accordingly, elevated secretion of active forms of these proteases in the GSAS/N5 cells (Fig. 5) likely accounts for high invasive (Fig. 1) and metastatic (27) activities of the cells.

We have shown that NF-κB level was elevated as GSAS cell metastasis passage advanced (Fig. 3B). The highly metastatic GSAS cells, particularly GSAS/N5, secrete more TNF-α (Fig. 4) together with increased expression of TNFR1 (Fig. 2A), the receptor for soluble TNF-α (22). The increase of TNF-α release brings about further NF-κB activation via TNFR1 signaling pathway in the cells and resultant elevation of MMPs secretion. As an autocrine TNF-α from tumor cells stimulated by this cytokine was reported (38), TNF-α release from the highly metastatic cells is possibly amplified in an autocrine manner. These findings suggest an auto-activation mechanism for TNF-α-triggered NF-κB activation in these cells. This may be an underlying mechanism by which GSAS/N3 and GSAS/N5 cells exert high invasive and metastatic activities. Such a positive feedback between NF-κB and TNF-α was reported in leukemia-initiating cells and contributes to leukemia progression (32). The present study suggests that this mechanism is present in the oral cancer cells and associated with enhanced secretion of active MMPs, augmenting invasion and probably metastasis of the malignant cells. In addition, the stimulative effect of TNF-α on the epithelial-mesenchymal transition (38) may also be augmented through the auto-activation mechanism, participating in the progression of the highly metastatic cancer cells.

Angptl4 is a member of proteins which are structurally similar to the angiopoietins but they do not bind to the angiopoietin receptors, namely the tyrosine kinase with immunoglobulin-like and EGF-like domain 1 (Tie1) and the endothelial-specific receptor tyrosine kinase (TEK or Tie2) (39). Angptl4 is a critical mediator in the transmigration process (40,41) and promotes transendothelial migration of cancer cells through the upregulation of vascular endothelial adhesion molecule 1 (VCAM-1) expression on endothelial cells (42). The increase of VCAM-1 on endothelial cells facilitates the attachment of cancer cells in the circulation to the vessels, allowing subsequent extravasation and tumor establishment. Clinical studies showed correlation of Angptl4 expression with venous and lymphatic invasion in human squamous cell carcinoma (43). Moreover, Angptl4 was reported as a lung metastasis gene in breast cancer (44). Considering these Angptl4 activities to support cancer progression, a high expression of the molecule (Table II and Fig. 6) in GSAS/L cells seems to be associated with metastasis of the OSCC cells, particularly to lungs, in addition to the enhanced invasiveness (Fig. 7).

In the highly metastatic OSCC cells, the elevated TNFR1 signaling pathway enhanced resultant NF-κB activation that was amplified through the auto-activation mechanism, leading to increase of active MMP-2 and MMP-9 secretion and invasive activity. Angptl4 mRNA was multiplied 25-fold in GSAS/L cells in comparison with GSAS/N5 cells, suggesting an involvement of this molecule in lung metastasis. The high expression level of Angptl4 was predictive of poor prognosis and prognostic of poor survival in patients with tongue cancer (45). These results may indicate that TNF-α, TNFR1 and Angptl4 were therapeutic targets of OSCC, including tongue cancers. Thus, blocking the TNF-α-TNFR1 system and Angptl4 with antibodies and receptor antagonists is possibly effective on the treatment of OSCC, inhibiting progression of the cancer.

