Humanized Anti-Interleukin-6 Receptor Antibody Suppresses Tumor Angiogenesis and In vivo Growth of Human Oral Squamous Cell Carcinoma

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Abstract

Purpose: The biological effect of interleukin-6 (IL-6) signaling in oral squamous cell carcinoma (OSCC) and whether IL-6 receptor (IL-6R)-mediated signaling can be a therapeutic target for OSCC are unclear. The aim of this study was to investigate the effects of inhibition of IL-6R-mediated signaling on OSCC progression and to evaluate the availability of tocilizumab, a humanized anti-human IL-6R antibody, as a therapeutic agent for OSCC.

Experimental Design: We evaluated expression levels of IL-6 and IL-6R in 58 OSCC tissues and 4 OSCC cell lines by real-time quantitative reverse transcription-PCR and/or immunohistochemistry. We investigated the effects of tocilizumab on OSCC growth in vitro and in xenografts. Xenografts were analyzed by immunohistochemistry for phosphorylated signal transducer and activator of transcription 3 (pSTAT3), Ki-67, and CD31, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was done.

Results: Expression levels of IL-6 at both mRNA and protein levels in OSCC tissues were significantly higher than those in normal mucosal tissues. In addition, OSCC cell lines expressed higher levels of both IL-6 and IL-6R mRNA than did HaCaT keratinocytes. Tocilizumab significantly reduced in vivo growth of SAS cells with a drastic reduction of STAT3 phosphorylation in tumor cells in mice. Inhibition of IL-6 signaling significantly decreased vascular endothelial growth factor mRNA expression in SAS, and microvessel density and vessel diameter in SAS tumors in tocilizumab-treated mice.

Conclusions: Therapeutic approaches targeting IL-6R by tocilizumab may be effective for OSCC treatment by at least inhibiting angiogenesis. (Clin Cancer Res 2009;15(17):5426–34)

Oral cancer, predominantly oral squamous cell carcinoma (OSCC), is the most common head and neck neoplasm, affecting about 270,000 people worldwide in 2002 (1). In Japan, OSCC is relatively common, accounting for >5,500 deaths in 2003 (2). Despite advances in early detection, diagnosis, and treatment of head and neck squamous cell carcinoma (HNSCC), the 5-year survival for patients with HNSCC has remained at 50% for the past 30 years (3). Appropriate treatment of HNSCC remains one of the most difficult challenges in head and neck oncology. Surgical therapy can be mutilating and often has significant effects on swallowing, speech, and physical appearance. The additional chemotherapy to radiation treatment has shown efficacy in organ preservation in some sites in the head and neck, but has resulted in limited improvement in survival rates. The aforementioned facts underscore the need for the development of a novel therapeutic approach for patients with HNSCC.

It has been well documented that cancer can be promoted and/or exacerbated by inflammation and infections (4). Important components in this linkage are the cytokines produced by tumor cells as well as activated innate immune cells (4). One avenue that holds promise is modulation of the expression and the signal of proinflammatory and proangiogenic cytokines (4, 5). Interleukin-6 (IL-6) is a multifunctional cytokine that was originally characterized as a regulator of immune and inflammatory responses (6). Elevated expression of IL-6 has been detected in multiple tumors (6). Multiple studies have
Translational Relevance

The involvement of interleukin-6 (IL-6) in cancer has been quite controversial, and it is unclear whether IL-6 receptor (IL-6R)-mediated signaling can be a therapeutic target for oral squamous cell carcinoma (OSCC). In the present study, we showed that IL-6 mRNA levels were elevated in OSCC tissues and that OSCC cells highly expressed both IL-6R and IL-6 mRNA. In addition, ablation of IL-6R function by a humanized anti-human IL-6R antibody named tocilizumab, which is clinically effective against several inflammatory diseases, led to a substantial decrease in OSCC-related angiogenesis, suggesting the possibility of tocilizumab as a therapeutic strategy to suppress OSCC progression. Therapeutic approaches targeting IL-6R using tocilizumab could provide benefit for patients with OSCC.

documented high IL-6 levels in the serum of patients with certain carcinomas, including OSCC, and have correlated high IL-6 levels with a poor clinical prognosis (7–10). However, the biological activity of IL-6 signaling in OSCC cells and whether IL-6 receptor (IL-6R)-mediated signaling can be a therapeutic target for OSCC are unclear.

Monoclonal antibodies (mAb) to IL-6Ra-chain or receptor antagonists are currently available approaches to selectively block IL-6 biological activities in vivo (11, 12). Tocilizumab is a humanized anti-human IL-6R antibody engineered by grafting the complementarily determining regions of a mouse anti-human IL-6R antibody into human IgG1 to create a human antibody with a human IL-6R binding site (12). Tocilizumab binds to the IL-6-binding site of human IL-6R and competitively inhibits IL-6 signaling (13) and completely neutralizes IL-6 activities. A series of clinical studies has shown that inhibition of IL-6 signaling by tocilizumab is therapeutically effective in rheumatoid arthritis, juvenile idiopathic arthritis, Castleman’s disease, and Crohn’s disease (14–17). In all of these diseases, tocilizumab ameliorates inflammatory manifestations, and normalizes acute phase protein levels. Given its success in treating these diseases, tocilizumab may also prove useful in treating IL-6-related cancers.

In the present study, we analyzed expression levels of IL-6 and IL-6R in OSCC. In addition, we investigated the effect of inhibition of IL-6R-mediated signaling using tocilizumab on OSCC progression in a xenograft model and evaluated the availability of this antibody as a therapeutic agent for OSCC.

Materials and Methods

Antibodies and reagents. Chugai Pharmaceutical Co., Ltd. (Roche Group) kindly provided a humanized anti–IL-6R monoclonal antibody (IgG1 class), namely, tocilizumab. Goat polyclonal anti-human IL-6 antibody was obtained from R&D Systems, Inc. Antibodies against phosphorylated STAT3 (pSTAT3-Tyr705) and STAT3 were obtained from Cell Signaling. Rat monoclonal anti-mouse CD31 (clone MEC 13.3) and mouse monoclonal anti-human Ki-67 antigen (MIB-1) were purchased from BD Pharmingen and DAKO Cytomation, respectively.

In situ apoptosis detection kit was purchased from Takara Bio Inc.

Samples from patients. Patient initial biopsy specimens were obtained from 58 OSCC patients who subsequently underwent surgery with curative intent at the Department of Oral and Maxillofacial Surgery, Kumamoto University Hospital, from 1999 to 2004. The average age ± SD of patients was 66.7 ± 11.3 y, and 37 men and 21 women were enrolled. Samples of normal lower gingival mucosa were obtained from 35 healthy volunteers. Those tissue samples for real-time quantitative reverse transcription-PCR (qRT-PCR) were sharply excised, placed in sterile tubes, and immediately frozen in liquid nitrogen and then stored at -80°C until analysis. Tissues for immunohistochemistry were obtained from patients with OSCC, same as those samples for qRT-PCR except for one sample we could not obtain (n = 57), and normal lower gingival mucosal tissues (n = 10) were fixed with 10% formalin before processing. The study followed the guidelines of the ethical committee at Kumamoto University. The nature and aims of the study were explained to all subjects, who gave their informed consent for the research.

Cells and cell culture. All human OSCC cell lines, namely, SAS, HSC2, HSC3, and HSC4, were donated by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) and grown in RPMI 1640 medium (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (Gibco). Human HaCaT nonmalignant keratinocytes, kindly provided by Dr P. Boukamp, DKFZ, Heidelberg, Germany, were cultured in DMEM (Gibco), supplemented with 10% heat-inactivated fetal bovine serum. All the cells were grown in 5% CO2 at 37°C.

RNA isolation and qRT-PCR. Total RNA was isolated from each tissue specimen and treated cells by using RNeasy Mini Kit (Qagen). Total RNA (0.5 μg) was reverse-transcribed to cDNA by using ExScript RT reagent (Takara Bio Inc.) according to the manufacturer’s protocol. Each PCR was done with 2 μl of the cDNA and 0.2 μmol/L of each primer in a LightCycler System with SYBR Premix Ex Taq (Takara Bio Inc.). The following primers were used: IL-6 (forward: 5′-AAGCCAGACGGTAGTGCAATGAGTA-3′; reverse: 5′-TGTTCCCGACCCACTGTTGCT-3′); IL-6Ra (forward: 5′-TGACCTCAATGCGGTGTAAC-3′; reverse: 5′-CGTCTGCGATGACACGATGATG-3′); vascular endothelial growth factor (VEGF; VEGF-A: forward: 5′-ATGACGGAGCCTGGATGTG-3′; reverse: 5′-CTATATGCTGTCGCCTTGTGAC-3′); β2-microglobulin (β2-M; forward: 5′-CAGGGCATCTTCAAGGTCTA-3′; reverse: 5′-GATGT-GTAAACCGCAGCATA-3′). Each reaction (20-μl samples) was done under the following conditions: initialization for 10 s at 95°C, and then 45 cycles of amplification, with 5 s at 95°C for denaturation, and 20 s at 60°C for annealing and elongation. A standard curve was plotted for each primer probe established by using a serial dilution of pooled cDNA from tissues or cells. All standards and samples were analyzed in triplicate.

Tumor xenografts generation and tocilizumab treatment. Male CB17/ICR-scid/scid mice (SCID mice), each 7 wk old and weighting 25 g, were obtained from CLEA Japan and maintained in a specific pathogen-free environment at the Center for Animal Resources and Development, Kumamoto University. SAS cells were trypsinized, washed with serum-free RPMI1640, resuspended in PBS, and adjusted to a concentration of 1 × 107 cells/100 μL in PBS. Then the cell suspensions were injected s.c. into SCID mice. After 24 h, the tumor-inoculated mice were injected i.p. with 100 μL PBS containing 100 μg of tocilizumab (n = 7) or control IgG (Sigma; data not shown) or PBS alone (n = 5) and received the same treatment every 48 h for 20 d. In the other two groups, the treatment of the antibody stopped on day 19 (n = 5) or started on day 21 (n = 5) after tumor inoculation in each group. In these groups, only PBS was injected every 48 h during the period without the treatment of tocilizumab. Tumor development was followed in individual animals (n = 5–7/group) every other day by sequential caliper measurements of length (L) and width (W). Tumor volume was calculated by the formula L2W/2. The mice were sacrificed after 41 d postinoculation with cells, and the tumors were removed and weighed. For the appropriate analyses of the tumor, the mice treated with tocilizumab or PBS every 48 h from a day after inoculation with cells were sacrificed on day 16, and the tumors were removed and weighed. The pieces of tumor tissues were sharply excised, placed in
sterile tubes, and immediately frozen in liquid nitrogen. All tissue samples for qRT-PCR were stored at -80°C until analysis. For immunohistochemical analysis or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, a segment was excised and immediately embedded in oстиine carbamyl transferase compound in liquid nitrogen (for CD31 staining) or fixed in either 10% neutral buffered formalin (for Ki-67 staining) or zinc formalin fixative (for TUNEL assay).

**Proliferation assay.** The antiproliferative effect of tocilizumab on SCCs cells in vitro was measured by a means of the MTS assay using CellTiter 96 AQueous One Solution Reagent (Promega), as described previously (18). Briefly, cells (5,000/well) were incubated, in triplicate, in a 96-well plate in the presence or absence of indicated concentrations of tocilizumab or IL-6 in a final volume of 100 μL for 24 to 72 h. Then, 20 μL of CellTiter 96 AQueous One Solution Reagent were added to each well. After a 4-h incubation, absorbance was measured at 490 nm by using EMax Precision Micropalte Reader (Molecular Devices).

**Protein extraction and immunoblotting.** Cells treated with tocilizumab or PBS were then washed once in ice-cold PBS and then lysed by adding Celllytic M Cell Lysis/Extraction Reagent (Sigma) containing freshly added protease inhibitor cocktail (Sigma), 50 mmol/L NaF, and 1 mmol/L Na3VO4. After incubation for 15 min on a shaker on ice, cell lysate was removed from the dishes and centrifuged at 15,000 × g for 15 min to remove insoluble material. Supernatants were stored at -70°C until use. Protein concentration was determined by using BCA kit (Pierce Chemical Co.). Equal amounts of protein were fractionated via SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 (Sigma) in TBS (pH 7.4) and then incubated overnight at 4°C with anti-STAT3 (1:1,000) or anti-pSTAT3-Tyr 705 (1:1,000) or anti–β-actin (1:5,000) antibodies in 5% bovine serum albumin (Sigma) and 0.1% Tween 20 in TBS (pH 7.4). After the membranes were washed, they were incubated for 1 h in biotinylated secondary antibodies and in horseradish peroxidase–conjugated streptavidin for 1 h. After a washing, specific protein bands were detected by using the enhanced chemiluminescence system (Amerham Pharmacia Biotech).

**Transfection with short interference RNA (siRNA).** SAS cells (5 × 10⁴ cells) were transfected with IL-6 or IL-6R short interference RNA (siRNA; 25 pmol) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After incubation for 48 h, total RNA was isolated. SAS cells transiently transfected with control siRNA (25 pmol) were used as control. Chemical siRNA sequences were as follows: for IL-6, sense strand 5′-CCAGAUCGGACACUCUCAU-3′, antisense strand 5′-CGAGCUAGUGUCAUCUCUG-3′; for IL-6R, sense strand 5′-GAAUCGCUUG-CAAACUCAU-3′, antisense strand 5′-UGAAGAUCGCUUCAGUCG-3′. All siRNAs were purchased from Applied Biosystems.

**Immunohistochemistry.** Clinical tissue specimens and excised tumor tissue specimens from SCID mice were used in histologic and immunohistochemical analysis. Formalin-fixed or zinc formalin-fixed specimens were embedded in paraffin, cut into 5-μm sections, and mounted onto slides. Paraffin sections were dewaxed in xylene and rehydrated in descending concentrations of alcohol. Frozen sections were fixed with acetone at 4°C for 10 min after being cut. For Ki-67 and pSTAT3, sections were autobclaved in 10 mmol/L citrate buffer (pH 6.0) for 20 min at 121°C followed by cooling down for 20 min at room temperature. Endogenous peroxidase was blocked by incubating slides with 3% hydrogen peroxide for 30 min. After slides were washed with PBS for 5 min, nonspecific background staining was blocked by using nonspecific staining blocking reagent (DAKO) for 10 min, followed by overnight incubation at 4°C with antihuman IL-6 (1: 20), antihuman pSTAT3 (1: 100), Ki-67 (1: 100), and antimouse CD31 (1: 50) antibodies diluted in PBS containing 1% bovine serum albumin. After slides were rinsed with PBS for 5 min, they were incubated for 1 h with biotinylated IgG and then washed again followed by incubation for 1 h with horseradish peroxidase–conjugated streptavidin. Chromogen was developed with 3,3-diaminobenzidine (DAKO). All slides were lightly counterstained with hematoxylin for 30 s before dehydration and mounting. TUNEL assay was done using the manufacturer’s protocol. The negative controls were PBS and normal isotype–matched IgG to replace primary antibodies. All sections were also stained with H&E for histologic evaluation. Ki-67-positive nuclei and TUNEL-positive cells were counted at high power (×400) in 10 random fields per tumor and expressed as a percentage of the total number of cells observed in each individual tumor. Expression of IL-6 was evaluated according to semiquantitative scales. The intensity of staining was rated on a scale of – to ++, with + meaning negative staining and ++ representing maximum intensity staining. The percentage of positive cells was evaluated according to the following scale: -, negative staining; +, <10%; ++, 10-50%; and ++++, >50% of the epithelial cells stained positive.

**Quantification of tumor vascularity.** Tumor vascularity was assessed in sections stained with an antibody against the endothelial cell surface marker CD31. Individual microvessels were counted in 10 random fields per tumor at ×100. Any brown staining endothelial cells that were clearly separate in brown appearance were counted as individual vessels. The diameter of blood vessels was calculated using NIH Image software. Results were expressed as an average of the total number of microvessels observed or total length of vessels calculated in each individual tumor.

**Statistical analysis.** Statistical significance was defined as P < 0.01 for Wilcoxon’s test and P < 0.05 for Student’s t test. Bivariate analyses were done with Wilcoxon’s test to assess differences in IL-6 mRNA expression between OSCC samples and normal mucosa samples. Student’s t test was used to assess differences in IL-6 or IL-6R mRNA expression between the cell lines and to compare means in experiments in vivo with two groups. JMP software, version 5.1 for Windows was used for statistical analysis.

**Results**

**Enhanced expression of IL-6/IL-6R mRNA in OSCC.** We analyzed the expression levels of IL-6 mRNA in 58 OSCC samples and 35 normal gingival mucosa samples derived from healthy volunteers using qRT-PCR. The levels of IL-6 mRNA in OSCC tissues were significantly higher than those in normal mucosal tissues (P < 0.0001; Fig. 1A). In addition, IL-6 expression at protein level in 57 individual primary OSCC tissues and 10 normal mucosal tissues was analyzed by immunohistochemistry using a rabbit anti–IL-6 antibody. Consistent with the data of qRT-PCR, IL-6 immunoreactivity was detected in 95% of OSCC tissues whereas most of normal mucosal tissues had no reactivity (Fig. 1B and C). Moreover, most of OSCC showed higher score for staining intensity (Fig. 1C, left panel) and percentage of cells positive (Fig. 1C, right panel). We also compared the expression levels of IL-6 and IL-6R mRNA in four kinds of OSCC cell lines (SAS, HSC2, HSC3, and HSC4) with HaCaT nonmalignant keratinocytes. All of the OSCC cell lines strongly expressed IL-6 and IL-6R mRNA, whereas HaCaT cells did not (Fig. 1D). We also confirmed the same results for IL-6 protein secretion in these cell lines assessed by Western blot analysis (data not shown).

**Suppression of SAS tumor growth by tocilizumab in SCID mice.** To investigate whether inhibition of IL-6 signaling by tocilizumab suppresses OSCC progression, we used a xenograft model inoculated with SAS cells. We assessed the effect of tocilizumab on SAS tumor growth in the protocol as described in Materials and Methods. Tumor growth was assessed according to tumor volume. As a result, tocilizumab markedly reduced tumor growth in SCID mice (Fig. 2A). The mean tumor size in control SCID mice was around 6,900 mm³ at the 41st day.
posttransplantation, but that in SCID mice injected with tocilizumab reached merely 1,660 mm$^3$ ($P < 0.005$). A similar difference was observed when IgG was used as control (data not shown). Interestingly, even in the group in which SCID mice were injected with tocilizumab only until day 19, the growth curves of these tumors were similar to those in mice treated from first to last. Tumor weight on day 41 was almost reflected in tumor volume (Fig. 2B). We could find the effect of this antibody to suppress the growth of advanced tumors to some degree (Fig. 2A, $P = 0.039$; Fig. 2B, $P = 0.1$). To confirm whether the reduction of tumor growth may be attributed to the blocking of IL-6R–mediated signaling in SAS cells, we evaluated STAT3 phosphorylation, which occurs consecutively following IL-6 stimulation, by immunohistochemistry using antiphosphorylated STAT3 antibody. As shown in Fig. 2C, tocilizumab dramatically reduced STAT3 phosphorylation in SAS cells in the mice ($P < 0.005$) without altering the overall expression of STAT3 protein (data not shown).

**Inhibition of IL-6R–mediated signaling suppresses VEGF expression.** To investigate the mechanism underlying the reduced growth of SAS tumors by tocilizumab observed in SCID mice, we first examined the in vitro cell proliferation of OSCC cells by MTS assay. Tocilizumab reduced the phosphorylation of STAT3 in vitro consistent with in vivo (Figs. 2C and 3A). However, neither tocilizumab nor exogenous IL-6 influenced the proliferation rate of SAS cells independently of treatment time (Fig. 3B) and concentration (data not shown) under normal conditions and also in the stress condition of 0.5% serum (data not shown). The same results were also shown in the other OSCC cells, such as HSC2, HSC3, and HSC4, and in HaCaT keratinocytes (data not shown). Similar to in vitro data, the immunohistologic staining using anti–Ki-67 antibody and TUNEL assay revealed that SAS tumors in tocilizumab-treated mice exhibited negligible changes in mitogenic rate (Fig. 3C) and no change in apoptosis (Fig. 3D), compared with control tumors.

It is well documented that the recruitment of new blood vessels through the process known as angiogenesis is essential for the continued growth of tumors (19). Because our results suggested that significant inhibition of SAS tumor growth by tocilizumab was anchorage-dependent, we next investigated the
effect of tocilizumab on expression of VEGF, one of the most important factors in tumor angiogenesis (20). As shown in Fig. 4A, tocilizumab significantly reduced VEGF mRNA expression in SAS cells. Next, to investigate the involvement of IL-6 signaling in VEGF expression further, we evaluated VEGF mRNA expression in SAS cells transiently expressing IL-6 siRNA or IL-6R siRNA. The effect of IL-6 or IL-6R knockdown by siRNA in SAS cells was confirmed by qRT-PCR and Western blot analysis (data not shown). As expected, VEGF mRNA expression was significantly reduced by both IL-6 siRNA ($P < 0.01$) and IL-6R siRNA transfection ($P < 0.005$; Fig. 4B).

**Tocilizumab reduces angiogenesis in SAS tumors.** Our experimental data suggested that blocking IL-6 signaling in SAS cells led to an impairment of tumor angiogenesis. To test whether tocilizumab would actually affect tumor angiogenesis in vivo, SAS tumor tissues were evaluated immunohistochemically using CD31 staining for tumor-associated vessels and quantitatively assessed for microvessel density and diameter. As shown in Fig. 5, injection of tocilizumab drastically reduced both vessel density ($P < 0.05$) and diameter ($P < 0.01$).

### Discussion

In the present study, we first showed the intriguing possibility that targeting IL-6R by tocilizumab may have therapeutic value for the treatment of OSCC, a major type of HNSCC. Ablation of IL-6R function in OSCC tumors leads to a substantial decrease in tumor angiogenesis.

IL-6 is a pleiotropic cytokine that has widespread effects on hematopoietic lineages, such as induction of immunoglobulin production in B cells, T-cell proliferation and differentiation into cytotoxic T cells (6). Multiple studies have documented that high IL-6 level is observed in the serum of patients of several carcinomas and associated with poor prognosis (7–10, 21). Our data showed that IL-6 expression at both the mRNA and protein levels in OSCC tissues was significantly higher than those in normal oral mucosal tissues, and both IL-6 and IL-6R mRNA were increased in OSCC cell lines as compared with HaCaT keratinocytes (Fig. 1). Although the biological activity of IL-6 signaling in OSCC is quite unclear, the high mRNA levels of IL-6 (ranging from 240% of control levels in HSC3 to 1,060% in HSC4 cells) and IL-6R (from 518% in HSC4 to 3,120% in SAS cells) found in this study indicate the critical role of IL-6 signaling in human OSCC cells. Consistent with these data and a previous report (22), the intensity of anti-IL-6R antibody staining was much higher in SCC lesions than in adjacent epithelial cells in certain cases (data not shown). Thus, these data suggest that IL-6 signaling contributes to OSCC progression and/or carcinogenesis and is an available therapeutic target for OSCC treatment.

One interesting finding in this study is that in vivo growth of OSCC was significantly inhibited by targeting IL-6R (not IL-6) with a marked reduction of phosphorylated STAT3 in tumor cells. Therapeutic attempts in humans with mAbs against IL-6 have yielded disappointing results. Administration of a single anti-IL-6 mAb does not lead to efficient neutralization of its biological activities in vivo (23). In the majority of patients with diseases that may benefit from anti-IL-6 treatments, IL-6 production is much higher and therefore it is not conceivable that injectable doses of mAbs could lead to an efficient neutralization. On the other hand, a series of clinical studies has
Fig. 3. Effects of IL-6R–mediated signaling on proliferation and apoptosis of SAS cells. A, SAS cells were plated and grown for 24 h and then treated with 1 μmol/L tocilizumab for 6 h before harvesting. Lysate from SAS cells was immunoblotted by use of anti-pSTAT3, anti-STAT3, or anti–β-actin antibody. B, SAS cells were incubated with 1 μmol/L tocilizumab or 100 ng/mL IL-6 for the indicated periods and were then examined for viability via the MTS method. The Y-axis shows the changes in absorbance at 490 nm; bars, SD of triplicate samples. SAS tumor tissues excised on day 16 from mice injected with PBS alone or tocilizumab were immunohistochemically stained using an antibody to Ki-67 (C) or were assessed by TUNEL assay (D). Representative examples of tumor sections in each experimental condition are shown in the photomicrographs. Scale bars, 50 μm. Results are shown as the percentages of Ki-67- or TUNEL-positive cells in 10 high magnification fields per section. Each data point is derived from three tumors and corresponds to the mean ± SD. *, P < 0.05; n.s., not significant.
shown that inhibition of IL-6 signaling by tocilizumab, a humanized antihuman IL-6R antibody, is therapeutically effective in several inflammatory diseases. Thus, we attempted to use tocilizumab to block IL-6 signaling in the present study. In addition to the efficacy of tocilizumab to inhibit OSCC growth, we did not observe any significant side effect in terms of hematologic factors, biochemical factors, and weight of organs (data not shown). These results indicate a safety profile of tocilizumab treatment. In general, tocilizumab as a monotherapy and in combination, such as with methotrexate in case of rheumatoid arthritis, seems to be well tolerated (24).

As another important finding, we showed that blocking IL-6R in OSCC cells by tocilizumab led to a significant impairment of tumor angiogenesis. Like all solid tumors, HNSCC must develop direct and indirect mechanisms to induce the production of new blood vessels. In the present study, tocilizumab markedly decreased the number of invaded capillary vessels in tumors (Fig. 5). These data suggest that inhibition of IL-6R function by tocilizumab leads to an impairment of tumor angiogenesis and in turn suppresses in vivo growth of SAS tumors. Because tocilizumab does not react to mouse IL-6R and mouse IL-6 does not react to human IL-6R (25), tocilizumab seems to inhibit mainly the autocrine IL-6–IL-6R loop in tumor cells in the present xenograft model. Thus, we attempted to investigate the angiogenesis-related factors derived from SAS cells to address the mechanism by which tocilizumab inhibits tumor angiogenesis. Among several proangiogenic factors, VEGF is the most potent angiogenesis-inducing signal (20). In addition, increased levels of VEGF were found in HNSCC (and serum) and seem to induce tumor growth, metastasis, and treatment failure, such as radioresistance (9, 26, 27). Consistent with previous reports (28–33), VEGF expression was suppressed by inhibiting IL-6 signaling in the OSCC cell line SAS. It should be noted, however, that the reduction level of VEGF expression was not consistent with the marked decrease of CD31-positive cells. In addition, tocilizumab showed almost no effect on mitogenic rate and apoptosis of tumor cells (Fig. 3B, C, and D).

The involvement of IL-6 in the proliferation of cancer cells has been shown to be quite controversial (8) and the reasons for such a complexity are unclear. Our findings that xenograft tumor growth was significantly suppressed by tocilizumab despite the absence of antiproliferative effect could be supported by previous in vivo studies (34–36). Indeed, we additionally found that tocilizumab significantly inhibited expression of both IL-8 and MMP-9, known as the major angiogenic factors (refs. 37, 38; data not shown). Therefore, the reduction of angiogenesis shown in tocilizumab-treated tumors may result from regulation of multiple angiogenic factors, not only VEGF but also such as IL-8 and MMP-9. However, we cannot exclude
the involvement of mechanisms other than antiangiogenic effect. Taking notice that STAT3 activity in tumor cells was markedly reduced (Fig. 2C), it is possible that the alteration of multiple inflammatory steps and antitumor immunity induced by IL-6–STAT3 signaling may be attributed to tocilizumab (39, 40). Further investigation will be needed to elucidate the critical mechanism underlying tocilizumab-mediated growth inhibition of OSCC xenografts.

Finally, we found that tocilizumab markedly reduced OSCC growth by inhibition of IL-6R derived from human tumor cells in vivo, but IL-6 actually also acts in the tumor microenvironment via paracrine signaling and may promote tumor progression. In particular, IL-6 secretion may facilitate the immune evasion of cancer cells (41–45). In the context of angiogenesis, hypoxic stress has been shown to induce IL-6 expression in vascular cells and cardiomyocytes (46, 47), and IL-6 recently has recently been shown to enhance endothelial cell migration directly via IL-6R (32) or through macrophage infiltration (48). A recent study has shown that tocilizumab markedly reduced OSCLC tumors led to a substantial decrease in tumor angiogenesis, suggesting the possibility of tocilizumab as a therapeutic strategy to suppress OSCC growth. Recent clinical trials intended for several inflammatory diseases have established the safety and efficacy of tocilizumab (12–17), as also shown in the present study. A deeper understanding of the biological significance induced by IL-6R–mediated signaling in OSCC and the additional mechanisms of antitumor activity of tocilizumab, including how it can be combined with other treatment approaches such as chemotherapy and radiation therapy, should contribute to improve OSCC treatments.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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