Antitumor effects of ZD6474 on head and neck squamous cell carcinoma

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Abstract. Angiogenesis is required for tumor growth and metastasis and, therefore, represents a target for cancer treatment. While many factors have been implicated in promoting angiogenesis, vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis. ZD6474 is a potent VEGF receptor-2 (VEGFR-2) tyrosine kinase inhibitor which also has activity against the epidermal growth factor receptor (EGFR) tyrosine kinase. The purpose of this study was to investigate the sensitivity of head and neck squamous cell carcinoma (HNSCC) cell lines to ZD6474, and to evaluate its antitumor efficacy on HNSCC xenografts. This is the first demonstration of antitumor effects of ZD6474 on HNSCC. *In vitro* ZD6474 displayed antiproliferative effects on HNSCC cells and inhibition of VEGFR-2 and EGFR pathways. *In vivo* ZD6474 displayed antitumor activity, induced apoptosis and antiangiogenic activity on nude mice bearing an established xenograft of YCU-H891 cells. These results suggest that ZD6474 has the potential to inhibit two key pathways in tumor growth via inhibition of VEGF-dependent tumor angiogenesis and via inhibition of EGFR-dependent tumor cell proliferation.

Introduction

Tumor angiogenesis is a process leading to formation of blood vessels within tumors and is crucial for maintaining a supply of oxygen and nutrients to support tumor growth and metastasis (1). Therefore, tumor angiogenesis represents a for cancer treatment. While many factors have been implicated in promoting angiogenesis, vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis including induction of endothelial cell proliferation, migration, survival and capillary tube formation (2-5). Enhanced production of VEGF is generally correlated with increased neovascularization within tumors (1,6). Different mechanisms, notably hypoxia can increase VEGF expression in cancer cells (1,7).

VEGF binds to two distinct receptors on endothelial cells, i.e. flt-1 (VEGFR-1) and flk/KDR receptor (VEGFR-2) (7). VEGFR-2 is considered to be the dominant signaling receptor for endothelial cell permeability, proliferation, and differentiation (8). VEGF and its receptors are good targets for cancer therapy because VEGF receptors are highly specific for VEGF and are expressed in increased numbers primarily during periods of tumor growth (9). Several different strategies to inhibit VEGF signaling, such as anti-VEGF monoclonal antibodies, blocking monoclonal antibodies against the VEGFR-2 and selective inhibitors of the VEGFR-2 tyrosine kinase, are currently in preclinical and clinical development (10-17).

ZD6474 is a potent, low molecular weight inhibitor of VEGFR-2 tyrosine kinase activity (18), which significantly inhibits tumor growth in a broad range of established human cancer xenografts in nude mice and is currently undergoing clinical evaluation (19-22). ZD6474 also has additional activity against epidermal growth factor receptor (EGFR) tyrosine kinase (23,24). ZD6474 has potent antitumor activity by a direct antiangiogenic mechanism via inhibition of VEGFR-2 signaling in endothelial cells, and can also directly inhibit cancer cell growth by interfering with the EGFR autocrine pathway which is central to cancer growth and progression. Overexpression of EGFR and/or its ligands, transforming growth factor (TGF)-α and EGF has been reported in many human tumor types (25). Furthermore, ZD6474 could block neoangiogenesis more efficiently than more selective anti-VEGFR agents, because in addition to a direct inhibitory effect on VEGFR-2 signaling, it also has an indirect effect on angiogenesis via blockade of EGFR-induced paracrine production of angiogenic growth factors (19,23).

The antitumor effects of ZD6474 on HNSCC has not been reported. The purpose of this study was to investigate the cellular response of HNSCC to ZD6474 and to evaluate its antitumor efficacy on HNSCC xenografts. We examined the molecular mechanisms underlying the effect of ZD6474 on signaling pathway, cell growth inhibition, angiogenesis and apoptosis induction.

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Materials and methods

Drugs. ZD6474 and gefitinib (ZD1839, Iressa) were provided by AstraZeneca (Macclesfield, UK). The chemical structure of ZD6474 is shown in Fig. 1. For the antiproliferative assay, stock solutions (10 mM) were made in 100% DMSO and diluted with culture media. For antitumor activity study, ZD6474 was dissolved in sterile 1% polysorbate 80 diluted in deionised water.

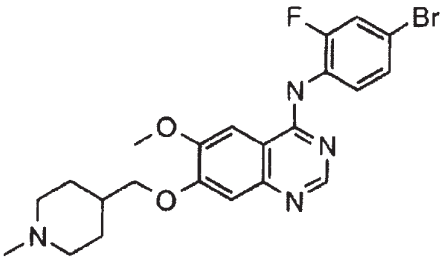

Figure 1. The structure of ZD6474.

Cell culture. Fifteen human HNSCC cell lines, and human epidermal carcinoma cell line A431 were examined in this study. The origins of these cell lines were oral floor (YCU-OR891), hypopharynx (YCU-H891), mesopharynx (YCU-M862, KCC-M871), larynx (KCC-L871, YCU-L891), tongue (KCC-T871, KCC-T873, YCU-T891, YCU-T892), maxillary sinus (KCC-MS871, YCU-MS861), and metastatic tumors from different tongue carcinomas (KCC-TCM901, KCC-TCM902, KCC-TCM903). These cell lines were established in the Department of Otolaryngology, Yokohama City University School of Medicine and the Research Institute, Kanagawa Cancer Center.

All cell lines were maintained in RPMI-1640 medium (Life Technologies Inc., Tokyo, Japan) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a 5% CO_2 atmosphere.

In vitro antiproliferative assays. The cell antiproliferative effect of ZD6474 was determined using MTT assay. These cell lines grown as suspensions were plated in 96-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA) and treated with drugs 24 h after seeding at $5x10^3$ cells/well. After 48 h of exposure to ZD6474, the MTT assay was carried out with Tetra Color One (Seigaku Co., Ltd. Tokyo, Japan). Relative growth inhibition was calculated compared with vehicle-treated control cells and IC_{50} values were determined as the drug concentrations showing 50% survival.

Western blot analysis. Fifteen human HNSCC and A431 cell lines were grown in a 100-mm plastic dish (Falcon) for 24 h. After removal of media, cells were washed twice with phosphate-buffered saline (PBS) and lysed with the lysis buffer [10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 5 mM mercaptoethanol, and 0.1 mM phenylmethylsulphonyl fluoride]. After removal of cell debris by centrifugation, protein concentration was determined. Equal amounts of proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. Membranes were reacted with (1:1000 diluted) primary antibodies to VEGFR-2 and EGFR, (Cell Signaling, Beverly, MA, USA). The reaction was developed using the ECL detection kit (Amersham Biosciences, UK) and exposed to film.

Kinase assay. YCU-H891 cells were serum starved for 24 h, and were stimulated by the addition of EGF (Sigma, St. Louis, MO) at various concentrations or VEGF (Sigma) at various concentrations. After a 10-min incubation, the cells were incubated for an additional 4 h in the presence of ZD6474 and then collected for Western blot analysis. They were lysed in lysis buffer and after removal of cell debris by centrifugation, they were separated by SDS gel electrophoresis, transferred to a nitrocellulose membrane. Membranes were reacted with (1:1000 diluted) primary antibodies to VEGFR-2, EGFR, mitogen-activated protein kinase (MAPK), STAT3, phospho-VEGFR-2, phospho-EGFR, phospho-MAPK and phospho-STAT3 (Cell Signaling). The reaction was developed using the ECL detection kit (Amersham Biosciences) and exposed to film.

In vivo antitumor activity studies. All *in vivo* experiments were carried out using 6-week-old female athymic nude (nu/nu) mice weighing 20-25 g (Oriental Yeast, Tokyo, Japan). Mice were maintained in a laminar flow room with constant temperature and humidity.

The animals were maintained and the experiments conducted at the Yokohoma City University School of Medicine, Laboratory Animal Facility. The experiments were conducted according to the guidelines for animal experiments set by the Animal Experiment Committee of Yokohama City Univesity School of Medicine.

YCU-H891 cells from *in vitro* cell culture were inoculated subcutaneously into the right flank of athymic mice ($1x10^7$ cells/mice). After 7 days, when established tumors of 50 to 100 mm^3 were detected, mice were treated i.p. with ZD6474 (25 or 50 mg/kg/day on days 1-5 of each week for 4 weeks). Control mice were given vehicle only. Each control or drug-treated group included seven mice bearing lateral subcutaneous tumors. Tumors were implanted on day 0, and tumor growth was followed by weekly measurements of tumor diameters with a Vernier caliper. Tumor weight (TW) was calculated according to the formula: TW (mg) = tumor volume (mm^3) = d^2 x $D/2$, where d and D are the shortest and the longest diameter, respectively.

Evaluation of apoptosis. Terminal deoxynucleotidyl transferase-mediated cUDP nick end-labeling (TUNEL) was done for evaluation of apoptosis. This was done with a commercial kit, 'Apoptosis *in situ* detection kit' (Wako Chemical, Osaka, Japan) for frozen sections. The TUNEL-positive cells based on the distribution of apoptotic cells were counted from a minimum of 5 microscopic fields from each section. The apoptotic index was shown as a percentage of TUNEL-positive cells relative to the total number of cells.

Evaluation of vessel density. To evaluate vessel density, an immunohistochemical technique was utilized. The vessels in

Table I. The antiproliferative activity of ZD6474.

HNSCC cell line	Primary site	IC_{50} (μg/ml) ZD6474
YCU-M862	Mesopharynx	8.5
YCU-MS861	Maxillary sinus	18.2
KCC-T871	Tongue	14.2
KCC-MS871	Maxillary sinus	10
YCU-H891	Hypopharynx	19.5
YCU-L891	Larynx	4.4
YCU-T891	Tongue	24.5
YCU-OR891	Oral floor	5
YCU-T892	Tongue	9.2
KCC-M871	Mesopharynx	3.8
KCC-L871	Larynx	26.4
KCC-T873	Tongue	22.6
KCC-TCM901	Lung[a]	8
KCC-TCM902	Lung[a]	7.7
KCC-TCM903	Lung[a]	8
A431		7.1

[a]Metastatic tumors from different tongue carcinomas. In the proliferation assay described in Materials and methods, tumor cells were exposed to ZD6474 for 48 h. Data are expressed as IC_{50}, the drug concentration (in μg/ml) that causes 50% inhibition of cell proliferation.

the tumor tissues were stained with an anti-von Willebrand Factor (vWF) antibody (1:200; Dako, Denmark). YCU-H891 tumors were harvested and immediately frozen in OCT medium for cryosection. Tumor sections at 6 μm were prepared and fixed in cold acetone. The sections were blocked with 2% goat serum and 1% bovine serum albumin in PBS and stained with the antibody. Slides were developed using 3,3'-diaminobenzidine substrate biotinylated peroxidase reagent (Vector Laboratories, Inc., Burlingame, CA). Vessel density was recorded as the number of point counts of vWF-positive vessels per field x200 magnification. Five fields per section, randomly selected from nonnecrotic area of tumors, were examined.

Statistical analysis. For statistical analyses of vessel density, apoptotic index and *in vivo* antitumor activity studies Student's paired t-test was used. $P<0.05$ was considered significant.

Results

Antiproliferative effects of ZD6474 on HNSCC cells. We first determined the antiproliferative effect of ZD6474 on HNSCC cells. Cells were cultured with media alone (control) or media containing ZD6474 (0-50 μM). As shown in Table I, the IC_{50} of ZD6474 ranged between 4.4 and 26.4 μM. HNSCC cells were more highly sensitive to ZD6474 compared with

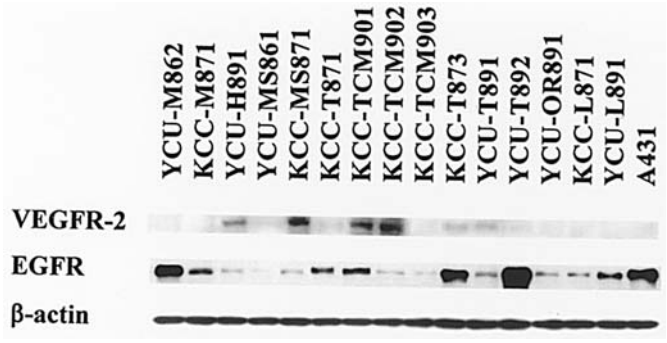

Figure 2. Expression of VEGFR-2 and EGFR in 15 HNSCC cell lines and A431 by Western blot analysis with anti-VEGFR-2 and anti-EGFR. ß-actin serves as control.

geftinib, the IC_{50} of geftinib ranged between 41.6 and 85.0 μM (data not shown).

Expression of VEGFR-2 on HNSCC cell lines with Western blotting. To clarify the correlation between the cellular sensitivity of HNSCC cells to ZD6474 and the VEGFR status, we performed Western blot analysis to determine the expression of VEGFR-2 on HNSCC cells growing in culture (Fig. 2). No correlation was found between the expression VEGFR-2 and the IC_{50} value of ZD6474. We also performed Western blot analysis to determine the expression of EGFR on HNSCC cells growing in culture. In addition, no correlation was found between the expression of EGFR and the IC_{50} value of ZD6474.

ZD6474 inhibits VEGFR-2 and EGFR-dependent intracellular signaling in HNSCC cells. We used YCU-H891 cells which expressed VEGFR-2 and EGFR to determine the effects of ZD6474 on intracellular singnaling under serum starvation conditions. In condition of serum starvation, VEGFR-2 did not show any detectable autophosphorylation, whereas the addition of VEGF activated the receptor in a dose-dependent manner (Fig. 3A). ZD6474 exposure inhibited the VEGF-dependent phosphorylation in a dose-dependent manner. MAPK and STAT3 are two major intracellular downstream signaling pathways. Suppression of phosphorylated MAPK was observed following treatment with ZD6474 in a dose-dependent manner. Under serum starvation condition, EGFR showed low levels of autophosphorylation and the addition of EGF increased the EGFR phosphorylation in a dose-dependent manner. ZD6474 inhibited the EGF-dependent phosphorylation of EGFR and MAPK in a dose-dependent manner. In contrast, the phosphorylation levels of STAT3 were not increased by EGF or VEGF, and were unaffected by ZD6474 (Fig. 3A and B).

In vivo studies of the antitumor effects of ZD6474 on YCU-H891. We investigated the *in vivo* antitumor activity of ZD6474 in nude mice bearing YCU-H891 squamous cell carcinoma xenografts. After 7 days, when established tumors of 50-100 mm^3 were detected, the group of 7 mice were treated with ZD6474 for 4 weeks. As shown as Fig. 4, significant inhibition of tumor growth was obtained after the ZD6474 treatment.

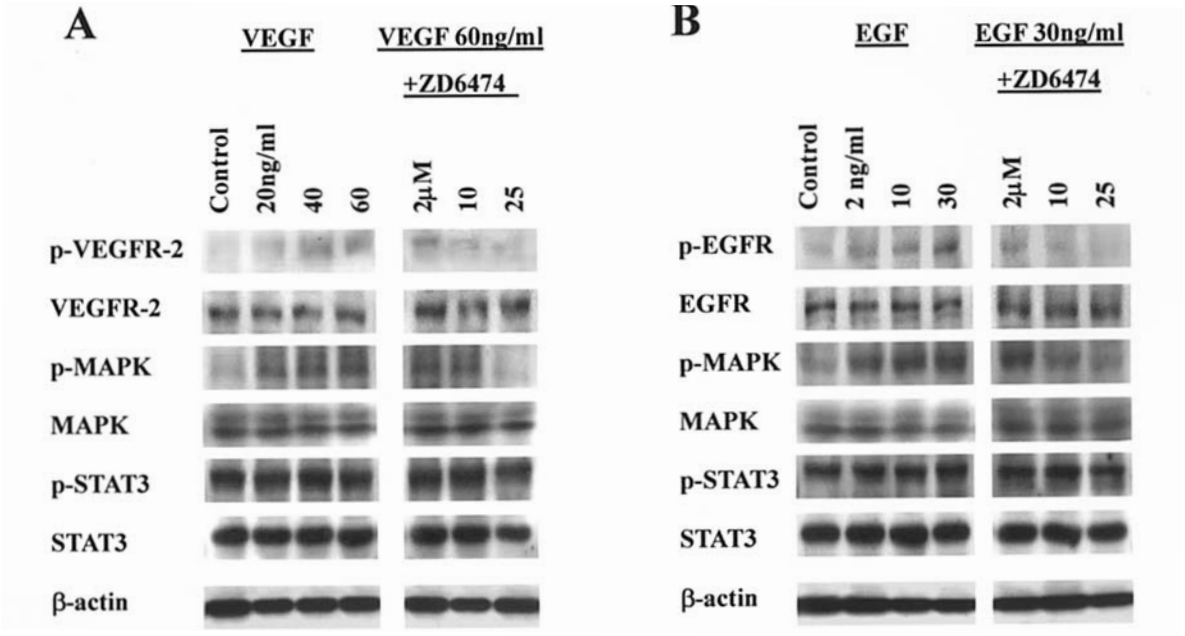

Figure 3. Effect of ZD6474 on cytokine-mediated phosphorylation of tyrosine kinase receptors *in vitro*. YCU-H891 cells were treated with ZD6474 for 4 h. Cells were stimulated for 10 min with either VEGF (A) or EGF (B). Phosphorylated and total protein levels were determined by Western blot analysis.

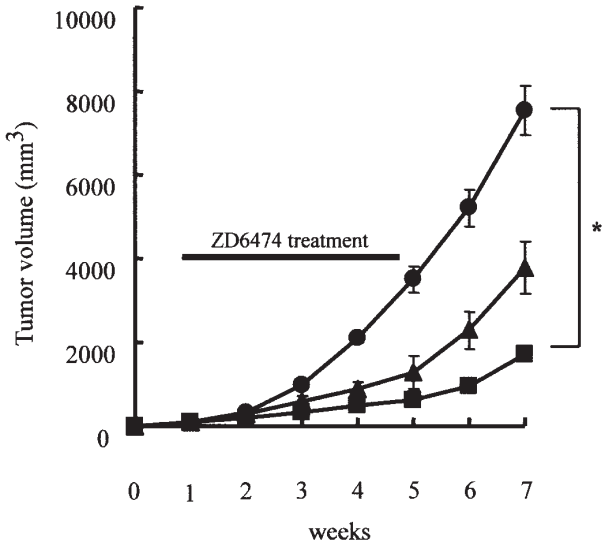

Figure 4. ZD6474 exerts a dose-dependent inhibitory effect on the growth of HNSCC, YCU-H891 tumor xenografts *in vivo*. ●, tumors treated with vehicle (1% polysorbate in deionized water); ▲, tumors treated with 25 mg/kg i.p. ZD6474; ■, tumors treated with 50 mg/kg i.p. ZD6474. Data presented are mean values (n=7 per group); error bars indicate SE. *P<0.05 versus control.

Inhibition of angiogenesis and induction of apoptosis by ZD6474. To determine the effects of ZD6474 treatment on tumor neovascularization and apoptotic cell death *in vivo*, two mice per group were sacrificed after 2 weeks of treatment. Tumors were then collected and analyzed by TUNEL method for apoptotic cell. Simultaneously, using antibodies against vWF, microvessel density was assessed in the most intense areas of neovascularization.

ZD6474 treatment resulted in an increased number of TUNEL-positive cells, and a quantitative analysis is summarized in Fig. 5B. The apoptotic index was significantly higher in tumors from mice treated at a dose of 50 mg/kg with ZD6474 compared to the 25 mg/kg group or untreated control mice.

The number of microvessels positive for anti-vWF in the group treated with ZD6474 50 mg/kg was significantly less than those in the group treated with ZD6474 25 mg/kg and in the control group (Fig. 5B).

Discussion

This is the first detailed study of ZD6474 on HNSCC *in vitro* and *in vivo*. In this study, ZD6474 proved to be an effective agent for suppressing growth of HNSCC cells *in vitro*. ZD6474 inhibited YCU-H891 tumor growth *in vivo* in a dose-dependent fashion. Together, these results indicate that ZD6474 has the potential to exert significant antitumor activity against HNSCC cells.

We demonstrated that ZD6474 inhibited VEGFR-2 and EGFR phosphorylation in a dose-dependent manner. These results are consistent with previous studies (23,24) and indicate that ZD6474 is also an EGFR tyrosine kinase inhibitor. The EGFR signaling pathway is a critical role of cancer progression, and overexpression of EGFR and/or its ligands, i.e., TGF-α and EGF, has been reported in various human tumors (25). EGF has been reported to activate VEGF production (26) and EGFR signaling has been further implicated in tumor progression, migration and angiogenesis (27,28). Therefore, ZD6474 may also inhibit angiogenesis via inhibition of EGFR-induced production of VEGF, which is a key stimulus for angiogenesis. We also demonstrated that ZD6474 inhibited phospho-MAPK, a major downstream signaling intermediate. VEGF stimulates the activation of Raf-1-MEK-MAP kinase cascade and VEGF-induced activation is mainly mediated by protein kinase C (PKC)-

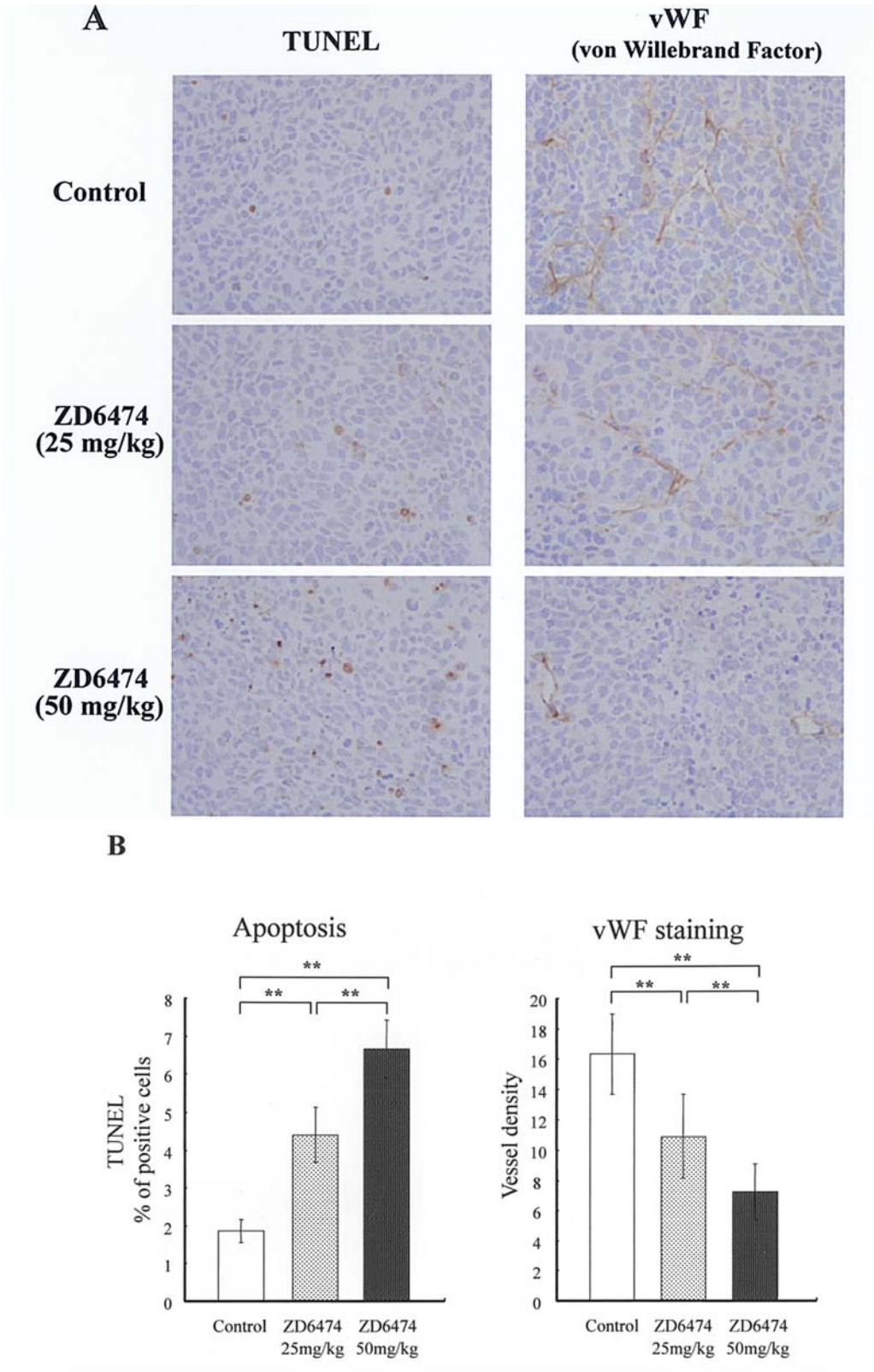

Figure 5. Tissue-based studies of YCU-H891 tumor xenografts treated with ZD6474 (25 mg/kg/d, 50 mg/kg/d). A, immunohistochemical analysis of tumor cell stained with an antibody to vWF (von Willebrand Factor) and apoptosis by TUNEL (x200). B, effect of ZD6474 on the apoptosis index (TUNEL) and the vessel density of YCU-H891 tumors *in vivo*. Data represent mean values (± SD). Significant difference by the Student's t-test ($^{**}P<0.01$).

dependent pathway, much more than by phospho-inositide 3-kinase (PI3K)-dependent pathway (29,30). In the present study of head and neck tumor cell lines, the levels of phosphorylated STAT3 were not increased by treatment with EGF or VEGF, and were unaffected by ZD6474.

To evaluate apoptotic events *in vivo*, the TUNEL method was utilized. After treatment with ZD6474, the number of TUNEL-positive tumor cells increased and the apoptotic index was significantly higher in the tumors treated with ZD6474 than that in the control. These results indicated that ZD6474 induced apoptotic events in HNSCC cells and had an antiproliferative effect on HNSCC cells. In addition, inhibition of EGFR signaling has been shown to induce selective apoptosis in tumor endothelial cells in an orthotopic model of human renal cell carcinoma metastasis in bone (31). Therefore, ZD6474 may promote endothelial cell apoptosis via inhibition of the EGFR pathway, although this was not examined in the present study.

To evaluate vessel density *in vivo*, the immunohistochemical staining was utilized. Vessels were detected with an anti-vWF antibody. After treatment with ZD6474, the number of vWF-positive endothelial cells decreased and the vessel density was significantly lower in the tumors treated with ZD6474 compared with control. These results indicated that ZD6474 inhibited angiogenesis in this HNSCC tumor model.

Based on the present results, ZD6474 may have dual antitumor effects by i) promoting tumor cell apoptosis via inhibition of the EGFR pathway, and ii) inhibiting VEGF-dependent tumor angiogenesis and VEGF-dependent endothelial cell survival.

Antiangiogenic agents acting through VEGF inhibition is an attractive therapeutic strategy because it may be less toxic than conventional cytotoxic therapy (32). Angiogenesis inhibitors may be the most effective when combined with standard cytotoxic chemotherapy because the cellular target for the angiogenesis inhibitors differs from that of cytotoxic agents. The combined treatment of angiogenesis inhibitors and cytotoxic agents may therefore induce tumor shrinkage without overlaps in mechanisms of resistance (1,33). In fact, bevacizumab, a recombinant humanized monoclonal antibody against VEGF, has clinical activity against metastatic colorectal cancer particularly in combination with fluorouracil-based chemotherapy (34). The results of a number of preclinical studies suggest that combining ZD6474 with certain anticancer agents may yield additional therapeutic benefits. The treatment with ZD6474 and paclitaxel has shown a greater inhibition on colon cancer tumor growth compared to those of either agent alone (23). The treatment with ZD6474 plus SC-236, a selective cyclooxygenase-2 (COX-2) inhibitor has caused a supra-additive inhibition on established tumor growth in human xenograft models of lung and colon cancer (35).

In conclusion, we show that *in vitro* ZD6474 had antiproliferative effects through inhibition of VEGFR-2 and EGFR pathways and that *in vivo* ZD6474 was a highly active antitumor agent for HNSCC by induction of tumor cell apoptosis and inhibition of VEGF-dependent angiogenesis. These results suggest that ZD6474 has the potential to inhibit two key pathways in tumor growth via inhibition of VEGF-dependent tumor angiogenesis and via inhibition of EGFR-dependent tumor cell proliferation. In future, the efficacy of ZD6474 in patients with HNSCC will be evaluated by the combined therapy with established therapeutic modalities.

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