Tumor Cell and Endothelial Cell Therapy of Oral Cancer by Dual Tyrosine Kinase Receptor Blockade

Orhan G. Yigitbasi,1 Maher N. Younes,1 Dao Doan,1 Samar A. Jasser,1 Bradley A. Schiff,1 Corazon D. Bucana,2 Benjamin N. Bekele,1,3 Isaiah J. Fidler,2 and Jeffrey N. Myers1,2

Departments of 1Head and Neck Surgery, 2Cancer Biology, and 3Biostatistics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Expression of the epidermal growth factor (EGF) and activation of its receptor (EGFR), a tyrosine kinase, are associated with progressive growth of head and neck cancer. Expression of the vascular endothelial growth factor (VEGF) is associated with angiogenesis and progressive growth of tumor. The tyrosine kinase inhibitor NVP-AEE788 (AEE788) blocks the EGF and VEGF signaling pathways. We examined the effects of AEE788 administered alone, or with paclitaxel (Taxol), on the progression of human head and neck cancer implanted orthotopically into nude mice. Cells of two different human oral cancer lines, JMAR and MDA1986, were injected into the tongues of nude mice. Mice with established tumors were randomized to receive three times per week oral AEE788, once weekly injected paclitaxel, AEE788 plus paclitaxel, or placebo. Oral tumors were resected at necropsy. Kinase activity, cell proliferation, apoptosis, and mean vessel density were determined by immunohistochemical immunofluorescent staining. AEE788 inhibited cell growth, induced apoptosis, and reduced the phosphorylation of EGFR, VEGFR-2, AKT, and mitogen-activated protein kinase in both cell lines. Mice treated with AEE788 and AEE788 plus paclitaxel had decreased microvessel density, decreased proliferative index, and increased apoptosis. Hence, AEE788 inhibited tumor vascularization and growth and prolonged survival. Inhibition of EGFR and VEGFR phosphorylation by AEE788 effectively inhibits cellular proliferation of squamous cell carcinoma of the head and neck, induces apoptosis of tumor endothelial cells and tumor cells, and is well tolerated in mice. These data recommend the consideration of patients with head and neck cancer for inclusion in clinical trials of AEE788.

INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common human neoplasm, with an estimated annual worldwide incidence of 500,000 new cases (1, 2). Despite developments in surgery, chemotherapy, radiotherapy, and combinations of treatment modalities, the long-term survival of patients with SCCHN has remained ~50% for the past 30 years (3). To improve the outcome for patients with SCCHN, novel therapeutic approaches are needed.

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor of the ErbB family that is expressed or highly expressed in a range of solid tumors, including head and neck cancers (4). EGFR activation induces activation of several downstream intracellular substrates, leading to mitogenic signaling and other tumor-promoting cellular activities (5). In human tumors, overexpression of this receptor correlates with a more aggressive clinical course (4).

Nevertheless, recent experimental evidence suggests that the cancer cells can escape from growth inhibition by using alternative growth pathways or by constitutive activation of downstream signaling effectors. For example, human A431 cancer cells xenografts can acquire resistance to anti-EGFR monoclonal antibodies (mAbs), such as C225 and hR3, by increased tumor-induced angiogenesis due to the constitutive overexpression of proangiogenic growth factors, such as vascular endothelial growth factor (VEGF; ref. 6).

VEGF, a mitogen specific for vascular endothelial cells, is considered to play a key role in angiogenic processes (7). VEGF binds to two distinct receptors on endothelial cells, flt-1 (VEGFR-1) and flk-1/KDR receptor (VEGFR-2), the latter of which is considered the dominant signaling receptor governing endothelial cell permeability, proliferation, and differentiation (8). Expression of these receptors in normal tissues is low and is up-regulated during the development of pathological conditions associated with neovascularization (9, 10). Inhibition of VEGF-induced angiogenic signals would be expected to selectively target tumor-associated vessels because division of endothelial cells in the normal vasculature is infrequent (8). Hence, inhibition of VEGF-mediated effects likely is an attractive alternative or complement to cytotoxic therapies.

Blockade of EGFR with specific mAbs or small molecule chemical inhibitors has been demonstrated to decrease tumor cell production of proangiogenic molecules (such as VEGF) and to inhibit tumor-associated angiogenesis (10, 11). A recent report documented that treatment combining an anti-EGFR antibody with an anti-KDR (VEGFR-2) antibody in a murine model of human colon cancer decreased tumor vascularity and increased tumor and endothelial cell apoptosis (12). Therefore, although EGFR/ErbB2 inhibitors can inhibit production of VEGF by the tumor cells, a more potent antitumor response can be achieved through the concomitant inhibition of VEGFR. Dual inhibitors, combining both activities in the same molecule, would therefore be attractive for the treatment of numerous solid tumors.

NVP-AEE788 (AEE788; molecular weight, M, 440,600) is an orally administered dual specific-kinase inhibitor targeting both the ErbB and VEGF receptors. It belongs to the class of the 7H-pyrrolo[2,3-d] pyrimidines. The efficacy of AEE788 in vitro against lung, bladder, and breast cancer cell lines has been demonstrated, and AEE788 has proved to be an effective antitumor compound in human tumor xenograft models (data on file at Novartis Pharma AG, Basel, Switzerland).

In the present study, we evaluated the therapeutic effect of the AEE788 administered alone or in combination with paclitaxel against established squamous cell carcinomas growing orthotopically within the tongue of athymic nude mice.

MATERIALS AND METHODS

JMAR and MDA1986 Cell Lines and Culture Conditions. For these studies, we used the invasive oral squamous carcinoma cell lines JMAR and MDA1986 (13). Cell lines were grown in DMEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, and a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in a mixture of 5% CO2 and 95% air. The cultures were free of Mycoplasma and pathogenic murine viruses. The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

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Requests for reprints: Jeffrey N. Myers, Department of Head and Neck Surgery, Unit 441, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. Phone: (713) 792-6920; Fax: (713) 794-4662; E-mail: jmyers@mdanderson.org.
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Measurement of Cell Proliferation. The antiproliferative effects of AEE788 against JMAR and MDA1986 cells were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that measures cell proliferation based on the ability of live cells to use MTT and convert it to a dark blue formazan (14). One thousand cells were plated into each well of a 96-well tissue culture plate. The cells were grown in DMEM supplemented with sodium pyruvate, essential amino acids, and 10% FBS. After a 24-hour attachment period, the cells were refed with medium (negative control with DMEM alone) or medium containing paclitaxel. After a 3-day incubation, the number of metabolically active cells was determined by MTT assay as measured by a MR-5000 96-well microtiter plate reader (Dynatech, Inc., Chantilly, VA) at an absorbance at 570 nm. Growth inhibition was calculated using the following formula: cytostasis (%) = (1 – (A/B)) × 100, where A is the absorbance of treated cells, and B is the absorbance of control cells.

Measurement of Cell Death. Cells were plated at a density of 2 × 10⁵ cells per well in 6-well plates (Costar, Cambridge, MA) and maintained for 24 hours before treatment with AEE788 and paclitaxel. Seventy-two hours later, the extent of cell death was determined by propidium iodide (PI) staining of hypodiploid DNA (15). For the PI staining, cells were resuspended in Nicoletti buffer 50 μg/mL PI (Sigma, St. Louis, MO) and 0.1% sodium citrate for 20 minutes at 4°C. Cells were then analyzed by flow cytometry, and the sub-G₀-G₁ fraction was measured with the Lysis program (Biosciences Clontech [BD], Franklin Lakes, NJ). The percentage of cells undergoing specific apoptosis was calculated by subtracting the percentage of cells that had undergone spontaneous apoptosis in the relevant controls from the total percentage of apoptotic cells in the study cultures (16).

Western Blotting. To determine whether AEE788 acted through its putative mechanism in vitro, we evaluated the effect of this agent on inhibition of EGFR-stimulated tyrosine phosphorylation of EGFR, VEGFR, mitogen-activated protein kinase (MAPK), and AKT in human JMAR and MDA1986 oral cancer cells. Under basal conditions in both serum-containing and serum-free media, both cells demonstrated a low level of autophosphorylation, which was enhanced after exposure to EGF for 15 minutes. These cell lines were plated onto 6-well plates at a concentration of 2 × 10⁵ cells/well and then incubated for 24 hours. On the next day, cells were incubated with serum-free media for 24 hours. The study cultures were treated with AEE788 at concentrations ranging from 0.1 to 10 μmol/L and controls with DMSO alone for 1 hour. The cells were then activated with EGF recombinant (40 ng/mL) for 15 minutes, washed with PBS, scraped with lysis buffer [1% Triton X-100, 20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl, 10% glycerol (v/v), 2 mmol/L EDTA, 1-mmol/L phenylmethylsulfonyl fluoride, 20 μmol/L aprotinin-leupeptin-trypsin inhibitor, and 2 mmol/L sodium orthovanadate], and centrifuged to remove insoluble protein. The samples were diluted in sample buffer [0.5 mmol/L Tris-HCl (pH 6.8), 10% SDS, 1 mol/L DTT, 10% (v/v) glycerol, and 1% bromophenol blue] and boiled. The proteins (100 μg per lane) were resolved on 10% SDS-PAGE onto 0.45-mmol/vol polysulfone difluoride membranes, which then were blocked with 5% (w/v) nonfat milk in 0.1% Tween 20 (v/v) in TBS (TBS-T) for 1 hour. The membrane was probed with anti-EGFR (1:3000 dilution) and anti-phospho-EGFR (Ty)₁₆₆₈, 1:2000 dilution) antibodies, respectively, incubated overnight in 1% milk TBST, washed three times with 1× TBS-L, and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated sheep antirabbit immunoglobulin (1:2000; Amersham, Inc., Arlington Heights, IL) in 1% nonfat milk. The blots were also probed with anti-phosphorylated AKT-Ser⁴⁷³ (Cell Signaling Technology, Beverly, MA), diluted 1:2000 in 1% nonfat milk TBST, phospho-EGFR (Ty)²₄⁸⁴ and phospho-VEGFR-2 (pY1045), and incubated with peroxidase-conjugated donkey antirabbit IgG (1:3000; Sigma) in 1% nonfat milk. Finally, all blots were probed with anti-β-actin (1:3000; Sigma) in 1% nonfat milk, followed by horseradish peroxidase-conjugated donkey antirabbit IgG (1:4000; Amersham, Inc.) in 1% nonfat milk. Protein bands were visualized by the enhanced chemiluminescence detection system (Amersham, Inc.; ref. 16).

Reagents. For in vivo administration, AEE788 was dissolved and administered to mice at a concentration of 50 mg/kg three times a week. Paclitaxel was obtained from Sigma and administered at 200 μg/week. The following antibodies were used: polyclonal rabbit anti-EGF, anti-VEGF, anti-EGFR, anti-VEGFR-2, and anti-activated EGFR (Santa Cruz Biotechnology, Santa Cruz, CA); anti-activated VEGFR-2 (Oncogene, Cambridge, MA); anti-activated AKT and anti-activated MAPK (Cell Signaling Technology); mouse anti-proliferating cell nuclear antigen clone PC-10 (DAKO A/S, Copenhagen, Denmark); rat anti-PEG-3100/PCAM-1 and rat anti-CD131 peroxidase-conjugated rat antirat IgG1 (PharMingen, San Diego, CA); peroxidase-conjugated F(ab')₂ goat antirabbit IgG (f(ab')₂), peroxidase-conjugated rat antirat IgG IgG (Jackson Laboratory, West Grove, CA); Alexa Fluor 594-conjugated rat antirat IgG, Alexa Fluor 488-conjugated goat antirabbit IgG (MoAbs, Eugene, OR); peroxidase-conjugated rat antimouse IgG2a (Serotec: Harlan Bioproducts for Science, Inc., Indianapolis, IN); horseradish peroxidase-conjugated goat anti-donkey anti-rabbit IgG (Sigma, St. Louis, MO), and human IgG (Sigma). Other reagents were Hoechst Dye 3342 615.9 (Hoechst, Warrington, PA); stable 3,3-diaminobenzidine (Research Genetics, Huntsville, AL), 3-amino-9-ethylcarbazole AEC (Biogenex Laboratories, San Ramon, CA), and Gill’s hematoxylin (Sigma). Prolong solution was purchased from Molecular Probes and pepsin from BioMeda (Foster City, CA).

PI and MTT were both purchased from Sigma. Stock solutions were prepared by dissolving 1 mg of each compound in 1 mL of PBS and filtering the solution to remove particles. The solution was protected from light, stored at 4°C, and used within 1 month. The enhanced chemiluminescence detection system was purchased from Amersham, and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was done with a commercial apoptosis detection kit (Promega, Madison, WI) with modifications as described below.

Animals and Maintenance. Male athymic nude mice (NCI-nu), ages 8 to 12 weeks, were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with Animal Care and Use Guidelines of The University of Texas M. D. Anderson Cancer Center under a protocol approved by the Institutional Animal Care Use Committee.

Orthotopic Injections of Oral Tumors. To produce tongue tumors, JMAR and MDA1986 oral cancer cells were harvested from subconfluent cultures. The cells were resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for the injections. A total number of 2 × 10⁵ cells were resuspended in 50 μL of HBSS and injected into the tongue with a 30-gauge hypodermic needle and a tuberculin syringe as described previously (17).

Once per week, the mice were weighed, and tongue tumors were measured with microwrapeters. Tumor volume was calculated using the formula V = (A(B²)/6), where A is the length of the longest dimension of the tumor, and B is the length of the tumor perpendicular to A. Mice were monitored for 42 days. Mice were killed after >25% loss of the recorded initial body weight in this period, and the others were killed on day 42, after which the body weight was determined and recorded. The tongues with tumors were resected; one half was fixed in formalin and embedded in paraffin, and the other half was embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −80°C. H&E staining confirmed the presence of tumor in each sample included in the analysis. Frozen and paraffin tissues were submitted for immunohistochemical analysis. Necropsy was performed in the mice to determine the extent of disease.

Therapy for Established Squamous Cell Carcinoma Tumors in Athymic Nude Mice. Mice were examined 7 days following injection of cells into the tongue. Mice with similarly sized tumors were randomized into one of four groups (n = 10 mice/group): group 1, the control group, received an oral diluent for AEE788 (90% polyethylene glycol 300 + 10% 1-methyl-2-pyrrolidinone) and i.p. HBSS; group 2, the AEE788 group, received AEE788 (50 mg/kg) by oral administration three times a week; group 3, the paclitaxel group, received paclitaxel by i.p. injection once per week (200 μg/week); and group 4, the combination therapy group, received both the oral AEE788 regimen of group 2 and the i.p. paclitaxel regimen of group 3 concomitantly.
Immunohistochemical-Immunofluorescent Determination of Proliferating Cell Nuclear Antigen (PCNA), CD31/PECAM-1, EGFR, Activated EGFR, VEGFR, Activated VEGFR, Activated MAPK, and Activated AKT. Specimens of the resected tongue tumors were processed for routine histologic and immunohistochemical analyses for markers of vascularization, survival, proliferation, and cell death. In vivo cell proliferation and apoptosis were evaluated using anti-PCNA antibodies and the TUNEL method, respectively. Paraffin-embedded tissues were used for identification of PCNA, EGF, VEGF, EGFR, and VEGFR-2. Sections (4 to 6-μm thick) were mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene, followed by treatment with a graded series of alcohol (100, 95, and 80% etomidate/double distilled H₂O v/v) and rehydrated in PBS (pH 7.5). Sections analyzed for PCNA and VEGFR-2 were heated in a microwave oven for 5 minutes; sections analyzed for VEGF and EGFR were incubated for 20 minutes with pepsin at 37°C for antigen retrieval as described previously (18, 19).

Frozen tissues were used for identification of CD31/PECAM-1, activated EGFR, activated VEGFR-2, activated MAPK, and activated AKT; TUNEL and double staining (CD31/TUNEL, CD31/ActEGFR, and CD31/ActVEGFR-2) were also performed. The sections were fixed in cold acetone (5 minutes), 1:1 acetone/chloroform (v/v; 5 minutes), and acetone (5 minutes) and washed with PBS. Immunohistochemical procedures were performed as described previously (20). A positive reaction was visualized by incubating the slides with stable 3,3’-diaminobenzidine for 10 to 20 minutes for CD31/PECAM-1 and all paraffin slides. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 minute, and mounted with Universal Mount (Research Genetics). A positive reaction for activated EGFR and activated VEGFR was visualized by incubating the slides for 1 hour with a 1:400 dilution of Alexa Fluor 488-conjugated goat antirabbit IgG at room temperature for 1 hour in the dark. For activated MAPK and activated AKT, a 1:600 dilution of Alexa Fluor 594-conjugated goat antirabbit IgG was used. Fluorescent bleaching was minimized by covering the slides with a mixture of 90% glycerol and 10% PBS. Control samples exposed to the secondary antibody alone showed no specific staining.

TUNEL assay was performed using an apoptosis detection kit (Promega) with the following modifications: samples were fixed with 4% paraformaldehyde (methanol-free) for 10 minutes at room temperature, washed twice with PBS for 5 minutes, and then incubated with 0.2% Triton X-100 for 15 minutes at room temperature. After two 5-minute washes with PBS, the samples were incubated with equilibration buffer for 10 minutes at room temperature. The equilibration buffer was drained, and the reaction buffer containing 44 μL of equilibration buffer, 5 μL of nucleotide mix, and 1 μL of terminal deoxynucleotidyl transferase (Promega kit) was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 hour, avoiding exposure to light. The reaction was terminated by immersing the samples in 2X SSC for 15 minutes. A positive reaction was visualized by incubating the slides with stable 3,3’-diaminobenzidine for 10 to 20 minutes for CD31/PECAM-1 and all paraffin slides. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 minute, and mounted with Universal Mount (Research Genetics). A positive reaction for activated EGFR and activated VEGFR was visualized by incubating the slides for 1 hour with a 1:400 dilution of Alexa Fluor 488-conjugated goat antirabbit IgG at room temperature for 1 hour in the dark. For activated MAPK and activated AKT, a 1:600 dilution of Alexa Fluor 594-conjugated goat antirabbit IgG was used. Fluorescent bleaching was minimized by covering the slides with a mixture of 90% glycerol and 10% PBS. Control samples exposed to the secondary antibody alone showed no specific staining.

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Samples were then washed three times for 5 minutes to remove unincorporated fluorescein-dUTP.

For quantification of endothelial cells, the samples were incubated with 1 μg/mL Hoechst stain for 2 minutes at room temperature. Fluorescent bleaching was minimized by treating slides with glycerol/PBS mounting medium containing 0.1 mol/L propyl gallate. Immunofluorescence microscopy was done with a ×100 objective on an epifluorescence microscope equipped with narrow band pass excitation filters mounted on a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured with a three-chip camera (Sony Corporation of America, Montvale, NJ) mounted on a universal microscope (Carl Zeiss, Tohnwood, NY) and Optimas image analysis software (Bioscan, Edmond, WA) installed on a Compaq computer with a Pentium chip, a frame grabber, an optical disk storage system, and a Mavigraph UP-D7000 digital color printer (Sony, Tokyo, Japan). To produce prints, images were additionally processed with Adobe PhotoShop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nuclei of apoptotic cells.

For the quantification analysis, five slides were prepared for each group, and two areas were selected in each slide. The percentage of stained cells among the total number of cells in each area and the average proportion of stained cells in each group were calculated and compared. For total TUNEL and PCNA expression, the cells were counted in 10 random 0.159-mm² fields at ×100 magnification. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 10 random 0.011-mm² fields at ×400 magnification. To quantify microvessel density (MVD), 10 random 0.159-mm² fields at ×100 magnification were captured for each tumor, and microvessels were quantified as described previously (21, 22).

Statistical Analysis. The Wilcoxon nonparametric test was used to test for differences in mice tumor volume and mice weight between each treatment group and control group on each day. To statistically model treatment, time, and treatment-by-time interactions on mouse tumor volume and mouse weight, a mixed model was used. Student’s t test from the mixed model was used to assess between-treatment differences. The linear mixed model with a compound symmetric correlation matrix was used to adjust for within-slide correlation (23).

RESULTS

AEE788 Inhibits In vitro Proliferation of SCCHN Cells and Sensitizes Them to Paclitaxel-mediated Toxicity. MTT assay was used to evaluate the effect of AEE788 on the proliferation of JMAR and MDA1986 cells. AEE788 inhibited cell growth of both cell lines, with an IC₅₀ of 0.287 μmol/L for the MDA1986 cells (Fig. 1A) and 0.8 μmol/L for the JMAR cells (data not shown) in medium supplemented with 2% serum. Moreover, treatment of the MDA1986 cells (Fig. 1B) and JMAR cells (data not shown) with AEE788 increased their sensitivity to paclitaxel but did not increase the level of inhibition achieved with paclitaxel at a high concentration.

AEE788 Induces Apoptosis of SCCHN Cells and Sensitizes Them to Paclitaxel-mediated Apoptosis. To assess the effects of AEE788 on the induction of apoptosis in the oral cancer cell lines JMAR and MDA1986, the cells were incubated in vitro with paclitaxel in the absence or presence of AEE788 and then evaluated for apoptosis with a flow cytometry-based assay. At 48 hours after the start of treatment with AEE788 at 4.5 μmol/L (JMAR) and 5.5 μmol/L (MDA1986), respectively, the percentage of apoptotic cells in the MDA1986 cell line (Fig. 1C) and JMAR cell line (data not shown) was 50%. The addition of 5 μmol/L AEE788 significantly increased tumor cell death. This concentration of AEE788 decreased the concentration of paclitaxel needed to induce 50% apoptosis from 2.2 to 1.0 nmol/L in the JMAR cell line (data not shown). At 5 nmol/L concentration, paclitaxel alone induced ~45% cell death. At the same paclitaxel concentration (5 nmol/L), when 0.5 μmol/L AEE788 was added, the percentage cells undergoing apoptosis increased to 78% (Fig. 1D).

AEE788 Inhibits EGF-induced EGFR, VEGFR, MAPK, and AKT Phosphorylation in SCCHN Cells. To determine whether AEE788 could inhibit EGF-stimulated growth and survival signaling pathways of MDA1986 and JMAR cells, the cells were stimulated in vitro with EGF and then treated for 1 hour with AEE788 at various concentrations. Cell lysis and Western blotting for phosphorylated forms of EGFR, VEGFR-2, AKT, and MAPK revealed inhibition of phosphorylation of all four of these kinases at 0.1 μmol/L, and complete inhibition of the activity of these kinases at 2 μmol/L. Total levels of EGFR and AKT remained unchanged by AEE788 treatment (Fig. 2). Results were similar in the JMAR cells using slightly higher concentrations of AEE788 (data not shown).

AEE788 Inhibits the Growth of Orthotopic SCCHN and Prolongs Survival in Nude Mice. To assess the effects of AEE788 on in vivo tumor growth, an orthotopic nude mouse model of SCCHN was used.

As shown in Table 1, in the MDA1986 orthotopic model, the tumor volume of mice treated with AEE788 alone or in combination with paclitaxel was significantly lower than that of the mice in the control group (P = 0.0048 for the AEE788 group and P = 0.0022 for the AEE788 plus paclitaxel group at day 21). Overall, the tumor volume was significantly lower in the groups treated with AEE788 alone, paclitaxel alone, and the combination of AEE788 and paclitaxel than in the control group (P < 0.0001, P = 0.0313, and P < 0.0001, respectively). Mouse tumor volume increased over time.
Similarly, in the JMAR orthotopic model, tumor volume was significantly lower in the combination-treated group than in the control group after day 14 (P = 0.0006 for day 21). After day 17, compared with the control group, the AEE788-treated group had significantly lower tumor volume (P = 0.0016 for day 21); at day 21, the paclitaxel group also showed significantly lower tumor volume (P = 0.0548; table not shown).

Overall, the groups receiving AEE788 alone or in combination with paclitaxel had significantly lower tumor volumes than did the control group (P < 0.001 for both study groups).

Two of the 10 mice in each of the combination treatment groups for both the JMAR and the MDA1986 orthotopic tongue models had no histologic evidence of tumors at the end of the study.

In the JMAR orthotopic model, the survival rate differed significantly between the treatment groups and the control group (all P < 0.05). Compared with the control group, mice treated with AEE788, paclitaxel, or the combination of AEE788 and paclitaxel had significantly increased survival rates, with P values of 0.0029, 0.0403, and 0.0009, respectively.

In the MDA1986 orthotopic model, the survival rate of the mice treated with AEE788 alone or in combination with paclitaxel was significantly greater than that of the mice in the control group (P < 0.0001 for both study groups; Fig. 3). There was no significant difference in survival rates between the group treated with paclitaxel alone and the control group.

**Immunohistochemical Analyses.** The results of immunohistochemical analysis showed that tumors from mice in all four treatment groups expressed similar levels of EGF, VEGF, EGFR, and VEGFR. Tumors from mice treated with AEE788 alone or the combination of AEE788 plus paclitaxel had decreased levels of staining for activated EGFR, activated VEGFR, activated AKT, and activated MAPK compared with the control and paclitaxel-treated groups. In addition, tumors from mice treated with AEE788 or combination therapy had decreased MVD, decreased PCNA levels, and increased TUNEL-positive cells. Double staining for CD31/TUNEL was performed with CD31 (red staining) and TUNEL (green staining). The percentage of apoptotic endothelial cells (yellow staining) was increased in the tumors from mice treated with AEE788 or AEE788 plus paclitaxel. Moreover, double staining for CD31/Act-EGFR and CD31/Act-VEGFR, which were performed with CD31 (red staining) and Act-EGFR and Act-VEGFR (green staining), revealed that only tumors from mice treated with AEE788 and AEE788 plus paclitaxel had decreased double staining for these markers, a finding consistent with decreased signaling through EGFR and VEGFR in endothelial cells (Fig. 4).

Quantitative immunohistochemical analyses were carried out to
Fig. 4. Inhibition of receptor and downstream-kinase phosphorylation and increase in tumor and endothelial apoptosis after treatment of oral cancer-bearing mice with AEE788 with or without paclitaxel. Tumors arising from MDA1986 cells that had been injected into nude mice were harvested and processed for histologic and immunohistochemical analyses 42 days after treatment with once-weekly i.p. injections of paclitaxel (200 μg/week), three-times-weekly oral administration of AEE788 (50 mg/kg), or a combination of both regimens, or HBSS (control). 

A. Immunohistochemical analysis with specific anti-EGF, anti-VEGF, anti-EGFR, and anti-VEGFR antibodies showed that tumors from all four treatment groups expressed similar levels of EGF, EGFR, VEGF, and VEGFR. B. Specific antibodies directed against tyrosine-phosphorylated activated EGFR and activated VEGFR were used to assess phosphorylation cascades. The tumors from mice treated with AEE788 alone or in combination with paclitaxel showed decreased phosphorylated activity of EGFR and VEGFR. Also, double staining for CD31/Act-EGFR and CD31/Act-VEGFR was performed with CD31 (red staining) and Act-EGFR and Act-VEGFR (green staining). C. Tumors were stained for PCNA, CD31/PECAM-1, TUNEL, CD31/TUNEL, activated AKT, and activated MAPK. Tumors from mice treated with AEE788 alone or in combination with paclitaxel had decreased expression of PCNA (decreased tumor cell proliferation) and CD31/PECAM-1 (decreased microvessel density), as well as increased apoptosis as determined by TUNEL. Double staining for CD31/TUNEL was performed with CD31 (red staining) and TUNEL (green staining). Apoptotic endothelial cells (yellow staining) was increased in the tumors treated with AEE788 alone or in combination with paclitaxel.
DUAL INHIBITION OF EGFR AND VEGFR IN SCCHN

**DISCUSSION**

AEE788 effectively inhibits cellular proliferation of head and neck cancer in vitro and in vivo. Used alone or in combination with paclitaxel, AEE788 induces tumor and endothelial cell apoptosis and is well tolerated in mice, providing a plausible basis for considering patients with head and neck cancer for clinical trials of AEE788.

EGFR elicits multiple biological effects in cells that overexpress the receptor, including proliferation, inhibition of apoptosis, invasion, and transformation (24). Comprehensive data over the past 20 years strongly support the role of EGFR and its ligands in the development and progression of SCCHN (25).

Several mAbs demonstrate antitumor efficacy against SCCHN cell lines and squamous cell carcinoma xenografts in nude mice (25). Of the EGFR-specific mAbs that have been developed to date, the human-murine chimeric mAb C225 has been studied most extensively. C225 dramatically enhances the in vitro and in vivo radiosensitivity of human SCCHN tumor cells and xenografts (26–28) and strongly support the role of EGFR and its ligands in the development of multitargeted therapies. Indeed, studies of preclinical models demonstrated that significant sustained antitumor activity can be obtained by combining anti-EGFR agents with other targeted therapy, such as inhibitors of VEGFR activity (46).

Although EGFR inhibitors can inhibit the production VEGF by tumor cells, a more potent antitumor response can be achieved through the concomitant inhibition of VEGFR. In the present study, we determined whether the simultaneous blockade of the EGFR and VEGFR signaling pathways by the novel EGFR and VEGFR tyrosine kinase inhibitor, AEE788, alone or in combination with paclitaxel could inhibit the growth of JMAR and MDA1986 cells injected into the tongues of nude mice. Our results showed that therapy with AEE788 inhibited cell growth, induced apoptosis, and reduced the phosphorylation of EGFR, VEGFR-2, AKT, and MAPK in both the tumor cells, a more potent antitumor response can be achieved through the concomitant inhibition of VEGFR. In the present study, we determined whether the simultaneous blockade of the EGFR and VEGFR signaling pathways by the novel EGFR and VEGFR tyrosine kinase inhibitor, AEE788, alone or in combination with paclitaxel could inhibit the growth of JMAR and MDA1986 cells injected into the tongues of nude mice. Our results showed that therapy with AEE788 inhibited cell growth, induced apoptosis, and reduced the phosphorylation of EGFR, VEGFR-2, AKT, and MAPK in both the tumor cells, a more potent antitumor response can be achieved through the concomitant inhibition of VEGFR.

Because multiple growth-controlling pathways may be altered in cancer cells, the combination of biological therapeutics targeting two or more such pathways should be tested in clinical settings to allow development of multitargeted therapies. Indeed, studies of preclinical models demonstrated that significant sustained antitumor activity can be obtained by combining anti-EGFR agents with other targeted therapy, such as inhibitors of VEGFR activity (46).

Although EGFR inhibitors can inhibit the production VEGF by tumor cells, a more potent antitumor response can be achieved through the concomitant inhibition of VEGFR. In the present study, we determined whether the simultaneous blockade of the EGFR and VEGFR signaling pathways by the novel EGFR and VEGFR tyrosine kinase inhibitor, AEE788, alone or in combination with paclitaxel could inhibit the growth of JMAR and MDA1986 cells injected into the tongues of nude mice. Our results showed that therapy with AEE788 inhibited cell growth, induced apoptosis, and reduced the phosphorylation of EGFR, VEGFR-2, AKT, and MAPK in both the tumor cells, a more potent antitumor response can be achieved through the concomitant inhibition of VEGFR.

**Table 2 Quantitative immunohistochemical analysis of MDA1986 tumors in tongues of nude mice**

<table>
<thead>
<tr>
<th>Treatment group *</th>
<th>PCNA †</th>
<th>TUNEL †</th>
<th>CD-31 †</th>
<th>TUNEL ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (HBSS)</td>
<td>19.04 ± 3.02</td>
<td>5.17 ± 4.06</td>
<td>11.92 ± 4.87</td>
<td>1.1 ± 0.99</td>
</tr>
<tr>
<td>Paclitaxel alone</td>
<td>16.69 ± 2.98</td>
<td>11.20 ± 7.37</td>
<td>7.90 ± 2.57</td>
<td>2.1 ± 1.05</td>
</tr>
<tr>
<td>AEE788 alone</td>
<td>4.52 ± 2.16 §</td>
<td>38.36 ± 10.59 §</td>
<td>1.17 ± 0.94 §</td>
<td>12.60 ± 3.47 §</td>
</tr>
<tr>
<td>AEE788 + paclitaxel</td>
<td>3.78 ± 2.44 §</td>
<td>48.58 ± 12.09 §</td>
<td>0.58 ± 0.49 §</td>
<td>16.1 ± 3.81 §</td>
</tr>
</tbody>
</table>

* MDA1986 tumors injected into nude mice were harvested and processed for histologic and immunohistochemical analyses 42 days after treatment with once-weekly i.p. injections of paclitaxel (200 μg/week), three-times-weekly oral administration of AEE788 (50 mg/kg), a combination of both drugs, or HBSS used as a control.
† Mean ± SD, ratio of positive cells/total cells determined from measurement of 10 random 0.159-mm² fields at ×400 magnification.
‡ Median of the ratio of apoptotic endothelial cells to total number of endothelial cells in 10 random 0.011-mm² fields at ×400 magnification.
§ P < 0.001 as compared with controls.
tivity in lesions from mice treated with AEE788 alone. These findings suggest that AEE788 can also directly inhibit the VEGFR.

In summary, we report that simultaneous blockade of the EGFR and VEGFR signaling pathway by the novel PTK inhibitor AEE788 alone and in combination with paclitaxel significantly inhibits oral carcinoma in nude mice. This inhibition of tumor growth is mediated by both direct antitumor effects and antiangiogenic effects. These data support the development of AEE788 for clinical use in the treatment of patients with SCCHN.

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Tumor Cell and Endothelial Cell Therapy of Oral Cancer by Dual Tyrosine Kinase Receptor Blockade

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