Inhibition of Epidermal Growth Factor Receptor and Vascular Endothelial Growth Factor Receptor Phosphorylation on Tumor-Associated Endothelial Cells Leads to Treatment of Orthotopic Human Colon Cancer in Nude Mice


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Abstract
The purpose of our study was to determine whether the dual inhibition of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) signaling pathways in tumor-associated endothelial cells can inhibit the progressive growth of human colon carcinoma in the cecum of nude mice. SW620CE2 human colon cancer cells growing in culture and orthotopically in the cecum of nude mice expressed a high level of transforming growth factor alpha (TGF-α) and vascular endothelial growth factor (VEGF) but were negative for EGFR, human epidermal growth factor receptor 2 (HER2), and VEGFR. Double immunofluorescence staining revealed that tumor-associated endothelial cells expressed EGFR, VEGFR2, phosphorylated EGFR (pEGFR), and phosphorylated VEGFR (pVEGFR). Treatment of mice with either 7H-pyrrrolo-[2,3-d]-pyrimidine lead scaffold (AEE788; an inhibitor of EGFR and VEGFR tyrosine kinase) or CPT-11 as single agents significantly inhibited the growth of cecal tumors (P < .01); this decrease was even more pronounced with AEE788 combined with CPT-11 (P < .001). AEE788 alone or combined with CPT-11 also inhibited the expression of pEGFR and pVEGFR on tumor-associated endothelial cells, significantly decreased vascularization and tumor cell proliferation, and increased the level of apoptosis in both tumor-associated endothelial cells and tumor cells. These data demonstrate that targeting EGFR and VEGFR signaling on tumor-associated endothelial cells provides a viable approach for the treatment of colon cancer.

Keywords: Endothelial cells, colon cancer, tyrosine kinase receptors, dual inhibition, AEE788.

Introduction
Colorectal carcinoma is the third most common cancer found in men and women in the United States and is responsible for the deaths of > 55,000 patients annually [1]. Despite the use of aggressive surgical resection and chemotherapy, nearly 50% of patients with colorectal carcinoma develop recurrent disease [2]. To foster the development of new approaches to therapy, a better understanding of the biology of colon cancer is critical. Because the genetic instability and biologic heterogeneity of neoplasms are the principal causes of the failure of systemic antitumor therapy, targeting the neovascularature of tumors has been explored as a way of attacking a more genetically stable and essential component of tumors [3,4].

Growth factors and their receptors play a pivotal role in the regulation of cancer progression and neovascularization [5,6]. In particular, epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) have been extensively investigated as targets for antineoplastic therapy. Transforming growth factor alpha (TGF-α) can bind to EGFR and stimulate downstream signaling cascades involved in cell proliferation (Ras/mitogen-activated protein kinase [MAPK]) and antiapoptosis (phosphatidylinositol 3-kinase [PI3K]/Akt) [7–9]. In addition, the overexpression of TGF-α and EGFR by many carcinomas correlates with the development of cancer metastasis, resistance to chemotherapy, and poor prognosis [9–11]. We have reported that the TGF-α/EGFR signaling network involving tumor cells and tumor-associated endothelial cells is a critical component in colon cancer progression [12].

Vascular endothelial growth factor (VEGF) is one of several proangiogenic factors whose receptors include VEGFR1 (Flt-1)

Abbreviations: AEE788, 7H-pyrrrolo-[2,3-d]-pyrimidine lead scaffold; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; MVD, microvessel density; PECAM-1, platelet–endothelial cell adhesion molecule 1; pEGFR, phosphorylated EGFR; PI3K, phosphatidylinositol 3-kinase; pVEGFR, phosphorylated VEGFR; RT-PCR, reverse transcriptase–polymerase chain reaction; TGF-α, transforming growth factor alpha; TUNEL, deoxyuridine 5-triphosphate nick-end labeling; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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and VEGFR2 (KDR/Fk-1) [13,14]. Hypoxia, an important stimulus for VEGF production by both normal and tumor cells, can stimulate the proliferation, differentiation, migration, and survival of endothelial cells [14]. The expression of VEGF is substantially increased in solid neoplasms, leading to an increase in microvascular density and poor prognosis [15]. VEGF acts not only as a mitogenic and permeability factor but also as an antiapoptotic survival factor by activating intracellular signaling such as MAPK and PI3K/Akt pathways [16–19]. Therefore, targeting the VEGF and VEGFR signaling pathways has been undertaken for the treatment of solid tumors. Several experimental approaches with a VEGFR tyrosine kinase inhibitor that mimics ATP or antibody against VEGF have been shown to inhibit angiogenesis and tumor progression in animal models [20,21].

Tumor cells cannot survive if they lack adequate supplies of oxygen and nutrients or cannot dispose of toxic molecules. Oxygen can diffuse from capillaries to a distance of only 150 to 200 μm. When this distance is exceeded, cell death follows [22–24]. The extent of angiogenesis, however, depends on the balance between proangiogenic and antiangiogenic factors released by tumor and host cells [25]. The expression of epidermal growth factor (EGF), VEGF, or their respective receptors correlates with angiogenesis and progressive growth in many human carcinomas [26–31]. The turnover rate of endothelial cells within these tumor-associated vessels is 20 to 2000 times faster than the rates within the vessels in normal organs [32]. The proliferation rate of endothelial cells within the vasculature of normal human organs has been reported to be < 0.01%, whereas this rate within tumor-associated vessels has been reported to be 2% to 9% [33]. One recent study reported that tumor-associated vessels express EGFR and lose ErbB3, whereas normal endothelial cells express ErbB3 and do not respond to TGF-α [34]. These results suggest that EGFR and VEGF tyrosine kinase inhibitor is effective for antivascular therapy because tumor-associated vessels are specifically targeted.

We therefore hypothesized that inhibition of the EGFR and VEGFR signaling pathways in tumor-associated endothelial cells of colon tumors inhibits tumor growth. To test this hypothesis, we used SW620 human colon cancer cells [35], which do not express EGFR, human epidermal growth factor receptor 2 (HER2), or VEGFR, but produce EGF, TGF-α, and VEGF, which can in turn induce paracrine activation of EGFR and VEGFR in tumor-associated endothelial cells. We treated nude mice with oral administrations of 7-pyrrolo [2,3-d]-pyrimidine lead scaffold (AEE788), an inhibitor of both EGFR and VEGFR tyrosine kinases [36], alone or combined with CPT-11. These treatments produced apoptosis in tumor-associated endothelial cells and inhibited the progressive growth of SW620 human colon cancer cells in the cecal wall of the mice.

Materials and Methods

Colon Cancer Cell Line and Culture Conditions

SW620 human colon cancer cells obtained from Dr. Gary Gallick [35] were maintained in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, a 2-fold vitamin solution (Life Technologies, Grand Island, NY), and a penicillin/streptomycin mixture (Flow Laboratories, Rockville, MD). 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 (assayed by Science Applications International Co., Frederick, MD) and were maintained for no longer than 12 weeks after recovery from frozen stocks.

In Vivo Selection of Highly Tumorigenic Variants from the SW620 Human Colon Cancer Cell Line

SW620 cells were injected into the cecal wall of nude mice. Three months after the injection, cecal tumors were harvested and treated with DNase and collagenase [37,38]. Cells were established in culture. Primary cultures were passaged in vitro two or three times and then cells were harvested by trypsinization and injected into the cecum of another set of nude mice. The selection cycle was repeated two times to yield cell lines designated SW620CE1 or SW620CE2.

Reagents

AEE788 (synthesized and provided by Novartis Pharmaceuticals, Basel, Switzerland) is a low–molecular weight ATP-competitive dual EGFR and VEGFR tyrosine kinase family inhibitor [36]. For oral administration (three times per week), AEE788 was dissolved in DMSO/0.5% Tween 80 and then diluted 1:20 in water. CPT-11 (Camptozar; Eli Lilly, Indianapolis, IN) was kept at room temperature and dissolved in 0.9% NaCl on the day of injection.

The following primary antibodies were purchased: rabbit anti-EGFR (SC03; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti–phosphorylated EGFR (pEGFR) (Tyr1173; Biosource, Camarillo, CA); rabbit anti-VEGFR2 (Flk-1, C1158; Santa Cruz Biotechnology); rabbit anti–phosphorylated VEGFR2 (pVEGFR2) (Flk-1, PC460; Oncogene, Boston, MA); rabbit anti-VEGFR1 (C17; Santa Cruz Biotechnology); rabbit anti–mouse F4/80 (MCAP497; Serotec, Raleigh, NC) and rat anti–mouse CD31 (BD PharMingen, San Diego, CA) for frozen samples; mouse anti–human TGF-α (GF10; Oncogene); mouse anti–human VEGF (23071D; BD PharMingen) and Ki-67 (MIB-1; DakoCytomation, Carpinteria, CA) for paraffin-embedded samples; and rabbit anti-EGFR (SC03; Santa Cruz Biotechnology), rabbit anti-pEGFR (Tyr1068; Cell Signaling Technology, Danvers, MA), and rabbit anti-VEGFR2 (Flk-1, C1158; Santa Cruz Biotechnology) for Western blot analysis.

The following secondary antibodies were used for colorimetric immunohistochemical analyses: peroxidase-conjugated goat anti–rabbit IgG; F(ab)2 (Jackson ImmunoResearch Laboratories, West Grove, PA); horseradish peroxidase (HRP)–conjugated donkey anti–sheep IgG (Sigma Chemical Co., St. Louis, MO); biotinylated goat anti–rabbit IgG (Biocare Medical, Walnut Creek, CA); streptavidin HRP (Dako); rat anti–mouse IgG2a HRP (Serotec, Harlan Bioproducts for Science, Indianapolis, IN); and goat anti–rat HRP (Jackson ImmunoResearch Laboratories).
The following fluorescent secondary antibodies were used: goat anti–rabbit Alexa 488 and goat anti–rat Alexa 594 (both from Molecular Probes, Inc., Eugene, OR). Terminal deoxynucleotidyltransferase–mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL) staining was done using a commercial apoptosis detection kit (Promega, Madison, WI) with modifications.

**Western Blot Analysis**

Cultures of SW620, SW620CE1, SW620CE2, and KM12C human colon cancer cells and SKOV3ip1 human ovarian cancer cells were washed twice with ice-cold phosphate-buffered saline (PBS); the cells were then scraped into PBS containing 5 mmol/l EDTA and 1 mmol/l sodium orthovanadate and centrifuged. The pellet was resuspended in lysis buffer (20 mmol/l Tris–HCl [pH 8.0], 137 mmol/l NaCl, 10% glycerol, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 20 mmol/l leupeptin, and 0.15 U/ml aprotonin) and centrifuged, and the supernatant was collected. The protein content of the samples was quantified spectrophotometrically. Aliquots of 30 μg of protein were subjected to electrophoresis on 7.5% polyacrylamide gels. The protein was then electrotransferred to nitrocellulose membranes (Millipore, Bedford, MA). After being blocked with 5% (w/v) nonfat milk in 0.1% Tween 20 in Tris-buffered saline (20 mmol/l Tris–HCl [pH 7.5], 150 mmol/l NaCl, and 0.1% Tween 20), the membranes were probed with a primary antibody (1:2000 dilution of rabbit anti–EGFR antibody [Santa Cruz Biotechnology]; 1:1000 dilution of rabbit anti–pEGFR antibody [Cell Signaling Technology]; 1:500 dilution of rabbit anti–VEGFR antibody [Santa Cruz Biotechnology]; and 1:1000 dilution of rabbit anti–pVEGFR antibody [Santa Cruz Biotechnology]). The membranes were then washed and treated with a secondary antibody conjugated to HRP (goat anti-rabbit at a 1:3000 dilution [Jackson ImmunoResearch Laboratories]). Protein bands were visualized using a commercially available chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

To evaluate the effect of EGF on the phosphorylation of EGFR, all tested cell lines were plated onto six-well plates at a concentration of 3 × 10⁵ cells/well and incubated for 24 hours. The next day, the cells were transferred to a serum-free medium and incubated for 24 hours. The cells were then activated for 15 minutes in the presence or absence of 10 ng/ml of recombinant human EGF (Santa Cruz Biotechnology) and then harvested and processed as described above.

**Enzyme-Linked Immunosorbent Assay for Human TGF-α Expression**

The level of TGF-α protein in culture supernatants was determined by using a quantitative immunometric sandwich enzyme-linked immunosorbent assay kit (Quantikine TGF-α ELISA kit; R&D Systems, Minneapolis, MN). The absorbance of the samples was compared with the standard curve.

**Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)**

The expression of EGF-R and VEGFR2 mRNA was assessed with RT-PCR analysis using 0.5 μg of total RNA extracted with an RNeasy kit (Qiagen, Hilden, Germany). The primer sets were as follows: human EGF-R, 5′–TTT-CGATAC-CCA-GGA-CCA-AGC-CAC-AGG-3′ (forward) and 5′–AAT-ATT-CTT-GCT-GGA-TGT-TCT-GTA-3′ (reverse); and human VEGFR2, 5′–CAT-CAC-ATC-CAC-TGG-TAT-T–3′ (forward) and 5′–GCC-AAG-CTT-GTA-CCA-TGT-G–3′ (reverse). β-Actin was also amplified for internal control. PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The experiment was performed three times.

**Effects of AEE788 on Murine Endothelial Cells**

To evaluate the effects of AEE788 on the activation of Akt and extracellular-regulated kinase (ERK) on endothelial cells, 4 × 10⁵ murine mesenteric endothelial cells established from the ImmortoMouse, as described previously [39] were seeded into six-well plates in 10% serum-enriched Dulbecco’s modified Eagle’s medium (DMEM). After 24 hours, the medium was replaced by serum-free DMEM and the cells were incubated for another 24 hours. To determine the effects of AEE788 on the activation of those signaling molecules, the endothelial cells were then pretreated for 1 hour with AEE788 (1 μM) or vehicle solution. The cells were then treated with serum-free DMEM or serum-free DMEM containing TGF-α (40 ng/ml), VEGF (40 ng/ml), or a combination of TGF-α and VEGF for 15 minutes.

Endothelial cells were washed with PBS three times and cell lysates were collected with 0.1 ml of lysis buffer (50 mM Tris–HCl [pH 7.5], 50 mM NaCl, 1% Triton X-100, 1 mM Na₃VO₄, and protease inhibitors). Proteins were quantified by the Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL). Western blot analyses were performed as described above with 20 μg of proteins. After blocking with 5% (w/v) nonfat dry milk in PBS containing 0.1% Tween-20 (Sigma) for 1 hour, either rabbit anti–ERK1/2 (1:1000 dilution, catalog #9101; Cell Signaling Technology, Inc.), rabbit anti–phospho-ERK1/2 (1:1000 dilution, catalog #9101L; Cell Signaling Technology, Inc.), rabbit anti-Akt (1:1000 dilution, catalog #9272; Cell Signaling Technology, Inc.), or rabbit anti–phospho-Akt (1:1000 dilution, catalog #9271L; Cell Signaling Technology, Inc.) was added and incubated overnight at 4°C with gentle shaking. Immunodetection was performed using the horseradish peroxidase–conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse at a 1:3000 dilution). Mouse anti-actin (1:10,000 dilution) was used to confirm the amount of proteins loaded. Protein bands were visualized using enhanced chemiluminescence kit (Amersham Biosciences).

**Animals and Orthotopic Implantation of Tumor Cells**

Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute’s Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained 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 U.S. Department of Agriculture, U.S.
Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

To produce cecal tumors, SW620CE2 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with a medium containing 10% fetal bovine serum; the cells were then washed once in a serum-free medium and resuspended in Hank’s balanced salt solution. Only suspensions consisting of single cells with > 90% viability were used. A total of 5 × 10^5 cells in 50 μl of Hank’s balanced salt solution were injected into the cecal wall of nude mice, as described previously [12,38].

Treatment of Established Human Colon Carcinoma Tumors Growing in the Cecum of Athymic Nude Mice

Fourteen days after the cecal injection when tumors were established, groups of 10 mice each were randomly assigned to receive one of the following four treatments: 1) oral administration of water diluted at 1:20 with DMSO/0.5% Tween 80 (diluent) three times per week and intraperitoneal (i.p.) injection of PBS once per week (control group); 2) administration of diluent by oral gavage three times per week and i.p. injection of 15 mg/kg CPT-11 once per week; 3) oral gavage of 50 mg/kg AEE788 three times per week and i.p. injection of PBS once per week; or 4) oral administration of water diluted at 1:20 with DMSO/0.5% Tween 80 (diluent) three times per week and intraperitoneal (i.p.) injection of PBS once per week (control group); 2) administration of diluent by oral gavage three times per week and i.p. injection of 15 mg/kg CPT-11 once per week; 3) oral gavage of 50 mg/kg AEE788 three times per week and i.p. injection of PBS once per week; or 4) oral administration of 50 mg/kg AEE788 three times per week and i.p. injection of 15 mg/kg CPT-11 once per week. All treatments were carried out for 5 weeks.

Necropsy Procedures and Histologic Studies

The mice were euthanized by methoxyflurane, and their body weight was recorded. On necropsy, tumors growing in the cecum and peritoneum were excised and weighed. For immunohistochemical and hematoxylin and eosin staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin and another was embedded in omenithine carbamyl transferase compound (Miles, Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −80°C. All macroscopically enlarged mesenteric lymph nodes were harvested, and the presence of metastatic disease was confirmed by histologic examination.

Immunohistochemical Analysis to Detect TGF-α and VEGF in Tumors

Paraffin-embedded tumors from mice in all treatment groups were immunostained for expression of TGF-α and VEGF. The sections were deparaffinized in xylene, treated with a graded series of alcohol (100%, 95%, and 80% ethanol/double-distilled water [v/v]), and rehydrated in PBS at pH 7.5. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. No antigen retrieval was done. Samples were exposed to protein blockers (5% normal horse serum and 1% normal goat serum in PBS) and incubated overnight at 4°C with each primary antibody at the appropriate dilution. After incubating for 1 hour at room temperature with peroxidase-conjugated goat anti–rabbit IgG, a positive reaction was detected by exposing the sample to stable 3,3'-diaminobenzidine (Open Biosystems, Huntsville, AL) for 5 to 10 minutes. Slides were counterstained with Gill’s hematoxylin.

Immunohistochemical Determination of Ki-67 Antigen and CD31/Platelet–Endothelial Cell Adhesion Molecule 1 (PECAM-1) and TUNEL

Paraffin-embedded tissues were used for immunohistochemical identification of Ki-67. Sections were deparaffinized and rehydrated in PBS, microwaved in water for 5 minutes for antigen retrieval, incubated at 4°C with mouse IgG1 anti–Ki-67 antibody overnight, and incubated for 1 hour at room temperature with a peroxidase-conjugated rat anti–mouse IgG1 antibody. Frozen tissues for the identification of CD31/PECAM-1 were sectioned (8–10 μm), mounted on positively charged slides, and air-dried for 30 minutes. Frozen sections were fixed in cold acetone (5 minutes), in acetone/chloroform (v/v, 5 minutes), and again in acetone (5 minutes) and were then washed in PBS. Immunohistochemical procedures were done as described previously [36]. For all parameters, we used a minimum of six samples.

For the quantification of microvessel density (MVD) in sections stained for CD31, 10 random 0.159-mm^2 fields at × 100 magnification were captured for each tumor, and microvessels were quantified according to a method described previously [10]. For the quantification of the expression of proliferating cell nuclear antigen, the number of positive cells was counted in 10 random 0.159-mm^2 fields at × 100 magnification.

Analysis of apoptotic cells was done by using a commercially available TUNEL kit (Promega) as described in detail previously [40]. For immunofluorescence microscopy, we used an epifluorescence microscope equipped with narrow band pass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY). We captured images by using a chilled, cooled, charge-coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, UK) on a computer (Macintosh; Apple Computer Inc., Cupertino, CA). Images were further processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). To quantify the apoptotic events, we counted the number of cells undergoing apoptosis in 10 random 0.159-mm^2 fields at × 100 magnification.

Double Immunofluorescence Staining for CD31/PECAM-1 and EGFR, pEGFR, VEGF, pVEGF, or TUNEL

Frozen sections of cecal tumors from nude mice were mounted on slides and fixed. Immunohistochemical procedures for CD31/PECAM-1 were done as described above. Samples were again blocked briefly in blocking solution (5% normal horse serum and 1% normal goat serum in PBS) and incubated at 4°C overnight with antibody against human EGFR, pEGFR, VEGFR, or pVEGFR. After the samples were washed in PBS, they were blocked briefly with blocking solution and incubated with streptavidin–Alexa 488 antibody for 1 hour. Samples were briefly incubated with Hoechst stain to visualize the nuclei. Endothelial cells were identified by red fluorescence, and EGFR, pEGFR, VEGFR, and pVEGFR
were identified by green fluorescence. The expression of growth factor receptors or phosphorylated receptors on endothelial cells was detected by colocalization of red and green fluorescence, which emitted yellow.

A TUNEL assay was done with the use of a commercial apoptosis detection kit as described above. TUNEL-positive apoptotic cells were detected by localized green fluorescence within the cell nuclei and endothelial cells were identified by red fluorescence. Apoptotic endothelial cells were identified by yellow fluorescence within the nuclei. The total number of apoptotic cells was quantified in 10 randomly selected microscopic fields and expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells (at × 400 magnification).

**Statistical Analysis**

We used the Mann-Whitney U test to compare the body weight of mice, tumor weight, the number of Ki-67–positive cells, the MVD (CD31/PECAM-1), and the number of TUNEL-positive cells.

**Results**

Expression of EGFR and VEGFR2 in SW620CE2 Human Colon Carcinoma Cell Lines Growing in Culture

In the first set of experiments, we examined the expression of EGFR and VEGFR by SW620CE2 human colon carcinoma cells growing in culture. KM12C human colon cancer cells [38] were used as a positive control for EGFR expression, and SKOV3ip1 human ovarian carcinoma cells [41] were used as a positive control for VEGFR2 and HER2 expression. The results of Western blot and RT-PCR analyses are shown in Figure 1. EGFR expression was detected in KM12C cells but not in SW620CE2 cells; consequently, the KM12C cells responded to EGF, whereas SW620CE2 cells...
cells did not (Figure 1A). HER2 (Figure 1B) or VEGFR2 (Figure 1C) expression was detected in SKOV3ip1 cells but not in SW620CE2 cells. The SW620 parental and selected variants expressed a high level of TGF-α (Figure 1D). SW620CE2 cells expressed low levels of EGFR but not VEGFR2 at the mRNA level (Figure 1E).

Inhibition of AKT and ERK Activation Pathways by AEE788 in Murine Colon Endothelial Cells

Murine endothelial cells established from the Immorto-Mouse were treated with AEE788 (1 μM) or vehicle solution for 1 hour and then stimulated with serum-free DMEM, TGF-α (40 ng/ml), VEGF (40 ng/ml), or a combination of TGF-α and VEGF for 15 minutes. Proteins were collected and Western blot was performed to determine the expression of phosphorylated Akt and phosphorylated ERK (Figure 2). The stimulation of endothelial cells with TGF-α significantly increased the phosphorylation of Akt and ERK. Treatment with VEGF activated Akt but not ERK. The combination of TGF-α and VEGF produced additive effects on Akt and ERK activation. Treatment with AEE788 significantly inhibited the activation of Akt and ERK (Figure 2).

Immunohistochemical Analysis of SW620CE2 Cecal Tumors

In the next set of experiments, we determined the expression of TGF-α, VEGF, EGFR, pEGFR, VEGFR, pVEGFR, and HER2 in SW620CE2 cecal tumors by immunohistochemical analysis. KM12C human colon cancer cells growing in the cecum of nude mice were used as a positive control for EGFR, and SKOV3ip1 human ovarian cancer cells growing in the peritoneal cavity of nude mice were used as a positive control for VEGFR and HER2. The SW620CE2 cells expressed TGF-α but not EGFR (Figure 3A). In contrast, KM12C cells expressed both TGF-α and EGFR (green). Dual localization of CD31 (red) and EGFR (green) demonstrated that, in both colon carcinomas, tumor-associated endothelial cells expressed EGFR and pEGFR (Figure 3A). The SW620CE2 cells expressed VEGF but did not express VEGFR1 or VEGFR2. In contrast, the SKOV3ip1 cells expressed both VEGF and VEGFR2 (green). In both neoplasms, tumor-associated endothelial cells expressed VEGFR2 and pVEGFR2 (Figure 3A). Dual localization for F4/80 (macrophages marker), CD31 (endothelial cell marker), and VEGFR1 revealed that both tumor-associated endothelial cells (yellow) and macrophages (yellow) expressed the VEGFR1 (Figure 3B). In the final set of immunohistochemical analyses (Figure 3C), we found that neither the SW620CE2 cells nor the tumor-associated endothelial cells expressed HER2. The SKOV3ip1 cells growing intraperitoneally but not the tumor-associated endothelial cells expressed HER2 (positive control).

Treatment of Human Colon Cancer Growing in the Cecum of Nude Mice

In the next set of experiments, we determined the therapeutic effects of AEE788, CPT-11, or the combination of AEE788 and CPT-11 on the growth of SW620CE2 human colon cancer cells implanted in the cecum of nude mice. SW620CE2 cells were injected into the cecal wall of nude mice. Two weeks later, when the tumors were established

Figure 2. Western blot analysis for expression of phosphorylated Akt and phosphorylated ERK on murine endothelial cells. Murine endothelial cells established from Immorto-Mouse were treated with AEE788 (1 μM) or vehicle solution for 1 hour and then stimulated for 15 minutes with serum-free DMEM, TGF-α (40 ng/ml), VEGF (40 ng/ml), or a combination of TGF-α and VEGF. Stimulation with TGF-α significantly increased the phosphorylation of Akt and ERK on endothelial cells. Stimulation with VEGF activated Akt but not ERK. The combination of TGF-α and VEGF produced additive activation of Akt and ERK. AEE788 treatment significantly inhibited the activation of Akt and ERK.
Treatment began. Five weeks later, all of the mice were euthanized and necropsies were performed. In the control and all three treatment groups, the incidence of cecal tumors was 100% (Table 1). None of the treatments produced loss of body weight. Treatment with CPT-11 alone or AEE788 alone significantly inhibited the growth of cecal tumors ($P < .01$ compared with control group). Treatment with oral administration of AEE788 and i.p. injection of CPT-11 produced the most significant inhibition in tumor growth ($P < .001$ compared with control). The gross appearance of the cecal tumors at the beginning of therapy and after 5 weeks of treatment is shown in Figure 4. The cecal tumors in control mice and in mice treated with CPT-11 were well vascularized. In contrast, the cecal tumors in mice treated with AEE788 alone and in those treated with AEE788 plus CPT-11 were small without gross evidence of vascularization.

Cell Proliferation (Ki-67), Apoptosis (TUNEL), and MVD in Cecal Tumors

Cell proliferation was evaluated by staining for Ki-67 (Figure 5A). In cecal tumors from control mice, the Ki-67 labeling index was $156 \pm 25$ (Table 2). A significantly lower Ki-67 labeling index was found in tumors from all three treatment groups. The combination of AEE788 and CPT-11 produced the largest reduction in Ki-67 labeling index, to $65 \pm 10$ ($P < .001$).
The mean number of apoptotic tumor cells was determined by TUNEL staining (Figure 5A). The number of apoptotic cells in cecal tumors from control mice was 1 ± 1 (Table 2). Treatment with CPT-11 alone or AEE788 alone increased the number of apoptotic cells to 9 ± 2 and 15 ± 2, respectively ($P < .001$). Treatment of mice with both CPT-11 and AEE788 induced the highest number of apoptotic cells (27 ± 8, $P < .001$).

The MVD in the cecal tumors was determined by immunohistochemical staining with antibodies against CD31. In cecal tumors from control mice, the MVD was 49 ± 4 (Figure 5A and Table 2). Treatment with AEE788 alone decreased the MVD to 16 ± 2, and treatment with AEE788 plus CPT-11 decreased the MVD even more, to 10 ± 2 ($P < .001$).

**Immunofluorescence Colocalization for CD31 and TUNEL**

To determine whether apoptosis of tumor-associated endothelial cells preceded the apoptosis of tumor cells, we examined cecal tumors of mice treated with AEE788 plus CPT-11 for 2 weeks. Dual localization for CD31/PECAM-1 (red) and TUNEL (green) (Figure 5B) clearly demonstrated...

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**Table 1.** Therapy for SW620CE2 Tumors Growing in the Cecal Wall of Nude Mice.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Tumor Incidence</th>
<th>Body Weight (g), Median (Range)</th>
<th>Cecal Tumor Weight (g), Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>26.7 (23.6–32.0)</td>
<td>0.15 (0.09–0.33)</td>
</tr>
<tr>
<td>CPT-11 (15 mg/kg)</td>
<td>10/10</td>
<td>28.4 (25.7–32.2)</td>
<td>0.09 (0.02–0.18)*</td>
</tr>
<tr>
<td>AEE788 (50 mg/kg)</td>
<td>10/10</td>
<td>26.0 (22.7–30.2)</td>
<td>0.05 (0.03–0.06)*</td>
</tr>
<tr>
<td>CPT-11 + AEE788</td>
<td>10/10</td>
<td>27.7 (23.4–30.9)</td>
<td>0.02 (0.01–0.05)</td>
</tr>
</tbody>
</table>

* $P < .01$ compared with control group.

$P < .001$ compared with control group.
that the majority of TUNEL-positive cells were endothelial cells (yellow). After 5 weeks of treatment with AEE788 plus CPT-11, apoptosis of tumor cells was evident (Figure 5A).

**Immunohistochemical Analysis of SW620CE2 Cecal Tumors**

To determine the biologic effects of treatment with AEE788 plus CPT-11, cecal tumors were immunohistochemically analyzed for the expression of TGF-α and VEGF. Treatment with AEE788 alone, CPT-11 alone, or AEE788

**Figure 5. Analysis for cell proliferation, apoptosis, and MVD.** (A) Cecal tumors from different treatment groups underwent immunohistochemical analysis for the expression of CD31 (MVD), Ki-67 (cell proliferation), and apoptosis (TUNEL). Note that treatment with AEE788 alone decreased the number of CD31-positive and Ki-67-positive cells and increased the number of TUNEL-positive cells; these changes were even more pronounced with the combination of AEE788 and CPT-11. (B) Double immunofluorescence staining was used for CD31/PECAM-1 (red) and TUNEL (green) in cecal tumors from mice treated with AEE788 and CPT-11 for only 2 weeks. The majority of apoptotic (TUNEL-positive) cells were endothelial cells.

**Table 2. Immunohistochemical Analysis of SW620CE2 Tumors Growing in the Cecal Wall of Nude Mice.**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Ki-67*</th>
<th>TUNEL*</th>
<th>CD31*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56 ± 25</td>
<td>1 ± 1</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>CPT-11 (15 mg/kg)</td>
<td>25 ± 16</td>
<td>9 ± 2</td>
<td>39 ± 6</td>
</tr>
<tr>
<td>AEE788 (50 mg/kg)</td>
<td>82 ± 18</td>
<td>15 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>CPT-11 + AEE788</td>
<td>65 ± 10</td>
<td>27 ± 8</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

*Mean ± SD labeling index.

1 $P < .001$ compared with control (Mann-Whitney U test).

2 $P < .01$ compared with control (Mann-Whitney U test).
plus CPT-11 did not alter the level of TGF-α (Figure 6A) and VEGF (Figure 6B) expressed on tumor cells. The expression of EGF was unaltered (data not shown). We used the double immunofluorescence staining technique to examine the expression of EGFR, pEGFR, VEGFR, or pVEGFR by CD31-positive tumor-associated endothelial cells. Tumor-associated endothelial cells from all treatment groups expressed EGFR and VEGFR. However, the phosphorylation of these receptors in tumor-associated endothelial cells was inhibited in tumors from mice treated either with AEE788 alone or with AEE788 plus CPT-11 (Figure 6, A and B). The number and diameter of blood vessels were clearly reduced in tumors from mice treated with AEE788 alone or AEE788 plus CPT-11 (Figure 6, A and B).

Figure 6. Double immunofluorescence staining for expression of EGFR, pEGFR, VEGFR, or pVEGFR in tumor-associated endothelial cells. (A) Tissue sections were stained with anti-CD31/PECAM1 antibody (red) and with anti-EGFR, pEGFR, VEGFR, or pVEGFR (green) as described in the Materials and Methods section. Colocalization of CD31 and EGFR, pEGFR, VEGFR, or pVEGFR appears as yellow fluorescence. Expression of EGFR and VEGFR by tumor-associated endothelial cells was found in tumors from all treatment groups. Phosphorylation of EGFR and VEGFR on endothelial cells was inhibited by treatment with AEE788 or AEE788 plus CPT-11. Note that treatment with AEE788 alone decreased the number and diameter of blood vessels and that this effect was even more pronounced with combined AEE788 and CPT-11.
Discussion

Our results demonstrate that, in colon carcinoma growing in the cecum of nude mice, pEGFR and pVEGFR on tumor-associated endothelial cells (and not on tumor cells) are the primary targets for therapy by AEE788, a tyrosine kinase inhibitor of both receptors. Previous studies from our laboratory revealed that the expression of pEGFR [12,40–43], pVEGFR [42–45], and platelet-derived growth factor receptor [45–47] on tumor-associated endothelial cells is dependent on the production of the relevant ligands by adjacent tumor cells. Moreover, targeting the tyrosine kinase receptors on tumor-associated endothelial cells resulted in a treatment for human renal cancer in the kidneys of nude mice [48] and for multidrug-resistant human prostate cancer in the prostate of nude mice [46].

The survival of all cells in the body depends on an adequate supply of oxygen and nutrients. The proliferation of tumor cells and the expansion of the tumor mass therefore require a new blood supply, i.e., angiogenesis [26,49]. The expression of EGF and VEGF and the activation of their respective receptors have been shown to correlate with angiogenesis and the progressive growth of human colon carcinoma [12]. Moreover, the activation of tyrosine kinase receptors on tumor-associated endothelial cells was reported to increase their resistance to anticycling chemotherapeutic agents [50]. Taken together, these findings suggest that the inhibition of EGFR and VEGFR signaling on tumor-associated endothelial cells in combination with an appropriate chemotherapeutic drug induces apoptosis of endothelial cells, leading to apoptosis of the surrounding tumor cells and stromal cells and thereby to the control of tumor growth.

Because EGFR and VEGFR are expressed on both tumor cells and tumor-associated endothelial cells, we carried out the present studies with SW620CE human colon cancer cells that do not express EGFR, HER2, VEGFR1, or VEGFR2 (Figures 1 and 3). These cells, however, produce TGF-α and VEGF in culture (Figure 1) and in vivo (Figure 3), and these ligands phosphorylate the respective receptors on tumor-associated endothelial cells in a paracrine manner (Figures 3 and 6). The inhibition of pEGFR and pVEGFR resulting in targeted therapy is therefore restricted to tumor-associated endothelial cells, and this orthotopic model for colon cancer is ideally suited to test our hypothesis.

In all in vivo studies, treatment began 2 weeks after the implantation of SW620CE2 cells into the cecum of nude mice, which produced well-established tumors (Figure 4). Blockade of the EGFR and VEGFR signaling pathways by the oral administration of the tyrosine kinase inhibitor AEE788 in combination with i.p. injections of CPT-11 inhibited further growth of these neoplasms. The major signaling pathways induced by the activation of tyrosine kinase receptors are MAPK and PI3K, which affect cell proliferation and inhibition of apoptosis [50–55]. In our studies, the administration of AEE788 plus CPT-11 inhibited the phosphorylation of EGFR and VEGFR on tumor-associated endothelial cells. Apoptosis of endothelial cells in the tumor vasculature was evident within 1 to 2 weeks of therapy (Figure 5B). Continued treatment with AEE788 plus CPT-11 led to a marked decrease in MVD, decreased the proliferation of tumor cells, and increased apoptosis of tumor cells (Figure 5A).

In conclusion, EGFR and VEGFR signaling in tumor-associated endothelial cells is an important regulator of colon cancer progression. Abrogating the signaling activation by a dual tyrosine kinase inhibitor in combination with conventional chemotherapy can induce significant apoptosis of tumor-associated endothelial cells leading to apoptosis of tumor cells. These data generated with tumor cells that do not express the EGFR, HER2, or VEGFR clearly indicate that targeting EGFR and VEGFR signaling in tumor vasculature provides a new approach to the treatment of colon cancer.

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References
