Dual Inhibition of Epidermal Growth Factor Receptor and Vascular Endothelial Growth Factor Receptor Phosphorylation by AEE788 Reduces Growth and Metastasis of Human Colon Carcinoma in an Orthotopic Nude Mouse Model


Departments of Cancer Biology, Pathology, and Medical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

We studied growth factors and their receptors in tumor cells and tumor-associated endothelial cells as the therapeutic targets in colon cancer. Immunohistochemical analysis of 13 surgical specimens of human colon adenocarcinoma revealed that both tumor cells and tumor-associated endothelial cells in 11 of the 13 specimens expressed the epidermal growth factor (EGF), transforming growth factor α (TGF-α), EGF receptor (EGFR), phosphorylated EGFR (pEGFR), vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR), and phosphorylated VEGF (pVEGFR). HT29 human colon cancer cells growing orthotopically in the cecum of nude mice expressed a high level of EGF, EGFR, pEGFR, VEGF, VEGFR, and pVEGFR. Double-immunofluorescence staining found that tumor-associated mouse endothelial cells also expressed pEGFR and pVEGFR. Tumors in mice treated for 5 weeks with oral AEE788 (an inhibitor of EGFR and VEGF tyrosine kinase) as a single agent or with CPT-11 alone were smaller (P < 0.01) and complete inhibition of lymph node metastasis. AEE788 alone or in combination with CPT-11 inhibited pEGFR, pVEGFR, and phosphorylated Akt expression on tumor-associated endothelial cells as well as on tumor cells. The combination therapy also significantly decreased microvessel density and tumor cell proliferation and increased the level of apoptosis in both tumor cells and tumor-associated endothelial cells. Collectively, these data suggest that the dual inhibition of EGFR and VEGFR signaling pathways in tumor cells and tumor-associated endothelial cells in combination with chemotherapy can provide a new approach to the treatment of colon cancer. (Cancer Res 2005; 65(9): 3716-25)

Introduction

Colorectal carcinoma is the second most common cause of cancer deaths in the United States and is responsible for the death of >55,000 patients annually (1). Despite aggressive surgical resection and chemotherapy, nearly 50% of the patients develop recurrent disease (2). Fluorouracil (5-FU) and leucovorin have been recognized as standard chemotherapy for colorectal cancer (3), and the topoisomerase I inhibitor CPT-11 has been shown to have activity against colorectal cancer that is refractory to standard chemotherapy with 5-FU and leucovorin (4, 5). Whereas the use of combined CPT-11/5-FU/leucovorin is approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic colon cancer, this regimen has a higher rate of treatment-associated deaths than do other modalities (4, 5). Clearly, there is a critical need to improve the treatment of this disease.

Growth factors and their receptors play a pivotal role in the regulation of cancer progression and neovascularization (6, 7). Epidermal growth factor (EGF) and transforming growth factor α (TGF-α) can bind to EGF receptor (EGFR) and stimulate downstream signaling cascades involved in cell proliferation [ras/mitogen-activated protein kinase (MAPK)] and antiapoptosis (phosphatidylinositol 3-kinase, PI3K/Akt; refs. 8, 9). Overexpression of EGF, TGF-α, and EGF by many carcinomas has been shown to correlate with the development of cancer metastasis, resistance to chemotherapy and hence poor prognosis (10–12). Inhibiting signaling pathways through EGFR represents a good strategy for therapeutic intervention. ZD1839 (Iressa, AstraZeneca, Macclesfield, United Kingdom), which has been approved by the FDA for treatment of non–small cell lung cancer (13), inhibits EGF-stimulated EGFR autophosphorylation in a broad range of EGFR-expressing human cancer cell lines. Cetuximab (IMC C225, Erbitux, ImClone, New York, NY), a monoclonal antibody targeting EGFR (14, 15), has been shown to induce apoptosis of colorectal cancer cells (16), and cetuximab in combination with irinotecan (in irinotecan-refractory and EGFR expressing metastatic colorectal cancers) produced a 22.9% response rate (17).

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) production is substantially increased in most type of cancers and leads to increased microvascular density (18). 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 (19–22). Therefore, targeting the VEGF and VEGFR signaling pathways has been undertaken for the treatment of solid tumors. Several experimental approaches with a VEGF tyrosine kinase inhibitor that mimics ATP or antibody against VEGF have been shown to inhibit angiogenesis and tumor progression in animal models (23, 24). Bevacizumab (Avastin, Genentech, Inc., South San Francisco, CA), a recombinant humanized monoclonal antibody to VEGF (25), has produced activity in clinical models (26). In a phase III trial for untreated advanced colorectal cancer, bevacizumab combined with chemotherapy significantly increased survival compared with chemotherapy alone (26).
Targeted Therapy of Colon Cancer with AEE788

Tumor cell proliferation and survival depend on vasculature to supply adequate oxygen and nutrients (27). The extent of angiogenesis depends on the balance between proangiogenic and antiangiogenic factors released by tumor cells and host cells (7, 28). Expression of EGF, VEGF, or their respective receptors has been shown to correlate with angiogenesis and progressive growth of human carcinomas of the colon (29), lung (30), esophagus (31), stomach (32), breast (33), and prostate (34). The turnover rate of endothelial cells within the tumor-associated vessels is 20 to 2,000 times faster than the rates of the vessels in normal organs (35). One recent detailed study of the multiple clinical specimen of human neoplasms reported that proliferation rate of endothelial cells within the vasculature of normal human organs has been reported to be <0.01%, whereas 2% to 9% of endothelial cells in tumor-associated vessels divide daily (36). Because the activation of the EGFR and VEGF receptor (VEGFR) signaling pathways inhibits apoptosis of cells (37), the dividing endothelial cells within neoplasms expressing EGF and VEGF can have increased resistance to anticycling agents.

We therefore hypothesized that the dual inhibition of EGFR and VEGF signaling pathways in colon cancer cells as well as in tumor-associated endothelial cells combined with chemotherapy could increase apoptosis. AEE788 (Novartis Pharma, Basel, Switzerland) is a novel synthesized small molecule inhibitor of both EGFR and VEGFR tyrosine kinases (38). In the present study, we investigated whether the oral administration of AEE788 combined with i.p. injection of CPT-11 can inhibit the progressive growth and metastasis of human colon cancer cells implanted orthotopically into the cecal wall of nude mice.

Materials and Methods

Colon cancer cell line and culture conditions. The human colon cancer cell line HT 29 was maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 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). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Reagents. AEE788 (Novartis Pharma), 7H-pyrrolo[2,3-d]pyrimidine lead scaffold, is a low molecular weight, ATP-competitive dual EGFR and VEGFR tyrosine kinase family inhibitor (38). AEE788 was stored at room temperature and diluted in DMSO before oral administration. CPT-11 (Camptozar, Eli Lilly, Indianapolis, IN) was kept at room temperature and dissolved in 0.9% NaCl on the day of injection. Primary antibodies were purchased from the following manufacturers: rabbit anti-phosphorylated VEGF (pVEGFR) 2/3 (Flk-1; Oncogene, Boston, MA); rabbit anti-pEGFR (Tyrl068; Cell Signaling, Beverly, MA) for Western blot analysis; rat anti-mouse CD31 (BD PharMingen, San Diego, CA); rabbit anti-EGFR (Upstate Biotechnology, Lake Placid, New York), rabbit anti-pEGFR (Tyrl068; Cell Signaling, Beverly, MA) for Western blot analysis. The mouse antiproliferating cell nuclear antigen (PCNA) clone PC 10 (Dako A/S, Glostrup, Denmark); rabbit anti-mouse, human, rat Akt, and phospho-Akt (Ser473; Cell Signaling). 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 (Biocare Medical, Walnut Creek, CA); streptavidin HRP (Dako A/S); 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 Alexa488 (Molecular Probes, Inc., Eugene, OR) and goat anti-rat Alexa 594 (Molecular Probes). Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining was done using a commercial apoptosis detection kit (Promega, Madison, WI) with modifications.

Expression of transforming growth factor α, epidermal growth factor, epidermal growth factor receptor, phosphorylated epidermal growth factor receptor, vascular endothelial growth factor, vascular endothelial growth factor receptor, and phosphorylated vascular endothelial growth factor receptor in surgical specimens of human colon adenocarcinoma. Thirteen human colon cancer specimens were obtained by informed consent from patients at the University of Texas M.D. Anderson Cancer Center. The specimens were frozen in liquid nitrogen for preparation of frozen sections or fixed in neutral-buffered formalin for paraffin-embedded sections within 30 minutes after surgical resection. Paraffin-embedded sections were processed to determine expression of TGF-α, EGF, EGFR, pVEGFR, VEGFR, VEGF, and pVEGFR. The sections were deparaffinized in xylene, treated with a graded series of alcohol (100%, 95%, 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, 1% normal goat serum in PBS) and incubated overnight at 4 °C with each primary antibody at the appropriate dilution. After incubation for 1 hour at room temperature with peroxidase-conjugated goat anti-rabbit IgG, a positive reaction was detected by exposure to stable 3,3-diaminobenzidine for 5 to 10 minutes. Slides were counterstained with Gill’s hematoxylin. The level of staining intensity was assigned a grade: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong, as described previously (39).

Western blot analysis. Serum-starved HT29 cells were treated for 120 minutes with AEE788 (0, 0.2, or 1.0 μmol/L) followed by an additional 15 minutes incubation in the presence or absence of recombinant human EGF (Santa Cruz Biotechnology; 1 or 10 ng/mL), washed, 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 μmol/L leupeptin, and 0.15 unit/mL aprotinin), centrifuged, and supernatant was collected. The protein content of samples was quantified spectrophotometrically, exactly as described previously (40).

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 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 NIH. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

To produce cecal tumors, HT29 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used. The cells were injected orthotopically into the cecal wall of nude mice as described previously (41).

Treatment of established human colon carcinoma tumors growing in the cecum of athymic nude mice. Fourteen days after the cecal injection, groups of mice were randomly assigned to receive one of the following four treatments (n = 10 mice each): (a) administration of water diluted at 1:20 with DMSO/0.5% Tween 80 (diluent) by oral gavage thrice...
per week and i.p. injection of PBS once a week (control group), (b) administration of diluent by oral gavage thrice per week and i.p. injection of CPT-11 25 mg/kg once weekly, (c) oral gavage of AEE788 (50 mg/kg) thrice per week and once-weekly i.p. injection of PBS, and (d) combination of oral AEE788 (50 mg/kg) and i.p. CPT-11 (50 mg/kg). The treatments continued for 5 weeks.

Necropsy procedures and histologic studies. The mice were killed on day 36 of treatment, and the body weight was recorded. Following necropsy, tumors growing in the cecum and peritoneum were excised and weighed. For immunohistochemical and H&E staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin and another part was embedded in ornithine carbamyl transferase compound (Miles, Elkhart, IN), rapidly frozen in liquid nitrogen and stored at −70°C. All macroscopically enlarged mesenteric lymph nodes were harvested, and the presence of metastatic disease was confirmed by histologic review.

Immunohistochemical analysis to detect epidermal growth factor, vascular endothelial growth factor, epidermal growth factor receptor, vascular endothelial growth factor receptor, phosphorylated epidermal growth factor receptor, and phosphorylated vascular endothelial growth factor receptor in tumors. Paraffin-embedded tumors from mice from all four treatment groups were immunostained for expression of EGFR, VEGF, EGFR, VEGFR, activated/phosphorylated EGFR, and activated/phosphorylated VEGFR, as described above.

Immunohistochemical determination of proliferating cell nuclear antigen and CD31/platelet-endothelial cell adhesion molecule 1 endothelial cell marker and terminal deoxynucleotidyl transferase–mediated nick-end labeling. Paraffin-embedded tissues were used for immunohistochemical identification of proliferating cell nuclear antigen (PCNA). Sections were deparaffinized and rehydrated in PBS as described previously, microwaved for 5 minutes at antigen retrieval, incubated at 4°C with the primary antibody overnight (mouse IgG2a anti-PCNA), and incubated for 1 hour at room temperature with a secondary antibody (peroxidase-conjugated rat anti-mouse IgG2a). Frozen tissues used for identification of CD31/platelet-endothelial cell adhesion molecule 1 (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 washed with PBS. Immunohistochemical procedures were done as described previously (40). For the quantification of mean vessel density (MVD) in sections stained for CD31, 10 random 0.159-mm² fields at ×100 magnification were captured for each tumor, and microvessels were quantified according to the method described previously (24). For quantification of PCNA expression, the number of positive cells was counted in 10 random 0.159-mm² fields at ×100 magnification.

Analysis of apoptotic cells was done by using a commercially available TUNEL kit (Promega) as described in detail previously (40). Immunofluorescence microscopy was done on epifluorescence microscope equipped with narrow band pass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY). Images were captured by using a chilled cooled charge-coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom) on a Macintosh computer. Images were further processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). To quantify the apoptotic events, the number of cells undergoing apoptosis was counted in 10 random 0.159-mm² fields at ×100 magnification.

Double immunofluorescence staining for CD31/platelet-endothelial cell adhesion molecule 1 and epidermal growth factor receptor, phosphorylated epidermal growth factor receptor, vascular endothelial growth factor receptor, phosphorylated vascular endothelial growth factor receptor, phosphorylated Akt, or terminal deoxynucleotidyl transferase–mediated nick-end labeling. Frozen sections of human colon cancer surgical specimens and cecal tumors from nude mice were mounted on slides and fixed. Immunohistochemical procedures for CD31 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 with antibody against human EGFR, pEGFR, VEGFR, pVEGFR, or phosphorylated Akt (pAkt) at 4°C overnight. After washes in PBS, samples were blocked briefly with blocking solution and incubated with the 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, pVEGFR, and pAkt were identified by green fluorescence. The presence of growth factor receptors, phosphorylated receptors, or pAkt in endothelial cells was detected by colocalization of red and green fluorescence, which seemed yellow.

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. The relationship between pEGFR and pVEGFR expression in human colon cancer specimens was evaluated by the Spearman rank correlation test. Associations between MVD and EGF or VEGF expression in human colon cancer specimens and the incidence of lymph node metastasis were evaluated by Fisher’s exact probability test. Body weight of mice, tumor weight, PCNA-positive cells, MVD (CD31/PECAM-1), TUNEL-positive cells, and ratio of CD31/PECAM-1/TUNEL colored cells were compared using the Mann-Whitney U test.

Results

Immunohistochemical analysis of clinical specimens of human colon cancer. Thirteen surgical specimens of human colon cancer were examined for expression of TGF-α, EGF, EGFR, pEGFR, and pVEGFR by immunohistochemical analysis. Seventy percent to 80% of the specimens were positively stained for these proteins (Table 1). Examples of a strongly stained specimen

<table>
<thead>
<tr>
<th>Patient</th>
<th>TGF-α</th>
<th>EGFR</th>
<th>pEGFR</th>
<th>VEGF</th>
<th>pVEGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table 1. Immunohistochemical analysis of TGF-α, EGF, EGFR, pEGFR, VEGF, VEGFR, and pVEGFR expression intensity in 13 human colon cancer specimens*
(patient 9) and a nonstaining specimen (patient 8) are shown in Fig. 1A. The Spearman rank correlation test revealed a significant positive correlation of pEGFR and pVEGFR expression levels (i.e., the majority of colon cancer specimens expressed similar levels of pEGFR and pVEGFR). Correlation between MVD versus EGF and VEGF expression levels determined by Fisher's exact probability test showed a significant positive correlation (data not shown). Analysis of the immunohistochemical data revealed that
tumors expressing EGF or TGF-α also express EGFR and phosphorylated EGFR. Similarly, tumors expressing VEGF also express VEGFR and phosphorylated VEGFR, suggesting an autocrine loop for activation of these receptors.

Epidermal growth factor receptor, vascular endothelial growth factor receptor, phosphorylated epidermal growth factor receptor, and phosphorylated vascular endothelial growth factor receptor on tumor-associated endothelial cells in colon cancer specimens. Next, we examined whether endothelial cells within colon cancers expressed EGFR, VEGFR, pEGFR, or pVEGFR. In tumors expressing EGF and VEGF ligands, the receptors and phosphorylated receptors were positively stained on tumor cells and tumor-associated endothelial cells. In contrast, in tumors that did not express the ligands, the tumor cells and tumor-associated endothelial cells were negative for the receptors (Fig. 1B).

Inhibition of epidermal growth factor receptor and Akt phosphorylation in human colon cancer cells by AEE788. HT29 human colon cancer cells were cultured in serum-free CMEM for 24 hours and incubated with DMSO or with different concentration of AEE788 for 2 hours followed by additional incubation in the presence or absence of recombinant human EGF for 15 minutes (1 or 10 ng/mL). The expression of EGFR and Akt was unaffected. Phosphorylation of EGFR and Akt was increased by EGF stimulation in a dose-dependent manner, and the phosphorylation of EGFR and Akt was inhibited by AEE788 at a concentration of 0.2 to 1.0 μmol/L (Fig. 1C). HT-29 cells growing in culture did not express detectable levels of VEGFR, and thus we were unable to determine the inhibitory effects of AEE788 on the phosphorylation of this receptor. In contrast, HT-29 growing in vivo expressed high levels of the VEGFR (see below).

Therapy of human colon cancer growing in the cecum of nude mice. The effects of AEE788, CPT-11, or the combination of AEE788 and CPT-11 on the growth and metastasis of HT-29 human colon cancer cells implanted in the cecum of nude mice were determined next. Tumor incidence was 100% in all treatment groups (Table 2). The oral administration of AEE788, the i.p. injection of CPT-11, or the combination of the two drugs did not significantly affect the body weight. Mice treated with saline had large tumors in the cecum and peritoneum with 80% incidence of regional (mesenteric) lymph node metastasis. Mice receiving CPT-11 had smaller tumors than did controls with 20% incidence of lymph node metastasis. Oral treatment with AEE788 also inhibited growth of tumors in the cecum and peritoneum (>50%) and reduced the incidence of lymph node metastasis to 70%. The combination treatment produced the largest inhibition of tumor growth in the cecum and peritoneum (P < 0.01) and completely inhibited lymph node metastasis (Table 2). A sample of normal cecum of mice, HT29 cells growing in the cecum of mice treated with saline, and HT29 cells growing in the cecum of mice treated with AEE788 and CPT-11 for 5 weeks as described in Materials and Methods is shown in Fig. 2. The tumor in mice treated with saline was large and highly vascularized. In contrast, mice treated with AEE788 and CPT-11 had small tumors without gross evidence of neovascularization.

Immunohistochemical analysis of HT29 cecal tumors. To determine the biological effects of the treatment with AEE788 and CPT-11, cecal tumors were harvested from the mice for immunohistochemical analysis. Tumors from all treatment groups were analyzed for the expression of EGF, EGFR, and pEGFR, as well as VEGF, VEGFR, and pVEGFR. Treatment with AEE788, CPT-11, or AEE788 plus CPT-11 did not alter the expression level of EGF, VEGF, EGFR, or VEGFR. The expression levels of pEGFR and pVEGFR, however, were significantly lower only in tumors from mice treated either with AEE788 alone or with AEE788 combined with CPT-11 (Fig. 3).

Epidermal growth factor receptor, vascular endothelial growth factor receptor, phosphorylated epidermal growth factor receptor, and phosphorylated vascular endothelial growth factor receptor on tumor-associated endothelial cells. The double immunofluorescence staining technique used to examine whether tumor-associated endothelial cells express EGFR, VEGFR, pEGFR, or pVEGFR showed that tumor-associated endothelial cells from all treatment groups expressed EGF and VEGF. In tumors expressing EGF and VEGF ligands, the receptors and phosphorylated receptors were positively stained on tumor cells and tumor-associated endothelial cells. In contrast, in tumors that did not express the ligands, the tumor cells and tumor-associated endothelial cells were negative for the receptors (Fig. 1B).

Table 2. Therapy of HT29 human colon cancer cells implanted in the cecum of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body weight in g (range)</th>
<th>Tumor incidence</th>
<th>Tumor weight (g)</th>
<th>Incidence of lymph node metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cecal tumor in g (range)</td>
<td>Peritoneal tumor in g (range)</td>
</tr>
<tr>
<td>Control</td>
<td>21.3 (18.1-26.0)</td>
<td>10/10</td>
<td>0.39 (0.16-0.58)</td>
<td>0.60 (0.19-0.98)</td>
</tr>
<tr>
<td>CPT-11</td>
<td>22.9 (19.3-26.6)</td>
<td>10/10</td>
<td>0.13 (0.09-0.27)*</td>
<td>0.32 (0.12-0.86)</td>
</tr>
<tr>
<td>AEE788</td>
<td>19.9 (18.2-24.3)</td>
<td>9/9</td>
<td>0.15 (0.06-0.34)*</td>
<td>0.30 (0.12-0.92)</td>
</tr>
<tr>
<td>CPT-11 + AEE788</td>
<td>22.0 (18.9-25.7)</td>
<td>9/9</td>
<td>0.05 (0.01-0.07)*</td>
<td>0.04 (0.02-0.27)</td>
</tr>
</tbody>
</table>

NOTE: HT29 cells (1 × 10⁶) were injected into the cecal wall of nude mice. Two weeks later, the mice were randomly assigned (n = 10) to receive water diluted 1:20 with DMSO/0.5% Tween 80 (diluent) by oral gavage thrice per week and i.p. injection of PBS once per week (control); CPT-11 (25 mg/kg) i.p. once per week and thrice per week administration of diluent by oral gavage; oral gavage of AEE788 (50 mg/kg) thrice per week and once weekly i.p. injection of PBS; oral gavage of AEE788 thrice per week and once weekly i.p. injection of CPT-11. The treatment continued for 5 weeks, and the mice were killed and necropsied. Body weight, tumor incidence and weight, and incidence of regional lymph node metastasis were recorded.

*P < 0.01.
\*P < 0.05.
Expression of phosphorylated Akt. pAkt was expressed by tumor cells and tumor-associated endothelial cells in tumors from control and CPT-11-treated mice. In tumors from mice treated with AEE788 or with AEE788 plus CPT-11, however, neither tumor cells nor tumor-associated endothelial cells expressed pAkt (Fig. 4B).

Cell proliferation (proliferating cell nuclear antigen), apoptosis (terminal deoxynucleotidyl transferase–mediated

Figure 2. Gross pathology of mouse cecum. HT29 cells were implanted into the cecum, and the mice were treated for 5 weeks with saline (control) or AEE788 (50 mg/kg, thrice/wk) and CPT-11 (25 mg/kg, i.p. injection). Arrow, cecal tumor.

Figure 3. Immunohistochemical staining for EGF, EGFR, pEGFR, VEGF, VEGFR, and pVEGFR. HT29 human colon cancer growing in the cecum of nude mice treated with saline (control), AEE788 (50 mg/kg, thrice/wk), CPT-11 (25 mg/kg; once/wk), and the combination of AEE788 and CPT-11 were sectioned and stained for expression of EGF, EGFR, p-EGFR, VEGF, VEGFR, and p-VEGFR as described in Materials and Methods. Tumors from control mice or mice treated with CPT-11 expressed pEGFR and pVEGFR. Treatment with AEE788 or with AEE788 plus CPT-11 decreased the phosphorylation of EGFR and VEGFR.
nick-end labeling), and mean vessel density. In tumors from saline-treated mice, the mean number of PCNA-positive cells (Fig. 5A) was 174 ± 26. A significant lower number of PCNA-positive cells were found in tumors from all other treatment groups. The combination of AEE788 and CPT-11 produced the largest reduction in PCNA-positive cells (Table 3).

In saline-treated tumors, the mean number of apoptotic tumor cells by TUNEL was minimal (1 ± 1). Tumors from mice in all other treatment groups had an increase in the number of apoptotic tumor cells, with the largest number in tumors from mice treated with the combination of AEE788 and CPT-11 (21 ± 6).

Tumors from mice treated with AEE788, CPT-11, or AEE788 combined with CPT-11 had a significantly lower MVD in immunohistochemistry with CD31 than those from mice treated with saline. The largest decrease in MVD was found in tumors from mice treated with the combination of AEE788 and CPT-11 (Fig. 5).

Immunofluorescence double staining for CD31/platelet-endothelial cell adhesion molecule 1 and terminal deoxynucleotidyl transferase–mediated nick-end labeling. Next, we determined whether the treatment of mice with AEE788 plus CPT-11 would increase apoptosis in endothelial cells by the CD31/TUNEL fluorescent double-labeling technique (Fig. 5D). In
tumors from mice treated with saline, the median percentage of apoptotic endothelial cells was 0%. In tumors from mice treated with AEE788 and CPT-11, the median percentage of apoptotic endothelial cells was 10% (range, 0-31%; Table 3).

Discussion

The expression levels of TGF-α, EGF, VEGF and their respective receptors have been shown to correlate with progressive tumor growth, development of metastasis, and resistance to chemotherapy by many carcinomas (10–12, 42). These results suggest that the inhibition of EGFR and VEGFR signaling could be a good approach to therapy of this disease. In the first set of experiments, we used immunohistochemical analysis to determine the frequency of TGF-α, EGF, VEGF, and their receptors and phosphorylated receptors in surgical specimens of human colon cancers. We found a significant correlation between the expression levels of pEGFR and pVEGFR, suggesting that inhibiting signaling by just one of the receptors may not be sufficient to inhibit tumor progression (24), whereas the inhibition of both pEGFR and pVEGFR by a dual kinase inhibitor such as AEE788 should provide a superior outcome. Support for this observation comes from a study with ZD6474, a VEGFR tyrosine kinase inhibitor with activity against EGFR (43) capable of inhibiting the growth of tumor cells with acquired resistance to therapy mediated against the EGFR (32). We also examined the expression of EGFR, VEGFR, and the phosphorylated receptors on tumor-associated endothelial cells. We found that these

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor cells</th>
<th>Endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCNA</td>
<td>TUNEL&lt;sup&gt;↑&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>174 ± 26</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>CPT-11</td>
<td>103 ± 24&lt;sup&gt;↑&lt;/sup&gt;</td>
<td>10 ± 3&lt;sup&gt;↑&lt;/sup&gt;</td>
</tr>
<tr>
<td>AEE788</td>
<td>99 ± 18&lt;sup&gt;↑&lt;/sup&gt;</td>
<td>8 ± 3&lt;sup&gt;↑&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPT-11 + AEE788</td>
<td>71 ± 15&lt;sup&gt;↑&lt;/sup&gt;</td>
<td>21 ± 6&lt;sup&gt;↑&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup>% TUNEL-positive endothelial cells in 10 random 0.01-mm² fields ×400 magnification.

<sup>↑</sup>Mean ± SD.

<sup>↑↑</sup>P < 0.01, compared with control (Mann-Whitney U test).

<sup>↑↑↑</sup>P < 0.05, compared with control (Mann-Whitney U test).
receptors and phosphorylated receptors were expressed on tumor-associated endothelial cells only when the tumors expressed the relevant ligands. These results agree with our previous findings that ligands released by tumor cells can up-regulate the expression of receptors on tumor-associated endothelial cells in a paracrine manner (37, 40). Similar to results of a previous report (42), MVD correlated with the level of angiogenic factors, such as EGF and VEGF (42). Collectively, these findings suggest that blockade of EGFR- and VEGF-dependent pathways on both tumor cells and tumor-associated endothelial cells may be an effective strategy to inhibit angiogenesis and hence the growth of colon cancer.

Blockade of EGFR signaling has been shown to inhibit cyclin-dependent kinase 2, to produce cell cycle arrest at the G1 phase, and to eventually increase apoptosis (44). VEGF plays a critical role in the early events in angiogenesis. These data indicate that inhibition of both EGFR and VEGFR signaling may induce cell cycle arrest and apoptosis and inhibit angiogenesis. In our present study, the oral administration of AEE788 inhibited the growth of HT29 cells implanted into the cecum of nude mice, lowering the proliferation rate of tumor cells as measured by PCNA staining and lowering MVD by apoptosis of both tumor cells and endothelial cells.

The combination of AEE788 and CPT-11 was the most effective treatment in inhibiting the progressive growth of primary cecal tumors as well as disseminated peritoneal tumors and regional lymph node metastasis. Similarly, this combination therapy yielded a higher incidence of apoptosis of tumor cells and tumor-associated endothelial cells, resulting in lower values for MVD and PCNA-staining positive cells compared with the other treatment groups. Several possible mechanisms could account for the increased efficacy of this combination for treatment of colon cancer. VEGF and EGF are survival factors that inhibit apoptosis. Akt, which has been shown to mediate cell survival via regulation of antiapoptotic proteins such as Bcl2, Bcl-XL, and nuclear factor κB, is one of the major downstream signaling targets of EGF and VEGF (22, 45, 46). In our experiments, we found decreased phosphorylation of Akt on tumor cells as well as on tumor-associated endothelial cells in tumors from mice receiving AEE788. The abrogation of Akt phosphorylation by AEE788 indicates that activation of Akt on tumor cells and tumor-associated endothelial cells is mediated by EGF and VEGF. With reduction of antiapoptotic resistance by AEE788, dividing tumor cells and endothelial cells are more sensitive to anticycling drugs, such as CPT-11. Indeed, the oral administration of AEE788 without i.p. CPT-11 blocked the phosphorylation of EGR-R, VEGFR, and Akt but did not produce significant therapeutic response, whereas the combination of oral AEE788 and i.p. CPT-11 produced the best outcome.

Endothelial cells in normal tissues have a long half-life and <0.01% divide daily. In contrast, 2% of endothelial cells in clinical specimen of human colon cancer were found to divide daily (36). The inhibition of EGFR and VEGFR signaling by AEE788 prevents the up-regulation of antiapoptotic proteins by endothelial cells (and tumor cells). Hence, the administration of an anticycling agent such as CPT-11 (47) can produce apoptosis in the dividing endothelial cells. Our data suggest that the apoptosis in tumor-associated endothelial cells correlates with the apoptosis of tumor cells and hence with the therapeutic response of the tumors.

In conclusion, EGFR and VEGFR signaling in both tumor-associated endothelial cells and the tumor cells themselves are important in the progression of colon cancer. Abrogating the signaling activation by a dual tyrosine kinase inhibitor in combination with conventional therapy can induce a significant decrease in proliferation of tumor cells and significant apoptosis of both tumor cells and endothelial cells. These data indicate that targeting the EGFR and VEGFR signaling in tumor vasculature with antiveinovascular therapy provides a new approach to the treatment of colon cancer.

Acknowledgments

Received 10/14/2004; revised 1/24/2005; accepted 2/15/2005.

Grant support: Cancer Center support grant CA16672; National Cancer Institute, NIH Specialized Programs of Research Excellence in Prostate Cancer grant CA90270; and University of Texas Frederick F. Becker Distinguished University Chair in Cancer Research (S.R. Hamilton).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Novartis Pharma for providing us with AEE788 and for critical discussions and support, Michael Worley for critical editorial review, and Lola López for expert assistance with the preparation of this article.

References

21. Huang GW, Yang LY, Lu WQ, Effects of PI3K and p38/ 
P44 MAPK on overexpression of vascular endothelial growth factor in hepatocellular carcinoma. World J 
endothelial growth factor regulates endothelial cell 
survival through the phosphatidylinositol 3-kinase/Akt 
signal transduction pathway. Requirement for Fk-1/ 
receptor inhibitor PTK787/ZK222584 [correction of 
ZK222548] combined with ionizing radiation on endo-
thelial cells and tumour growth. Br J Cancer 2001;85: 
2010–6.
growth of colon cancer carcinomatosis by antibodies to 
vascular endothelial and epidermal growth factor 
25. Ferrara N, Hillan KJ, Gerber HP, Novotny W. 
Discovery and development of bevacizumab, an anti-
VEGF antibody for treating cancer. Nat Rev Drug Discov 
Bevacizumab plus irinotecan, fluorouracil, and leuco-
27. Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate 
angiogenic biologic implications for antiangiogenic 
29. Iqbal S, Lenz HJ. Integration of novel agents in the 
29.
28.
27.
25.
24.
23.
22.
21.
20.
19.
18.
17.
16.
15.
14.
13.
12.
11.
10.
9.
8.
7.
6.
5.
4.
3.
2.
1.

angiogenesis: biologic implications for antiangiogenic 
treatment of colorectal cancer. Cancer Chemother 
30. McCarty MF, Wey J, Stoeltzing O, et al. ZD6474, a 
vascular endothelial growth factor receptor tyrosine 
kinase inhibitor with additional activity against epidermal 
growth factor receptor tyrosine kinase, inhibits ortho-
topic growth and angiogenesis of gastric cancer. Mol 
31. de Jong JS, van Diest PJ, van der Valk P, Baak JP. 
Expression of growth factor receptors, growth-inhibiting 
factors, and their receptors in invasive breast cancer. II. 
Correlations with proliferation and angiogenesis. 
32. Trojan L, Thomas D, Knoll T, Grobholz R, Alken P, 
Michel MS. Expression of proangiogenic growth factors 
VEGF, EGF and bFGF and their topographical relation 
to neovascularization in prostate cancer. Urol Res 
33. Holson B, Deneckamp J. Endothelial proliferation in 
tumours and normal tissues: continuous labeling 
34. Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate 
KH, Augustin HG. Heterogeneity of angiogenesis and 
blood vessel maturation in human tumors: implications 
10:784–93.
in G1 arrest mediated by an anti-epidermal growth 

factor receptor tyrosine kinase inhibitor, in human 
cancer cells with acquired resistance to antiepidermal 
growth factor receptor therapy. Clin Cancer Res 2004; 
10:784–93.
platelet-derived growth factor receptor enhances sur-

vival of murine bone endothelial cells. Cancer Res 2004; 
64:1200–10.
effects of CPT-11 on colon carcinoma cells: depen-
dence on p53 and MLH1 status. Int J Cancer 2002;101: 
23–31.
family epidermal growth factor receptor/Erbb2 and 
vascular endothelial growth factor receptor tyrosine 
kinase inhibitor with antitumor and angiogenic activity. 
and regulation of CD97 in colorectal carcinoma cell 
lines and tumor tissues. Am J Pathol 2002;161: 
1657–67.
40. Kim SJ, Uehara H, Karashima T, Shepherd DL, Killon 
JJ, Fidler JJ. Blockade of epidermal growth factor-
receptor signaling in tumor cells and tumor-associated 
endothelial cells for therapy of androgen-independent 
human prostate cancer growing in the bone of nude 
41. Morikawa K, Walker SM, Jessup JM, Fidler JJ. In vivo 
selection of highly metastatic cells from surgical 
specimens of different primary human colon carcino-
mas implanted into nude mice. Cancer Res 1986;46: 
1943–8.
42. Barozzi C, Ravaoli M, D’Errico A, et al. Relevance of 
biologic markers in colorectal carcinoma: a compara-
activity of ZD6474, a vascular endothelial growth 

factor receptor tyrosine kinase inhibitor, in human 
cancer cells with acquired resistance to antiepidermal 
growth factor receptor therapy. Nat Rev Cancer 2004; 
www.aacrjournals.org
Dual Inhibition of Epidermal Growth Factor Receptor and Vascular Endothelial Growth Factor Receptor Phosphorylation by AEE788 Reduces Growth and Metastasis of Human Colon Carcinoma in an Orthotopic Nude Mouse Model


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/9/3716

Cited articles
This article cites 42 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/9/3716.full#ref-list-1

Citing articles
This article has been cited by 21 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/9/3716.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.