

Antitumor effect of FP3 in combination with cetuximab on patient-derived tumor tissue xenograft models of primary colon carcinoma and related lymphatic and hepatic metastases

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Abstract. FP3 is an engineered protein which contains the extracellular domain 2 of vascular endothelial growth factor (VEGF) receptor 1 (Flt-1) and the extracellular domain 3 and 4 of VEGF receptor 2 (Flk-1, KDR) fused to the Fc portion of human immunoglobulin G1. Previous studies have demonstrated its antiangiogenic effects *in vitro* and *in vivo*, and its antitumor activity *in vivo*. Cetuximab is a monoclonal antibody against epidermal growth factor (EGF) receptor. Combined inhibition of VEGF and EGF signaling may act additively or synergistically. In this study, patient-derived tumor tissue (PDTT) xenograft models of primary colon carcinoma and lymphatic and hepatic metastases were established for assessment of the antitumor activity of FP3 in combination with cetuximab. Xenografts were treated with FP3 and cetuximab, alone or in combination. After tumor growth was confirmed, volume and microvessel density in tumors were evaluated. Levels of VEGF, EGFR and PCNA in the tumor were examined by immunohistochemical staining, and levels of related cell signaling pathway proteins were examined by western

blotting. FP3 in combination with cetuximab showed significant antitumor activity in three xenograft models (primary colon carcinoma, lymphatic metastasis and hepatic metastasis). The microvessel density in tumor tissues treated with FP3 in combination with cetuximab was lower compared to that of the control. Antitumor activity of FP3 in combination with cetuximab was significantly higher than that of each agent alone in two xenograft models (colon carcinoma lymphatic metastasis and hepatic metastasis). This study indicated that addition of FP3 to cetuximab significantly improved tumor growth inhibition in the PDTT xenograft models of colon carcinoma lymphatic and hepatic metastases. Combination anti-VEGF (FP3) and anti-EGFR (cetuximab) therapies may represent a novel therapeutic strategy for the management of metastatic colon carcinoma.

Introduction

Therapies directed against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) have shown their clinical benefit in the treatment of cancer (1-8). Combined inhibition of VEGF and EGFR may act additively or synergistically.

Cetuximab (Erbix[®]), a chimeric immunoglobulin G subclass 1 monoclonal antibody that blocks the binding of EGF to its receptor, inhibits cell proliferation, tumor neoangiogenesis and metastatic potential, and promotes tumor cell apoptosis. Clinical activity has been demonstrated when given as a single agent in patients with previously treated colorectal cancer (CRC) and has been approved in CRC without *KRAS* mutation (4).

FP3 (also named as KH902 or KH903) is an engineered protein which contains the extracellular domain 2 of VEGF receptor 1 (Flt-1) and extracellular domain 3 and 4 of VEGF receptor 2 (Flk-1, KDR) fused to the Fc portion of human

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immunoglobulin G1 (9,10). Previous studies indicated that FP3 has promise as a local antiangiogenic treatment of human choroidal neovascularization (CNV)-related age-related macular degeneration (AMD) (9,11-13). In subsequent studies, it was demonstrated that FP3 has an inhibitory efficacy in VEGF-mediated proliferation and migration of human umbilical vein endothelial cells, and in VEGF-mediated vessel sprouting of rat aortic ring *in vitro* (10). It was also demonstrated that FP3 has an antitumor effect in an NSCLC cell line (A549) xenograft model (10) and patient-derived tumor tissue (PDTT) xenograft models of gastric carcinoma (14) and colon carcinoma with lymphatic and hepatic metastases (15) in nude mice.

In the present study, we evaluated the antitumor activity of FP3 in combination with cetuximab in a series of patient-derived tumor tissue (PDTT) xenograft models of primary colon carcinoma and lymphatic and hepatic metastases.

Materials and methods

Reagents and drugs. Anti-Akt, anti-ERK, and anti-MAPK, well as phosphorylation-specific antibodies against Akt (Ser³⁰⁸ and Ser⁴⁷³), ERK (Thr²⁰²/Tyr²⁰⁴), and MAPK (Thr¹⁸⁰/Tyr¹⁸²) as well as an antibody against PCNA were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against VEGF and EGFR were purchased from Epitomics, Inc. (Burlingame, CA); against platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) (rat monoclonal, clone MEC 13.3) from BD Pharmingen (San Diego, CA); and against α -smooth muscle actin (α -SMA, rabbit polyclonal) from Abcam (Cambridge, UK). Fluorescent (Cy3- or FITC-conjugated) secondary antibodies (goat anti-rat or anti-rabbit) were purchased from Jackson ImmunoResearch (West Grove, PA). The antibody against GAPDH and the horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The chemiluminescence detection system was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Cetuximab (Erbix[®]) was purchased from Merck, Inc. (Merck, Germany). FP3 was kindly provided as a gift from Kanghong Biotechnology, Inc. (Konghong, Chengdu, China).

Patient and tissue samples. Tumor specimens were obtained at initial surgery from a 40-year-old female colon carcinoma patient with lymphatic and hepatic metastases. Prior written informed consent was obtained from the patient and the study received ethics board approval at the First Affiliated Hospital, College of Medicine, Zhejiang University. The patient had not received chemotherapy or radiation therapy before surgery. The histological type was determined according to WHO criteria. The tumor was diagnosed as mucinous adenocarcinoma (T3N2M1).

Establishment of xenografts and treatment protocol. Four- to six-week-old female BALB/c nude mice purchased from Slaccas (Slaccas Laboratory Animal, Shanghai, China) were housed in a barrier facility and acclimated to 12-h light-dark cycles for at least 3 days before use. The use of experimental animals adhered to the 'Principles of Laboratory Animal Care' (NIH publication #85-23, revised in 1985). All experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University (approval ID:

SYXK(ZHE)2005-0072). PDTT xenograft models of human colon carcinoma with lymphatic and hepatic metastases were established as previously described (14-17).

PDTT xenografts of primary colon carcinoma, lymphatic metastasis and hepatic metastasis from the second mouse-to-mouse passage (the third generation, G3) were allowed to grow to a size of 200 mm³, at which time mice were randomized into three cohorts. The cohort of xenografts of primary colon carcinoma; the cohort of xenografts of colon carcinoma lymphatic metastasis; and the cohort of xenografts of colon carcinoma hepatic metastasis. Each cohort included four groups of treatment, with 10 mice in each group. The treatment groups included: i) control (100 μ l saline); ii) FP3, 15 mg/kg, *i.v.*, twice/week; iii) cetuximab, 10 mg/kg in 200 μ l, *i.p.*, twice/week; and iv) FP3, 15 mg/kg, *i.v.* and cetuximab, 10 mg/kg in 200 μ l, *i.p.*, twice/week. Mice were treated during 21 days, monitored twice/week for signs of toxicity, and weighed once a week. Tumor size was evaluated twice a week by caliper measurements using the following formula: tumor volume = (length x width²)/2. Relative tumor growth inhibition (TGI) was calculated by relative tumor growth of treated mice divided by relative tumor growth of control mice (T/C). Experiments were terminated on Day 30.

Immunohistochemistry. Selected tumor specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 5 μ m were cut, dewaxed, rehydrated, and subjected to antigen retrieval. After blocking endogenous peroxidase activity, the sections were incubated with primary antibodies against EGFR (1:100), VEGF (1:100) and PCNA (1:100) overnight at 4°C. Immunohistochemistry was performed using the streptavidin-biotin peroxidase complex method (Lab Vision, Fremont, CA). The slides were examined and pictures were captured using an Olympus BX60 (Olympus, Japan). Sections known to stain positively were incubated in each batch and negative controls were also prepared by replacing the primary antibody with preimmune sera.

Fluorescence immunohistochemistry. Mice with similar tumor sizes were anesthetized with ketamine (87 mg/kg) plus xylazine (13 mg/kg) injected intramuscularly. The chest was opened rapidly, and the vasculature was perfused for 3 min at a pressure of 120 mmHg with fixative [4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS), pH 7.4] from an 18-gauge cannula inserted into the aorta via an incision in the left ventricle. Blood and fixative exited through an opening in the right atrium. After the perfusion, the implanted tumor was removed and placed into fixative for 2 h at 4°C. Specimens were then rinsed several times with PBS, infiltrated overnight with 30% sucrose, embedded in OCT medium and frozen for cryostat sectioning. Cryostat sections 8 to 10 μ m in thickness were brought to room temperature, air dried overnight, then fixed in acetone for 10 min. Slides were allowed to air dry for 30 min and were washed three times for 5 min each in PBS. Samples were then incubated in 5% BSA in PBS for 30 min at room temperature to block nonspecific antibody binding. Next, the sections were incubated with two primary antibodies (CD31, 1:100; and α -SMA, 1:200) overnight at room temperature in humidified chambers diluted in PBS. After several rinses with PBS, specimens were incubated for 1 h at

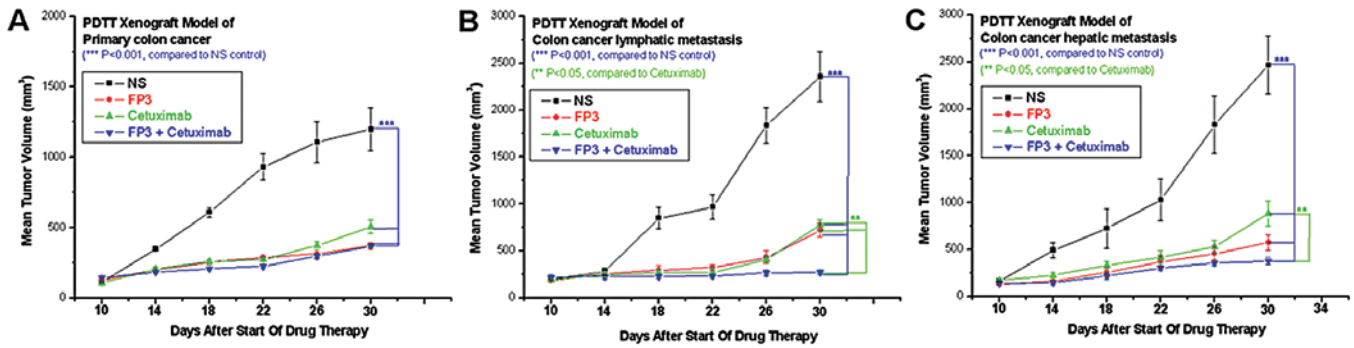


Figure 1. Response curves of FP3 and cetuximab in the PDDT xenograft models of (A) primary colon carcinoma, (B) lymphatic metastasis, and (C) hepatic metastasis. Ten mice per group were treated with the corresponding agent according to Materials and methods. Data shown are means \pm SEM. The differences between control tumor volumes, FP3-treated, cetuximab-treated, and FP3 and cetuximab-treated tumor volumes were analyzed using one-way ANOVA. *** $P<0.001$ vs. control; ** $P<0.05$ vs. cetuximab. Experiments were repeated at least two times with similar results.

room temperature with fluorescent (Cy3- or FITC-conjugated) secondary antibodies (goat anti-rat or goat anti-rabbit) diluted (1:200) in PBS. Specimens were rinsed again with PBS, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Tissue sections were examined and digitally photographed using a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with single, dual, and triple fluorescence filters and a low-light, externally cooled, three-chip charge-coupled device (CCD) camera (480x640 pixel RGB-color images, CoolCam; SciMeasure Analytical Systems, Atlanta, GA) and saved as TIFF files.

Western blotting. Protein expression profiles were analyzed by western blotting as previously described (15). Briefly, lysates for immunoblotting were prepared by adding lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 0.02% sodium azide, and 0.1% SDS] containing protease and phosphatase inhibitors (Sigma, St. Louis, MO) to the tumor tissue homogenized in fluid nitrogen. After centrifugation at 15,000 rpm at 4°C for 10 min, the supernatants were collected, and the protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Protein extracts of tumor lysates (30 μ g) were added to a loading buffer [10 mmol/l Tris-HCl (pH 6.8), 1% SDS, 25% glycerol, 0.1 mmol/l mercaptoethanol, and 0.03% bromophenol blue], boiled, and separated on 8% to 12% (w/v) polyacrylamide gels in the presence of SDS. Molecular weights of the immunoreactive proteins were estimated based on the relative migration with colored molecular weight protein markers (Amersham Pharmacia Biotech, Piscataway, NJ). After electrophoresis, the protein blots were electro-transferred to PVDF membranes (Millipore, Billerica, MA). Then, the membranes were blocked at room temperature with 5% nonfat milk in TBS [10 mmol/l Tris-HCl (pH 7.5), 0.5 mol/l NaCl, and 0.05% (v/v) Tween-20] buffer for 1 h. The primary antibodies were diluted at 1:1,000 and the membranes were incubated with primary antibodies overnight at 4°C. The antibodies used were anti-Akt, anti-ERK, anti-MAPK, anti-EGFR, and phosphorylation-specific antibodies against Akt (Ser³⁰⁸ and Ser⁴⁷³), ERK (Thr²⁰²/Tyr²⁰⁴) and MAPK (Thr¹⁸⁰/Tyr¹⁸²). The next day, the membranes were washed and incubated for 1 h at room temperature with rabbit immunoglobulin G-horseradish peroxidase-conjugated secondary

antibodies (Santa Cruz Biotechnology, Inc.), at a final dilution of 1:5,000. After washing thrice with TBS, antibody binding was visualized using enhanced chemiluminescence detection system (SuperSignal West Pico; Pierce Biotechnology, Inc.) as described by the manufacturer and autoradiography. To show equal protein loading, the blots were stripped and reprobed for GAPDH. This experiment was repeated three times with similar results.

Statistical analysis. Drug sensitivity data are presented as mean \pm SEM and analyzed by SPSS 16.0 software. Difference among means of the groups was determined with one-way ANOVA and were considered to be statistically significant if $P<0.05$.

Results

Antitumor activity of FP3 in combination with cetuximab.

It is a prerequisite to ascertain the molecular basis for the response to cetuximab in metastatic colon carcinoma. The mutation status of *KRAS* gene exon 1 at codons 12 and 13 in primary colon carcinoma and its lymphatic and hepatic metastases for establishment of the xenografts has been ascertained (15). A previous study revealed that the *KRAS* gene status in the three tumor sites were all wild-type (15). We firstly evaluated the antitumor activity of FP3 in combination with cetuximab in PDDT xenografts of primary colon carcinoma, lymphatic metastasis and hepatic metastasis. FP3 was administered i.v. at 15 mg/kg twice a week for 3 weeks and cetuximab was administered i.p. at 10 mg/kg twice/week. On Day 22 (21 days after the treatment was initiated), there were statistically significant differences in tumor volume between the control and the groups treated with cetuximab, FP3 and FP3 in combination with cetuximab ($P<0.001$) (Fig. 1) in all three xenograft models. No body weight-related toxicity was found in any group (data not shown). There were no statistically significant differences in tumor volume between the group treated with FP3 in combination with cetuximab and the groups treated with FP3 and cetuximab alone in xenografts of primary colon carcinoma (Fig. 1A). However, there were statistically significant differences in tumor volume between the group treated with FP3 in combination with cetuximab and the groups treated with cetuximab

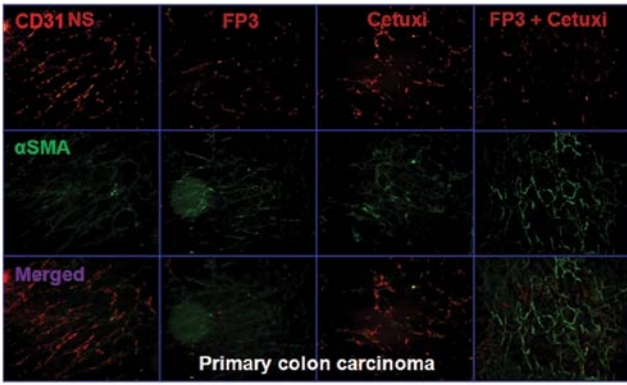


Figure 2. FP3 and FP3 in combination with cetuximab decreased vascular structure in the xenograft model of primary colon carcinoma. The vasculature was examined by angiography with immunostaining for endothelial cells (using anti-CD31 antibody; bar, 100 μ m), and pericytes (using anti- α -SMA antibody; bar, 100 μ m). Cetuxi, cetuximab.

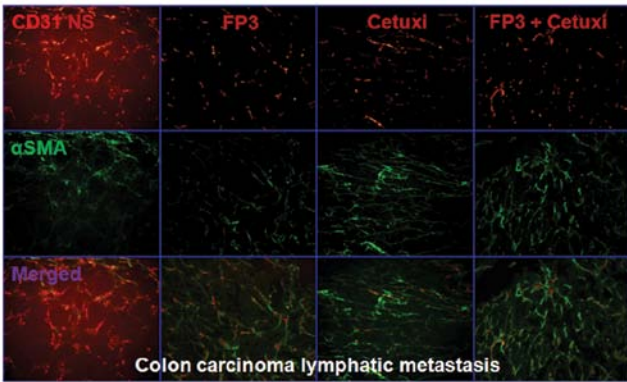


Figure 3. FP3 and FP3 in combination with cetuximab decreased vascular structure in the xenograft model of colon carcinoma lymphatic metastasis. The vasculature was examined by angiography with immunostaining for endothelial cells (using anti-CD31 antibody; bar, 100 μ m), and pericytes (using anti- α -SMA antibody; bar, 100 μ m). Cetuxi, cetuximab.

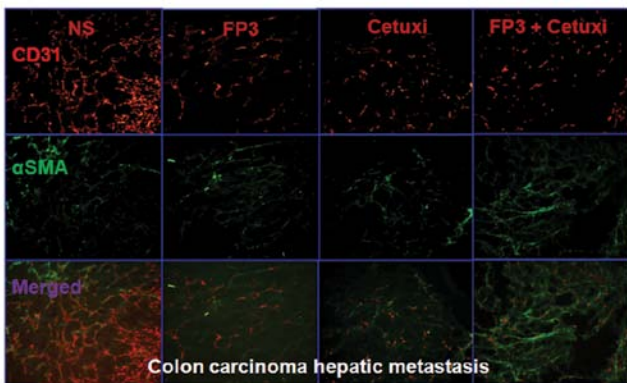


Figure 4. FP3 and FP3 in combination with cetuximab decreased vascular structure in the xenograft model of colon carcinoma hepatic metastasis. The vasculature was examined by angiography with immunostaining for endothelial cells (using anti-CD31 antibody; bar, 100 μ m), and pericytes (using anti- α -SMA antibody; bar, 100 μ m). Cetuxi, cetuximab.

alone ($P < 0.05$) (Fig. 1B) in xenografts of colon carcinoma lymphatic metastasis. There were also statistically significant differences in tumor volume between the group treated with

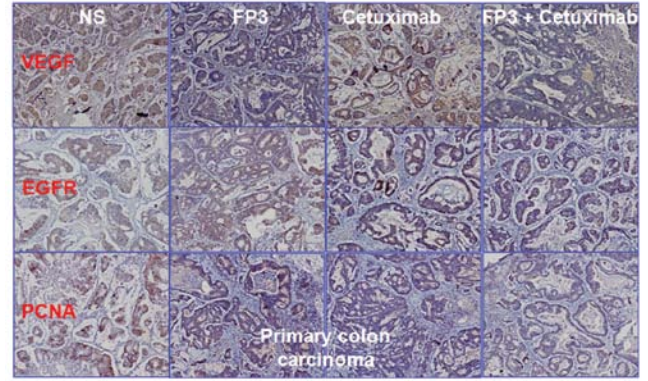


Figure 5. Effects of FP3 and FP3 in combination with cetuximab on the expression of VEGF, EGFR and PCNA in the PDTT xenograft models of primary colon carcinoma. Original magnification, x100.

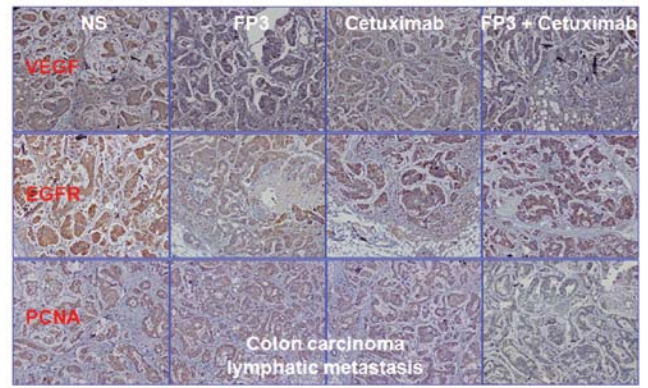


Figure 6. Effects of FP3 and FP3 in combination with cetuximab on the expression of VEGF, EGFR and PCNA in the PDTT xenograft models of colon carcinoma lymphatic metastasis. Original magnification, x100.

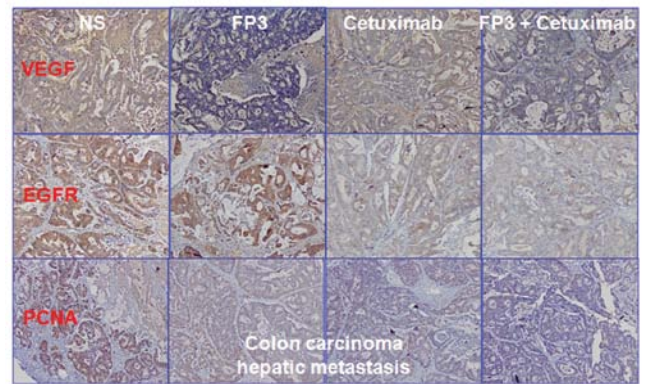


Figure 7. Effects of FP3 and FP3 in combination with cetuximab on the expression of VEGF, EGFR and PCNA in the PDTT xenograft models of colon carcinoma hepatic metastasis. Original magnification, x100.

FP3 in combination with cetuximab and the groups treated with cetuximab alone ($P < 0.05$) (Fig. 1C) in xenografts of colon carcinoma hepatic metastasis.

Effect of FP3 in combination with cetuximab on tumor-associated angiogenesis. To evaluate the effects of FP3 on tumor-associated angiogenesis in PDTT xenografts of primary colon carcinoma, lymphatic metastasis and hepatic metastasis,

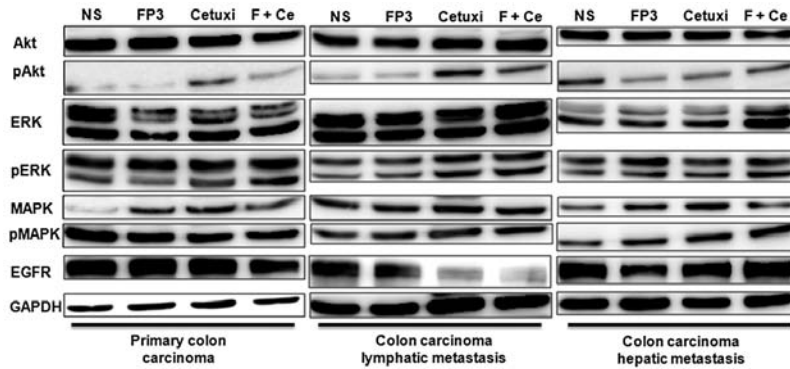


Figure 8. Immunoblotting data of the proteins Akt, pAkt (Ser³⁰⁸ and Ser⁴⁷³), ERK, pERK (Thr²⁰²/Tyr²⁰⁴), MAPK, pMAPK (Thr¹⁸⁰/Tyr¹⁸²), EGFR and GAPDH (as a loading control) showing the effects of FP3 and FP3 in combination with cetuximab on the protein expression in the PDTT xenograft models of primary colon carcinoma and its corresponding lymphatic and hepatic metastases. Cetuxi, cetuximab; F, FP3; Ce, cetuximab.

selected tumors were sectioned and immunostained with antibodies to CD31 and α -SMA, so that the vasculature could be visualized (Figs. 2-4). This analysis revealed that the vasculature was nearly absent in FP3-treated and FP3 in combination with cetuximab-treated xenografts. FP3 and FP3 in combination with cetuximab (treatment for 21 days) almost completely blocked tumor-associated angiogenesis, with the stunted tumors being largely avascular (Figs. 2-4). In contrast to the FP3-treated and FP3 in combination with cetuximab-treated tumors, tumors in saline-treated mice and cetuximab-treated mice not only were much larger but also had a very high vascular density (Figs. 2-4). These results indicate that FP3 administration reduces xenograft size and concurrently causes decreased microvessel growth, and the antiangiogenic effects were mainly induced by FP3 not by cetuximab.

Effect of FP3 in combination with cetuximab on tumor cell proliferation. We next measured cell proliferation in the treated tumors of PDTT xenografts of primary colon carcinoma, lymphatic metastasis and hepatic metastasis. By immunohistochemical staining, we found that VEGF expressions in FP3-treated and FP3 in combination with cetuximab-treated tumors were significantly suppressed (Figs. 5-7). We also found that EGFR expressions in cetuximab-treated and FP3 in combination with cetuximab-treated tumors were significantly suppressed (Figs. 5-7). PCNA expressions in FP3-treated, cetuximab-treated, and FP3 in combination with cetuximab-treated tumors were significantly suppressed (Figs. 5-7). We also measured the levels of related cell signaling pathway proteins by using western blotting. However, we found that the expression levels of related cell signaling pathway proteins such as Akt, pAkt, ERK, pMAPK in treated tumor tissues of three xenograft models were different (Fig. 8).

Discussion

The primary purpose of this study was to determine the effects of selective blockade of the VEGF and EGFR on tumor angiogenesis and growth in the PDTT xenografts of primary colon carcinoma, lymphatic and hepatic metastases. A secondary goal was to assess whether combination therapy with FP3 and cetuximab would demonstrate additive or synergistic effects on these variables.

Therapies directed against VEGF and EGFR have shown their clinical benefit in the treatment of cancer (1-8). VEGF is a multifunctional cytokine that increases microvascular permeability and directly stimulates endothelial cell growth and angiogenesis (18). VEGF is a potent and specific mitogen for endothelial cells, activates the angiogenic switch *in vivo*, and enhances vascular permeability (19). Enhanced expression of VEGF has been observed in human cancer cell lines and in cancer patients with different malignancies including colorectal, breast, non-small cell lung, and ovarian cancers and is directly correlated with increased neovascularization, as measured by microvessel density within the tumor (19). The increasing understanding of the biological mechanisms of tumor-induced angiogenesis has stimulated the development of agents able to interfere with the molecules involved in this process (9,20-22).

EGFR is a prominent member of the HER growth factor receptor family. Signaling through this receptor activates a cascade that leads to proliferation, migration, survival signals, and tissue remodeling. The EGFR is overexpressed in a variety of cancers. Overexpression may range from 10 to 80% in cancer. Its expression has been associated with poor survival (23). On the basis of the current knowledge of tumor biology, there is a rationale to combine targeted therapies that block different growth factor pathways. Preclinical studies show that inhibition of both EGFR and VEGF pathways produces additive or synergistic antitumor effects (24,25).

In our previous study, we successfully established the PDTT xenograft model of colon carcinoma with lymphatic and hepatic metastases (15). The ideal biological characteristics of such PDTT xenograft models as described previously (15) made us postulate that these PDTT models could be used to ascertain the antitumor activity of FP3 in primary colon carcinoma and its corresponding lymphatic and hepatic metastases. In the present study, we demonstrated the antitumor activity of FP3 in combination with cetuximab in the PDTT xenograft models of primary colon cancer and its corresponding lymphatic and hepatic metastases. The antitumor activity of FP3 in combination with cetuximab was significantly higher than that of cetuximab monotherapy in three xenograft models. In addition, we investigated the mechanisms of the combination effects of FP3 and cetuximab in the three xenograft models.

FP3 is a humanized fusion protein which combines ligand binding elements taken from the extracellular domains

of VEGF receptors 1 and 2 with the Fc portion of IgG1. It is aimed to bind to all forms of VEGF-A (15). As in many previous studies (10-15), the present study also demonstrated that FP3 decreased microvessel density in the tumor tissues of the xenograft models. In tumors expressing VEGF, tumor growth would be more dependent on angiogenesis regulated by VEGF. Cetuximab is a humanized chimeric mouse monoclonal antibody directed against the EGFR. EGFR activates a cascade that leads to proliferation, migration, survival signals, and tissue remodeling. Therefore, we investigated the levels of VEGF and EGFR after treatment with FP3 and cetuximab to possibly explain the mechanisms of the effects of FP3 and cetuximab in combination. However, no significant decrease in tumor VEGF was demonstrated after treatment with cetuximab and no significant decrease in tumor EGFR was demonstrated after treatment with FP3, suggesting that the combination effects are a result of mechanisms other than an alteration in the levels of VEGF in tumors treated with cetuximab or in the levels of EGFR in tumors treated with FP3.

The unregulated nature of tumor angiogenesis leads to the production of structurally and functionally abnormal vasculature, characterized by a number of different features, such as increased vessel density, diameter, length and tortuosity, abnormally high interstitial fluid pressure, and increased vascular permeability (26,27). These abnormalities prevent the effective delivery of therapy to the tumor. For example, the entry of large molecules, such as chemotherapeutic agents, into the tumor would be impeded and hypoxia results from inconsistent oxygen supply within the tumor, producing regions that would be resistant to radiotherapy and some cytotoxic agents.

The common belief that antiangiogenic therapy eradicates tumor vasculature, thus depriving the tumor of oxygen and nutrients necessary for survival was now challenged by some studies, which suggested that treatment with antiangiogenic agents can transiently reverse some of the abnormalities of tumor vessels or 'normalize' the tumor vasculature for a short period of time, thereby providing a window of opportunity for improving drug delivery and enhancing sensitivity to conventional chemotherapy and radiation treatment (22,28-31). Based on the results achieved previously and in the present study, it is hypothesized that FP3 may also have the potential to 'normalize' the tumor vasculature which is unique to this class of agents. The potential vessel normalization effect of FP3 in solid tumors may help make tumor cells more sensitive to cytotoxic chemotherapy, radiotherapy, and maximize the effectiveness of the overall cancer treatment strategy. In terms of clinical significance, normalization of the tumor vasculature by FP3 may also maximize efficacy of concomitant therapy.

In conclusion, this study has demonstrated that FP3 can increase the efficacy of cetuximab in the PDTT xenografts of colon carcinoma lymphatic and hepatic metastases. Based on these results, the combination of FP3 and cetuximab merit further clinical investigation in patients with metastatic colon carcinoma.

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