Epidermal Growth Factor Receptor Blockade with C225 Plus Gemcitabine Results in Regression of Human Pancreatic Carcinoma Growing Orthotopically in Nude Mice by Antiangiogenic Mechanisms


ABSTRACT

Both epidermal growth factor receptor (EGF-R) signaling mechanisms and angiogenesis have been evaluated as independent targets for therapy of human pancreatic carcinoma, but a link between the two processes has been identified only recently. This study evaluated whether EGF-R blockade therapy with anti-EGF-R antibody C225 inhibits pancreatic carcinoma growth and metastasis in an orthotopic nude mouse model via tumor-mediated angiogenesis and whether gemcitabine potentiates this effect. In vitro treatment of human pancreatic carcinoma L3.6pl cells with C225 inhibited EGF-R autophosphorylation, producing a maximum of 20% cytostasis. Treatment with C225 plus gemcitabine resulted in additive cytotoxic effects that increased with increasing gemcitabine concentrations. Dose-dependent decreases in expression of the angiogenic factors vascular endothelial growth factor and interleukin 8 were observed in the C225-treated cells (mRNA and protein levels). In L3.6pl tumors established in the pancreas of nude mice, systemic therapy with C225 alone and C225 in combination with gemcitabine resulted in growth inhibition, tumor regression, and abrogation of metastasis; median tumor volume was reduced from 538 to 0.3 and to 0 mm³, respectively. Gemcitabine treatment alone reduced median tumor volume from 538 to 152 mm³. Liver metastases were present in 50% of the controls, 30% of the gemcitabine-treated animals, and 20% of C225-treated animals. No macroscopically visible liver metastases were observed in the combination treatment group. As early as 11 days after C225 treatment, the median percentage of proliferating cell nuclear antigen-positive cells was substantially reduced compared with gemcitabine treatment alone (26% versus 73%, respectively) versus controls (92%), correlating with in vivo blockade of EGF-R activation. Similarly after 11 days treatment, production of vascular endothelial growth factor and interleukin 8 was significantly lower in C225 and C225 plus gemcitabine-treated tumors versus gemcitabine-treated and control tumors. Significant differences in microvessel density were observed 18 days after C225 or combination treatments (but not gemcitabine alone) in direct correlation with the difference in percentage of apoptotic endothelial cells, as visualized by double immunofluorescence microscopy. These experiments indicate that therapeutic strategies targeting EGF-R have a significant antitumor effect on human L3.6pl pancreatic carcinoma growing in nude mice which is mediated in part by inhibition of tumor-induced angiogenesis, leading to tumor cell apoptosis and regression. Furthermore, this effect is potentiated in combination with gemcitabine.

INTRODUCTION

Cancer of the exocrine pancreas continues to be a major unsolved health problem, its incidence rates being virtually identical to mortality rates (1–5). It is estimated that only 1–4% of patients with adenocarcinoma of the pancreas will be alive 5 years after diagnosis (1). This is attributable in large part to difficulties in diagnosis, the aggressiveness of pancreatic cancers, early metastasis, and the lack of effective systemic therapies (2–4). Pancreatic cancer metastasizes early to regional lymph nodes, and subclinical liver metastases are present in the majority of patients at diagnosis (1–4).

Single agents or combination chemotherapy have little reproducible impact on patient survival or quality of life in advanced pancreatic adenocarcinoma (1–4, 6). Recently, gemcitabine (2’,2’-difluorodeoxycytidine), a deoxycytidine analogue (5–8), demonstrated modest improvements in response rate, and median survival in patients with advanced pancreatic cancer was similar to those treated with 5-fluorouracil (1–5, 7, 8). However, despite these results, the median survival for
patients with metastatic disease continues to be <6 months. Although the development of alternative gemcitabine schedules and chemotherapy combinations continues (6, 9), study of novel targets and strategies are required to build on the results with gemcitabine.

Among the potential targets are angiogenesis and EGF-R3-mediated proliferation, both of which are fundamental to the development and progression of human pancreatic carcinoma. Each has been independently evaluated as a target for therapy, but the link between the two, involving EGF-R effects on angiogenesis, has been identified only recently (10–12). Human pancreatic cancers overexpress EGF-R (13, 14), and binding of its ligands, EGF, AR, or TGF-α, produced in an autocrine manner, can activate signal transduction pathways that regulate cell proliferation and anchorage-independent growth (14–21). A statistically significant decrease in survival was observed in pancreatic cancer patients overexpressing the EGF-R and at least one of its ligands (18). These data indicate that overexpression of the EGF-R and its ligands contributes to the malignant phenotype in this disease.

A crucial step in the process of pancreatic tumor progression is the production of neovascularization in and around the tumor (22–24). Under homeostasis, the microvasculature is maintained in a quiescent state with acquisition of the angiogenic phenotype in solid tumors dependent on the net outcome of stimulatory and inhibitory factors by the tumor and its microenvironment (22–24). These factors have not been fully elucidated for pancreatic cancer; however, VEGF, IL-8, and bFGF are expressed by human pancreatic carcinoma cells (25–27). After vascular induction by these factors among others, the rate of tumor growth increases exponentially (22–25). Interestingly, two of the most potent inducers of VEGF, a major angiogenesis stimulator for human cancers (28), are EGF and TGF-α (29–31). Recent data from our laboratory also suggests that therapy with either EGF-R protein tyrosine kinase inhibitors (32) or C225 (12), an anti-EGF-R antibody, inhibits human bladder carcinoma growth and metastasis by mechanisms affecting tumor-induced neovascularization secondary to the down-regulation of tumor cell-produced angiogenic factors including VEGF. This effect is not specific to one tumor type (10, 11).

The purpose of the present study was to evaluate whether down-regulating EGF-R signaling pathways by the anti-EGF-R antibody C225 inhibits pancreatic tumor growth and metastasis and whether this effect is potentiated when used in combination with gemcitabine. In our study, C225 treatment resulted in growth inhibition, tumor regression, and abrogation of metastasis. C225 treatment suppressed tumor VEGF and IL-8 production, both in cell culture and in tumors growing orthotopically in nude mice. The down-regulation of these angiogenic factors preceded the involution of blood vessels, as shown by double immunofluorescence microscopy for apoptotic endothelial cells, suggesting a cause and effect. Furthermore, this effect is potentiated in combination with gemcitabine.

MATERIALS AND METHODS

Cells and in Vitro Culture Conditions. L3.6pl human pancreatic cancer cells were established from COLO 357 fast-growing cells by injecting them into the pancreas of nude mice; hepatic metastases were harvested and reinjected into the pancreas. This cycle was repeated three times. The resulting L3.6pl cells produced significantly higher incidence and number of lymph node and liver metastases than parental cells (26). Cells were maintained on plastic in DMEM supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, l-glutamine, and 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), incubated in 5% CO₂–95% air at 37°C. The cultures were free of Mycoplasma and pathogenic murine viruses (as assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 8 weeks after recovery from frozen stocks.

Animals and Orthotopic Implantation of Tumor Cells. Male athymic nude mice (NCr-nu) were obtained from the animal production area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and NIH, and their use in these experiments was approved by the Institutional Animals Care and Use Committee. The mice were used when they were 8–10 weeks of age.

To produce tumors, tumor cells were harvested from subconfluent cultures by treatment with 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 single-cell suspensions with >90% viability were used for injections. Orthotopic tumor cell injections were performed as described (26). The mice were killed 5–6 weeks thereafter, and the size and weight of the primary tumors, the incidence of regional (celiac and paraaortic) lymph node metastasis, and the number of liver nodules were determined. Histopathology confirmed the nature of the disease.

Therapy of Established Human Pancreatic Carcinoma Tumors Growing in the Pancreas of Athymic Nude Mice. Treatment was initiated 7 days after tumor cell injection, when tumors were palpable. Five animals sacrificed at this time point had a median tumor volume of 18 mm³ (range, 16–18 mm³). To evaluate the therapeutic effect of gemcitabine alone in this model, an initial in vivo dose-response experiment was performed. Five mice/group were treated biweekly with 500, 250, 125, 62, 31, 15.5, and 7.5 mg/kg gemcitabine by i.p. administration, resulting in 65, 58, 55, 46, 36, 31, and 0.2% growth inhibition of L3.6pl tumors, respectively, compared with controls. For the studies herein, a dose of 250 mg/kg gemcitabine

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3 The abbreviations used are: EGF-R, epidermal growth factor receptor; AR, amphiregulin; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; IL, interleukin; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; Mab, monoclonal antibody; MVD, microvessel density; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PECAM, platelet/endothelial cell adhesion molecule; DNA-PK, DNA-dependent protein kinase.
was used. Mice were randomly separated into four groups (16 animals/group): C225, biweekly treatment with 1 mg/injection; gemcitabine, biweekly treatment 250 mg/kg/injection; C225 plus gemcitabine, C225 administration on Tuesday and Friday and gemcitabine administration on Wednesday and Saturday; and control, an equivalent volume of 0.9% NaCl on the same schedule as the combination therapy group. All groups received treatment by i.p. injection. Treated mice were closely monitored for any signs of progressive disease and sacrificed if they became moribund. Mice in all groups were sacrificed 32 days after tumor cell implantation. To evaluate the temporal effect of these treatments on different biological parameters, two mice/group were sacrificed 11, 18, and 30 days after initiation of therapy. The experiment was repeated using gemcitabine (125 mg/kg) alone and in combination with C225, with similar results (data not shown).

**In Vitro Effects of C225 Alone and in Combination with Gemcitabine.** Tumor cell cytostasis was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium assay as described (12, 32–34).

**ELISA.** To evaluate VEGF and IL-8 protein expression after treatment with C225, 2000 cells/38-mm² well were plated in 96-well microtiter plates in 0.2 ml of supplemented DMEM, 95, and 80% ethanol/double-distilled H₂O (v/v) and rehydrated in PBS (pH 7.5). Sections analyzed for PCNA were microwaved 5 min for “antigen retrieval” (36). All other paraffin-embedded tissues were treated with pepsin (Biomed, Foster City, CA) for 15 min at 37°C and washed with PBS. Frozen tissues used for identification of CD31/PECAM-1 and activated EGF-R were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were fixed in cold acetone (5 min), acetone/chloroform (v/v; 5 min), and acetone (5 min), and washed with PBS.

All samples were incubated with 3% hydrogen peroxide in methanol (v/v) for 12 min to block endogenous peroxidase, washed with PBS (pH 7.5), and incubated in protein blocking solution [5% normal human serum/0.5% normal goat serum in PBS (v/v)] for 20 min. Sections analyzed for activated EGF-R were pretreated with goat antirat IgG F(ab)₂ fragment (1:10 dilution in PBS) for 4–6 h. The sections were incubated with the primary antibody in a humidified chamber for 15–18 h at 4°C, rinsed with PBS, and incubated in protein blocking solution for 10 min. Sections were then incubated for 60 min with the corresponding peroxidase-conjugated secondary antibody [1:200 (v/v)] for 1 h at ambient temperature. Positive reaction was visualized by incubating the slides with stable 3,3′-diaminobenzidine for 10–20 min (Research Genetics, Huntsville, AL) or 3-amino-9-ethylcarbazole (Biogenex Laboratories, San Ramon, CA) after CD31 staining. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 min (Sigma Chemical Co., St. Louis, MO), and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining. The positive reaction after staining for activated EGF-R was enhanced with osmium tetroxide [4% aqueous solution (v/v); Electron Microscopy Sciences, Fort Washington, PA] at a 1:100 dilution in double-distilled H₂O after incubation with 3,3′-diaminobenzidine.

**Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptotic Cells).** Frozen tissue sections fixed and treated as above were incubated for 18 h with rat monoclonal anti-CD31 antibody (1:100) at 4°C, rinsed with PBS, and incubated with goat antirat IgG conjugated to Texas Red (1:200; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) at a 1:100 dilution in double-distilled H₂O after incubation with 3,3′-diaminobenzidine.

**Immunohistochemical Determination of VEGF, IL-8, PCNA, CD31/PECAM-1, and EGF-R.** Paraffin-embedded tissues were used for identification of VEGF, IL-8, EGF-R, and PCNA. Four to six-μm-thick sections were mounted on positively charged Superfrost slides (Fisher Scientific, Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylen, followed by treatment with a graded series of alcohol [100, 95, and 80% ethanol/double-distilled H₂O (v/v)] and rehydrated in PBS (pH 7.5). Sections analyzed for PCNA were microwaved 5 min for “antigen retrieval” (36). All other paraffin-embedded tissues were treated with pepsin (Biomed, Foster City, CA) for 15 min at 37°C and washed with PBS. Frozen tissues used for identification of CD31/PECAM-1 and activated EGF-R were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were fixed in cold acetone (5 min), acetone/chloroform (v/v; 5 min), and acetone (5 min), and washed with PBS.

All samples were incubated with 3% hydrogen peroxide in methanol (v/v) for 12 min to block endogenous peroxidase, washed with PBS (pH 7.5), and incubated in protein blocking solution [5% normal human serum/0.5% normal goat serum in PBS (v/v)] for 20 min. Sections analyzed for activated EGF-R were pretreated with goat antirat IgG F(ab)₂ fragment (1:10 dilution in PBS) for 4–6 h. The sections were incubated with the primary antibody in a humidified chamber for 15–18 h at 4°C, rinsed with PBS, and incubated in protein blocking solution for 10 min. Sections were then incubated for 60 min with the corresponding peroxidase-conjugated secondary antibody [1:200 (v/v)] for 1 h at ambient temperature. Positive reaction was visualized by incubating the slides with stable 3,3′-diaminobenzidine for 10–20 min (Research Genetics, Huntsville, AL) or 3-amino-9-ethylcarbazole (Biogenex Laboratories, San Ramon, CA) after CD31 staining. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 min (Sigma Chemical Co., St. Louis, MO), and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining. The positive reaction after staining for activated EGF-R was enhanced with osmium tetroxide [4% aqueous solution (v/v); Electron Microscopy Sciences, Fort Washington, PA] at a 1:100 dilution in double-distilled H₂O after incubation with 3,3′-diaminobenzidine.

**Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptotic Cells).** Frozen tissue sections fixed and treated as above were incubated for 18 h with mouse monoclonal anti-CD31 antibody (1:100) at 4°C, rinsed with PBS, and incubated with goat antirat IgG conjugated to Texas Red (1:200; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) for 60 min at ambient temperature in the dark. After sections were washed with PBS containing 0.1% Brij (v/v), TUNEL was performed using a commercial kit according to the manufacturer’s instructions with the following modifications (Promega Corp., Madison, WI). Samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at ambient temperature, washed with PBS, and then permeabilized by incubating with 0.2% Triton X-100 in PBS (v/v) for 15 min. The samples were incubated with equilibration buffer (from the kit); it was drained off, and reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase was added to the sections and incubated in a humidified chamber for 1 h at 37°C in the dark. The reaction was terminated by immersing the samples in 2× SSC (SSC: 30 mM NaCl/3 mM sodium citrate, pH 7.2) for 15 min, followed by three washes to remove unincorporated fluorescein-dUTP. Background reactivity was determined by processing slides in the absence of terminal deoxynucleotidyl transferase (negative control). Maximum reactivity was observed by
preincubating the tissue sections with DNase I and served to confirm the quality of the specimen.

Nuclei were stained with Hoechst Dye 3342 (MW, 615.9; 300 μg/ml). Fluorescent bleaching was minimized by treating slides with an enhancing reagent (Prolong; Molecular Probes, Eugene, OR). Immunofluorescence microscopy was performed using a ×40 objective (Zeiss Plan-Neofluar) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a cooled CCD camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom) on a Macintosh computer. Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells. Apoptotic endothelial cells were quantitated and expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm² fields at ×400. For total TUNEL expression, apoptotic events were quantified in 10 random 0.159-mm² fields at ×100 and divided by the total number of cells/field.

Quantification of MVD, PCNA, TUNEL, and Absorbance. For the quantification of MVD, 10 random 0.159-mm² fields at ×100 were captured for each tumor using a Sony three-chip camera (Sony Corporation of America, Montvale, NJ) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas Image Analysis software (Bio- scan, Edmond, WA) installed in a Compaq computer with Pentium chip, a frame grabber, an optical disc storage system, and a Sony color printer. Microvessels were quantified as described (12, 37).

For the quantification of the immunohistochemical reaction intensity, the absorbance of 100 VEGF- and IL-8-positive tumor cells in 10 random 0.039-mm² fields at ×200 of the differently treated tumor tissues was measured using the Optimas Image Analysis software (12, 26, 27). The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the immunohistochemical reaction. VEGF and IL-8 cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and was expressed as a ratio of tumor cell expression to normal pancreatic gland expression relative to the control (26, 27). For the quantification of PCNA expression, the number of positive tumor cells was quantitated in 10 random 0.159-mm² fields at ×100 and divided by the total number of cells/field.

Reagents. All antibodies were purchased from commercial sources as listed: rabbit anti-VEGF/VPF (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit antihuman IL-8 (1:25; Biosource International, Camarillo CA), rat antimouse IgG1 (PharMingen, San Diego, CA), mouse anti-PCNA clone PC 10 (1:50; Dako A/S, Copenhagen, Denmark), mouse antihuman EGF-R IgG1 (activated form; 1:25; Chemicon, Temecula, CA), which reacts specifically with the activated and phosphorylated human EGF-R and does not react with other phosphorylated proteins (32, 35, 38–40), monoclonal mouse antihuman IgG1 EGF-R clone 30 (1:80; Biogenex, San Ramon, CA), peroxidase-conjugated F(ab′)2 goat antirabbit IgG F(ab′)2 fragment, peroxidase-conjugated goat antimouse IgM F(ab′)2 fragment, AffiniPure Fab fragment goat antiamouse IgG (H+L), peroxidase-conjugated goat antirat IgG (H+L), and Texas Red-conjugated goat antirat IgG (1:200; Jackson Research Laboratories, West Grove, CA), peroxidase-conjugated rat antimouse IgG2a (1:75; Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN), monoclonal anti-phosphotyrosine MAb 4G10 (1:2000) and polyclonal sheep antihuman EGF-R (1:700; Upstate Biotechnology, Lake Placid, NY), peroxidase-conjugated donkey antiperoxidase IgG and human IgG (Sigma Immunochensicals), and Hoechst Dye 3342 5, 615.9 (300 μg/ml; Hoechst, Warrington, PA). Chimeric anti-EGF-R Mab C225 was provided by ImClone Systems, Inc. Gemicitabine (HCl, 2′,2′-difluoro- deoxyoxycytidine) was purchased from Eli Lilly & Company (Indianapolis, IN).

Statistical Analysis. Pancreatic tumor weights, expression intensities of VEGF and IL-8, and quantification of the number of PCNA, TUNEL, CD31, and apoptotic tumor and endothelial cells were compared by unpaired Student’s t test.

RESULTS
C225 Inhibits EGF-induced Autophosphorylation of EGF-R in L3.6pl Human Pancreatic Carcinoma Cells in Vitro. We first determined whether C225 inhibits EGF-stimulated tyrosine phosphorylation of the EGF-R in L3.6pl human pancreatic carcinoma cells. L3.6pl cells growing in vitro under serum-free conditions in the presence of EGF for 15 min exhibited high levels of autophosphorylated EGF-R (MW, 170,000 band) as detected using anti-phosphotyrosine antisera on Western blots of anti-EGF-R immunoprecipitated cell lysates (Fig. 1). Pretreatment of the cells with C225, specific for the EGF-R, for 1 h followed by treatment with EGF (15 min) abrogated this effect in a dose-dependent manner (1–10 μg/ml; Fig. 1). The M

![Fig. 1 Inhibition of EGF-induced autophosphorylation of EGF-R in L3.6pl human pancreatic cancer cells by C225. Tyrosine phosphorylation induced in L3.6pl cells by 40 ng/ml EGF for 10 min was inhibited by C225 in a dose-dependent manner. L3.6pl pancreatic carcinoma cells growing in vitro under serum-free conditions were stimulated for 10 min with EGF (40 ng/ml) in the presence or absence of C225 (0–10 μg/ml). Cells were washed and lysed, and insoluble proteins in the lysate were immunoprecipitated with an anti-EGF-R Mab, separated by 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and sequentially probed with antiserum specific to phosphotyrosine and EGF-R (see “Materials and Methods”). The immunoreactive proteins were detected by incubating the blot with the corresponding peroxidase-conjugated IgG and visualized using the ECL system. Densitometric quantitation of the ratio of the areas between the MW, 170,000 phosphotyrosine- and the MW, 170,000 EGF-R-specific bands were compared in each case with the untreated controls, arbitrarily assigned the value of 1.0.

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170,000 band was confirmed as EGF-R by Western blot analysis using independent anti-EGF-R antisera (Fig. 1). Northern blot analyses demonstrated high levels EGF-R-specific transcripts in L3.6pl cells that did not vary in response to treatment with either EGF or C225 treatment (data not shown).

**In Vitro Antiproliferative Effect of C225 Treatment Alone and in Combination with Gemcitabine on L3.6pl Cells.** We then determined whether the treatment of L3.6pl cells with C225 alone or in combination with gemcitabine would inhibit cell proliferation *in vitro*. At concentrations ranging from 1 to 12.5 μg/ml, C225 produced minimal alterations in cellular metabolic activity compared with untreated controls (Fig. 2A). The maximum inhibition observed was 20% after 72 h of continuous exposure of 12.5 μg/ml in either 1 or 5% serum-containing medium. The combination of C225 (2.5 μg/ml) and gemcitabine (0–64 ng/ml) produced an additive cytotoxic effect at gemcitabine concentrations below IC_{50} (Fig. 2B). No synergistic effects were observed *in vitro*.

**In Vivo Inhibition of Growth and Metastasis of Established Human Pancreatic Carcinoma in the Pancreas of Athymic Nude Mice.** Seven days after implantation of L3.6pl cells into the pancreas, groups of nude mice were randomized to receive treatment with saline (control), gemcitabine (250 mg/kg), C225 (1 mg), or C225 plus gemcitabine. Biweekly i.p. treatment for 4 weeks did not produce cumulative toxicity or change in body weight but did significantly reduce tumor incidence and metastasis compared with control animals (Table 1). The incidence of macroscopically visible pancreatic tumors was 0 of 10 and 0 of 9 in the C225 and C225 plus gemcitabine treatment groups, respectively, compared with 10 of 10 in the gemcitabine-treated and control groups (Table 1). Upon histological examination, three of nine and three of five macroscopic tumor-free animals in the C225 plus gemcitabine and C225 treatment groups, respectively, showed microscopic disease (Fig. 3; see H&E). All mice in the gemcitabine and control treatment groups showed progressive disease. Independent experiments using gemcitabine at 125 mg/kg demonstrated similar results (see “Materials and Methods”). The observed differences in pancreatic tumor weights and median tumor volume in the C225 and C225 plus gemcitabine groups suggest regression because the treated tumors were smaller than those when therapy was initiated (see “Materials and Methods”).

Treatments also reduced lymph node and liver metastases in treated compared with control animals (Table 1). No macroscopic visible liver metastases were observed in the C225 plus gemcitabine treatment group (Table 1).

**Analysis of Total EGF-R and Activated EGF-R in L3.6pl Pancreatic Tumor Sections of Treated versus Control Mice.** We next determined the EGF-R content and level of activated EGF-R (tyrosine phosphorylated form) in L3.6pl tumors growing in the pancreas of nude mice after therapy with saline (control), gemcitabine, C225, or C225 plus gemcitabine. Immunohistochemical analysis for the activated and tyrosine phosphorylated EGF-R (32, 35, 38–40) in L3.6pl tumors growing in the pancreas of nude mice demonstrated specific immunoreactivity both peripherally and centrally in tumors from control and gemcitabine treated mice, whereas immunoreactivity was significantly reduced in C225 and C225 plus gemcitabine-treated tumors (Fig. 3, *act. EGF-R*). In contrast, the identical tumors showed similar levels of immunoreactivity for total EGF-R (does not distinguish activated from inactivated) regardless of treatment group (Fig. 3, *EGF-R*). A431 epidermoid carcinoma, known not only to overexpress EGF-R but also to exhibit a high degree of EGF-R autophosphorylation (32, 35), demonstrated high immunoreactivity for both total and activated EGF-R (data not shown). These results suggest C225 or combination treatments inhibit EGF-R function.

**In Vivo Inhibition of Proliferation (PCNA) and Increase of Apoptosis (TUNEL) after C225 Blockade of EGF-R.** We next analyzed the effect of C225 treatment alone and in combination with gemcitabine on tumor cell proliferation *in vivo*.
Tumor cell proliferation was reduced as measured by the percentage of PCNA positive versus total cells (per microscopic field) in each tumor section 11, 18, and 30 days after initiation of C225 or C225 plus gemcitabine treatments (Fig. 4; Table 2). A significant difference was also observed in tumors (18 days after therapy) from mice treated with C225 (34%) or C225 plus gemcitabine (7%) compared with gemcitabine alone or control tumors (69 or 94%, respectively; Fig. 4; Table 2). These results indicate an antiproliferative effect of C225 treatment alone that is further augmented in combination with gemcitabine (P < 0.008).

Determination of tumor cell apoptosis as measured by the percentage of TUNEL-positive versus total number of cells (per microscopic field) demonstrated a significant increase in the

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**Table 1** Therapy of L3.6pl pancreatic tumors in nude mice with C225 and gemcitabine

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Incidence of macroscopic tumors</th>
<th>Total pancreas weight</th>
<th>Median tumor volume</th>
<th>Median body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pancreas tumor</td>
<td>Liver metastasis</td>
<td>Regional LN metastasis</td>
<td>mg (range)</td>
</tr>
<tr>
<td>Control</td>
<td>10/10</td>
<td>5/10</td>
<td>10/10</td>
<td>923 (660–1371)</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>10/10</td>
<td>3/10</td>
<td>6/10</td>
<td>297 (205–485)</td>
</tr>
<tr>
<td>C225</td>
<td>5/10</td>
<td>2/10</td>
<td>8/10</td>
<td>119 (97–157)</td>
</tr>
<tr>
<td>C225 + gemcitabine</td>
<td>0/9</td>
<td>0/9</td>
<td>1/9</td>
<td>130 (93–173)</td>
</tr>
</tbody>
</table>

* L3.6pl cells (1 × 10⁶) were implanted into the pancreas. Therapy began on day 7, when median tumor size was 18 mm³. C225 (1 mg), Gemcitabine (250 mg/kg), alone or in combination, was injected i.p. biweekly for 4 weeks. Control mice received saline. Tumor weight and metastases were evaluated at necropsy at day 32. One experiment of two is shown.

† Number of tumor-positive mice per number of mice receiving injections.

‡ Number of mice with visible nodules (>1 mm in diameter) per number of mice receiving injections.

§ Number of mice with enlarged regional lymph nodes (LN) per number of mice receiving injections.

¶ P < 0.001 (unpaired Student’s t test) control versus all three therapy groups.

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| **Fig. 3** Immunohistochemical analysis of activated and total EGF-R in tumor sections of L3.6pl cells growing in the pancreas of nude mice 30 days after therapy. Tumor sections were immunostained with Mab antibodies specific to activated EGF-R (tyrosine phosphorylated) or total EGF-R as described (32–36). Tumor sections from control (Contr.) and gemcitabine (Gem)-treated mice show immunoreactivity specific for both activated EGF-R (act. EGF-R) and total EGF-R (EGF-R), whereas tumor sections from C225 and C225 plus gemcitabine (C+G)-treated animals show no change in total EGF-R immunostaining but undetectable immunostaining for activated EGF-R. |
percentage of TUNEL-positive tumor cells in tumor sections 11, 18, and 30 days after each treatment compared with controls (Fig. 4; Table 2). The results suggest that treatment with C225 results in not only cytostasis but also cytotoxicity in vivo, correlating with inhibition of tumor growth and regression.

**Down-Regulation of VEGF and IL-8 in L3.6pl Human Pancreatic Cancer Cells Growing in Vitro after Treatment with C225.** Because C225 treatment in vitro resulted in a 20% maximal cytostatic effect and treatment in vivo of established tumors not only decreased tumor cell proliferation but also increased apoptosis, we investigated the mechanism for this differential effect. Recent studies have demonstrated that an indirect effect in which tumor cell production of angiogenic factors is inhibited, thereby affecting tumor-induced angiogenesis, resulting in tumor regression (10–12). ELISA detected a dose-dependent down-regulation of VEGF (48%; $P < 0.04$) and IL-8 (20%; $P < 0.05$) protein production in conditioned media of subconfluent L3.6pl pancreatic carcinoma cells after contin-

**Fig. 4** PCNA and TUNEL immunohistochemistry of L3.6pl human pancreatic tumors growing in nude mice after 18 days of therapy. Tissue sections were analyzed for expression of PCNA (to show cell division) and TUNEL (to show apoptosis). Decreased immunoreactivity to PCNA is observed in tumors from C225 and C225 plus gemcitabine (Gem.) compared with gemcitabine-treated and control (Contr.) animals. Increased DNA fragmentation (apoptosis) identified by localized green fluorescence was observed in specimens from each treatment group compared with controls.
Table 2  Immunohistochemical analysis of tumor cell proliferation (anti-PCNA) and apoptosis (TUNEL) in tumors from animals treated for 11, 18, and 30 days

<table>
<thead>
<tr>
<th>Therapy</th>
<th>% PCNA/Total cellsa</th>
<th>Pb</th>
<th>% TUNEL/Total cellsa</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 days</td>
<td>92 (87–98)</td>
<td></td>
<td>1.4 (1–7)</td>
<td></td>
</tr>
<tr>
<td>18 days</td>
<td>94 (79–98)</td>
<td></td>
<td>0.9 (0–3)</td>
<td></td>
</tr>
<tr>
<td>30 days</td>
<td>72 (31–94)</td>
<td></td>
<td>1.1 (0.5–2)</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 days</td>
<td>73 (51–80)</td>
<td>0.01</td>
<td>10 (3–13)</td>
<td>0.006</td>
</tr>
<tr>
<td>18 days</td>
<td>69 (31–75)</td>
<td>0.01</td>
<td>15 (8–21)</td>
<td>0.005</td>
</tr>
<tr>
<td>30 days</td>
<td>56 (37–67)</td>
<td>NSa</td>
<td>30 (18–39)</td>
<td>0.005</td>
</tr>
<tr>
<td>C225</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 days</td>
<td>26 (9–85)</td>
<td>0.04</td>
<td>8 (2–21)</td>
<td>NS</td>
</tr>
<tr>
<td>18 days</td>
<td>34 (26–52)</td>
<td>0.04b</td>
<td>17 (7–18)</td>
<td>0.004d</td>
</tr>
<tr>
<td>30 days</td>
<td>30 (14–41)</td>
<td>0.02d</td>
<td>39 (25–41)</td>
<td>0.004</td>
</tr>
<tr>
<td>C225 + gemcitabine</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 days</td>
<td>19 (13–30)</td>
<td>0.000001</td>
<td>7 (5–14)</td>
<td>0.02</td>
</tr>
<tr>
<td>18 days</td>
<td>7 (3–23)</td>
<td>0.000001</td>
<td>27 (21–43)</td>
<td>0.009</td>
</tr>
<tr>
<td>30 days</td>
<td>4 (0–6)</td>
<td>0.004</td>
<td>39 (36–68)</td>
<td>0.009d</td>
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</tbody>
</table>

a PCNA- or TUNEL-positive cells were counted in 10 microscopic fields per tumor section at ×100 (0.159 mm²). The total number of cells/field and the number of PCNA- or TUNEL-positive cells in that field were determined to calculate the median percentage in each tumor section.

b Control versus the therapy shown.

c NS, not significant.

d For C225 versus C225 plus gemcitabine, P < 0.008 (unpaired Student’s t test).

Table 3  In vitro inhibition of VEGF and IL-8 protein production by C225

<table>
<thead>
<tr>
<th>Protein</th>
<th>C225</th>
<th>0 µg/ml</th>
<th>1.25 µg/ml</th>
<th>2.5 µg/ml</th>
<th>5.0 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td>909 ± 141</td>
<td>486 ± 11b</td>
<td>499 ± 75</td>
<td>455 ± 15b</td>
<td>906 ± 10</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>328 ± 11</td>
<td>251 ± 32b</td>
<td>274 ± 78a</td>
<td>255 ± 32a</td>
<td>323 ± 12</td>
</tr>
</tbody>
</table>

a L3.6pl cells were cultured in the presence or absence of C225 for 48 h in 5% serum containing medium. VEGF and IL-8 were measured by ELISA. Values are the mean ± SD of triplicate cultures and have been normalized to account for cell number.

b For 5 µg/ml C225 versus control, P < 0.05 (unpaired Student’s t test).

Continuous exposure to C225 for 48 h (Table 3). No effect was observed on cell-associated bFGF protein production (data not shown). Northern blot analysis directly correlated with the ELISA results, demonstrating a 40 and 20% decrease in steady-state mRNA transcripts for VEGF and IL-8, respectively, after treatment with C225 (5 µg/ml for 48 h) compared with controls (data not shown).

In Vivo Inhibition of VEGF and IL-8 Protein Production in Vivo and MVD after Therapy with C225 or C225 Plus Gemcitabine. Because treatment with C225 down-regulates VEGF and IL-8 production in vitro, we evaluated whether therapy with C225 alone or in combination with gemcitabine inhibits angiogenesis in vivo. Production of VEGF and IL-8 as evaluated by immunohistochemistry after 11, 18, and 30 days of therapy was significantly reduced in C225- and C225 plus gemcitabine-treated tumors versus tumors from gemcitabine-treated or control animals (P < 0.002; Fig. 5; Table 4). Furthermore, no significant reduction in bFGF protein expression was seen after treatments with either C225 or C225 plus gemcitabine in comparison to gemcitabine-treated or control tumors (data not shown).

C225 and C225 plus gemcitabine resulted in the involution of tumor-induced neovascularity (Fig. 5; Table 4). MVD was significantly reduced in tumors treated for 18 and 30 days with C225 and C225 plus gemcitabine. No significant difference in MVD was observed in tumors from gemcitabine-treated and control animals (Fig. 5; Table 4). The down-regulation of VEGF and IL-8 protein production observed as early as 11 days after therapy preceded the reduction in MVD (Table 4). This temporal sequence suggests that the reduction in the levels of VEGF and IL-8 within the tumor contribute to the involution of neovessels observed after treatment.

Endothelial Cell Apoptosis. Double-staining immunofluorescence was used to evaluate the percentage of apoptotic endothelial cells (per microscopic section) in tumors from animals receiving the different treatments for 11, 18, and 30 days (Fig. 6). The highest significant difference was observed 18 days after treatment with C225 (69% median apoptotic endothelial cells; P < 0.00004) or C225 plus gemcitabine (62% median apoptotic endothelial cells; P < 0.0004) compared with control tumors (0% median apoptotic endothelial cells; Fig. 6; Table 4). Gemcitabine treatment did not enhance endothelial cell apoptosis compared with controls (Fig. 6; Table 4). These data directly correlate with the observed reduction in MVD (Table 4). The down-regulation of VEGF and IL-8 protein production by tumor cells, therefore, preceded the reduction in MVD, suggesting that the decline in VEGF and IL-8 directly contributed to the reduction in neovascularity as a result of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but an actual involution of the neovascularity, leading to tumor cell apoptosis and regression of established tumors.

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DISCUSSION

Our studies confirm that systemic administration of the chimeric anti-EGF-R MAb C225 inhibits the growth and metastasis of human pancreatic cancer established in the pancreas of athymic nude mice. We have shown that therapy with C225 has a significant antitumor effect, mediated in part by inhibition of angiogenesis and potentiated by its combination with gemcitabine. These data also indicate the antitumor effect of C225 versus gemcitabine in this model system. This effect was even more pronounced for eradication of liver and lymph node metastases. The observation that down-regulation of the angiogenic stimulus of the tumor cells inhibits the host angiogenic response emphasizes the complexity of tumor-host interactions. These experiments demonstrate that inhibition of angiogenesis characterizes, in part, the antitumor effect of therapy directed at inhibiting EGF-R signaling pathways in human pancreatic tumor cells. The down-regulation of VEGF and IL-8 protein production by tumor cells preceded the reduction in MVD, suggesting that the decline in VEGF and IL-8 directly contributed to the reduction in neovascularity as a result of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but an actual involution of the neovascularature, leading to tumor cell apoptosis and regression of established tumors.

EGF-R and its ligands (including EGF, AR, and TGF-α) are commonly overexpressed in pancreatic cancer, and their expression is associated with decreased survival duration (13, 18, 19). This coexpression of both receptor and ligand and the fact that EGF, AR, and TGF-α are mitogens for pancreatic cancer cell lines have led to the hypothesis that an EGF-R-dependent autocrine loop contributes to the malignant phenotype (14, 17, 20, 21). Its importance in the biology of pancreatic cancer is supported by in vitro experiments targeting either EGF-R or its ligands (41–43). In addition, Schmiegel et al. (43) demonstrated a longer median survival in unresectable pancreatic cancer patients treated with the anti-EGF-R Mab 425. One complete response was observed in a patient who remained in an unmaintained remission for 3 years (43). These results suggest that the EGF-R is a potential therapeutic target in pancreatic carcinoma.

In the present study, we describe a new therapeutic approach for pancreatic cancer, the combination of anti-EGF-R antibody C225 and gemcitabine. Whereas gemcitabine inhibits DNA synthesis by cells in S-phase and by blocking cell cycle progression through G1/S, blockade of EGF-R signaling pathways results in cell cycle arrest at the G1/S checkpoint. The induction of G1 arrest by C225 is associated with the inhibition of cyclin-dependent kinase 2 activity and increased levels of cyclin-dependent kinase inhibitor p27KiP1 (44, 45). In fact, C225...
has been shown recently to augment the antitumor activity of several chemotherapeutic agents and irradiation in different mouse xenograft models (10, 14, 45–49). The molecular pathways for this effect are unclear but may be mediated by several different mechanisms, including those affecting DNA repair (41, 46, 48, 50, 51), multidrug resistance (52, 53), cell cycle checkpoint control (45), or angiogenesis (10–12, 58). For example, C225, but not EGF, triggered a specific physical interaction between the internalized EGF-R and DNA-PK, implicated in the repair of DNA double strand breaks, in a variety of cell types (51). This association significantly reduced the level of DNA-PK in the nucleus and concomitantly increased DNA-PK levels in the cytosol, suggesting that EGF-R blockade and down-regulation by C225 may impair DNA repair.

Our data indicate a significant regression of established human pancreatic carcinoma growing orthotopically in the pancreas of nude mice after administration of C225, which could not be explained by the modest 20% cytostatic effect observed after in vitro treatment of the same highly metastatic L3.6pl human pancreatic cancer cells. The in vivo treatment effect was also significantly potentiated when C225 was combined with gemcitabine. This discrepancy may be explained, at least in part, by inhibition of the host angiogenic response. Indeed, our in vivo and in vitro data confirm a down-regulation of tumor cell-produced VEGF and IL-8 (but not bFGF) after treatment with C225. This down-regulation preceded the reduction in MVD, suggesting that the decline in VEGF and IL-8 directly contributed to the reduction in neovascularity as a direct result of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of endothelial MVD, suggesting that the decline in VEGF and IL-8 directly contributed to the reduction in neovascularity as a direct result of endothelial cell apoptosis. These data confirm a down-regulation of tumor cell-produced VEGF and IL-8 (but not bFGF) after treatment with C225. This down-regulation preceded the reduction in MVD, suggesting that the decline in VEGF and IL-8 directly contributed to the reduction in neovascularity as a direct result of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of endothelial cell apoptosis.

The capacity of C225 to modulate tumor cell cycle distribution may also play a central role in regulating the increased sensitivity to chemotherapeutic agents and irradiation. This may involve cell cycle checkpoint control as an activator of cell death (45). EGF-R blockade results in cellular arrest at the G1 restriction point, and cells damaged by chemotherapy or radiation typically arrest in G2/M to repair DNA alterations. Mendelsohn (45) has hypothesized that tumor cells die if they consecutively ignore two checkpoint signals (e.g., activated by EGF-R blockade and cytotoxic drug or radiation treatment). Interestingly, nonmalignant endothelial cells, which obey checkpoint control signals, may be less susceptible to the cytotoxic effects of these combination treatments. These data indicate that inhibition of EGF-R signaling may act through several potential mechanisms to sensitize tumor cells to cytotoxic agents or radiation.

Our data indicate a significant regression of established human pancreatic carcinoma growing orthotopically in the pancreas of nude mice after administration of C225, which could not be explained by the modest 20% cytostatic effect observed after in vitro treatment of the same highly metastatic L3.6pl human pancreatic cancer cells. The in vivo treatment effect was also significantly potentiated when C225 was combined with gemcitabine. This discrepancy may be explained, at least in part, by inhibition of the host angiogenic response. Indeed, our in vivo and in vitro data confirm a down-regulation of tumor cell-produced VEGF and IL-8 (but not bFGF) after treatment with C225. This down-regulation preceded the reduction in MVD, suggesting that the decline in VEGF and IL-8 directly contributed to the reduction in neovascularity as a direct result of endothelial cell apoptosis. These data also indicate that therapy with C225 and C225 plus gemcitabine, but not gemcitabine alone, caused not merely an inhibition but in fact an involution of the neovasculature, leading to tumor cell apoptosis and regression of established tumors. This is the first report to our knowledge demonstrating that therapy with C225 leads to apoptosis of endothelial cells within pancreatic tumors as determined by a double-labeling immunofluorescence procedure for CD31 and TUNEL.

This premise is further strengthened by the recent studies of Koch et al. (55) and Benjamin et al. (54, 58) using a genetic system designed for conditional VEGF expression in either a xenografted glioma or well-established, slow-growing human prostate cancers. Their data demonstrate the dependence of immature vessels on VEGF for survival and prove that this can be exploited to an existing tumor vasculature and hence the dependent tumor mass (54, 55, 56). Antiangiogenic therapy therefore capitalizes on the concept that the proangiogenic processes in a tumor are more exaggerated in intensity and local-
A shift to an antiangiogenic phenotype can result from a suppression of proangiogenic mechanisms and/or enhancement of the opposing antiangiogenic processes.

The mechanisms by which EGF-R signaling pathways regulate VEGF and IL-8 are unclear. However, it is established that activation of EGF-R signaling pathways by EGF and TGF-α up-regulate VEGF (31, 58). Furthermore, it is known that after stimulation of EGF-R signaling pathways, ras and raf are activated, resulting in phosphorylation of c-fos and c-jun, which leads to increased AP-1 transcriptional activity (54, 58–61). Increased AP-1 activity leads to transcription of genes with AP-1 binding sites in their promoter including VEGF and IL-8 (58, 60, 62). Experiments in progress are analyzing the effect of EGF-R blockade on the specific transcription factors involved in VEGF and IL-8 regulation in the human L3.6pl pancreatic carcinoma cells.

In summary, our experiments indicate that therapeutic strategies targeting EGF-R signaling have a significant antitumor effect on human L3.6pl pancreatic carcinoma growing in nude mice and the effect is mediated by inhibition of tumor-induced angiogenesis, which in turn leads to tumor cell apoptosis and regression. Furthermore, this effect is potentiated in combination with gemcitabine. These experiments indicate that normalization of angiogenesis related biomarkers such as CD31 expression, and apoptotic cells (detected by TUNEL) in human pancreatic tumors.
VEGF, IL-8, or MVD may be clinically useful (in addition to other markers including EGF-R activation status, PCNA, and TUNEL for tumor or endothelial cells) to demonstrate regression or eradication of cancer after EGF-R-directed therapy. Analysis of these biomarkers should be included in clinical trials for evaluating this form of therapy. Finally, combination of this approach with gemcitabine may provide increased benefit in pancreatic carcinoma patients.

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REFERENCES


Epidermal Growth Factor Receptor Blockade with C225 Plus Gemcitabine Results in Regression of Human Pancreatic Carcinoma Growing Orthotopically in Nude Mice by Antiangiogenic Mechanisms


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