Advances in Brief

Enhanced Antitumor Activity of Anti-epidermal Growth Factor Receptor Monoclonal Antibody IMC-C225 in Combination with Irinotecan (CPT-11) against Human Colorectal Tumor Xenografts

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Abstract

Colon carcinomas frequently express the epidermal growth factor receptor (EGFR), and this expression correlates with more aggressive disease and poor prognosis. Previous studies have shown that EGFR blockade by monoclonal antibody IMC-C225 can inhibit the growth of human colon carcinoma tumor cells *in vitro* and xenografts of these tumors in athymic mice. In this report, we have studied the *in vivo* activity of IMC-C225 combined with the topoisomerase I inhibitor irinotecan (CPT-11) using two models of human colorectal carcinoma in nude mice. IMC-C225 was tested at a dose of 1 or 0.5 mg administered q3d. CPT-11 was administered at a dose of 100 mg/kg/week or a maximum tolerated dose of 150 mg/kg/week. Treatment with the combination of IMC-C225 (1 and 0.5 mg) and CPT-11 (100 mg/kg) significantly inhibited the growth of established DLD-1 and HT-29 tumors compared with either CPT-11 or IMC-C225 monotherapy ($P < 0.05$). Combination therapy with IMC-C225 (1 mg) and the MTD of CPT-11 (150 mg/kg) resulted in a regression rate of 100 and 60% of established DLD-1 and HT-29 tumors, respectively. In a refractory tumor model, combined treatment with IMC-C225 and CPT-11 significantly inhibited the growth of CPT-11 refractory DLD-1 and HT-29 tumors, whereas either agent alone did not control tumor growth. Histological examination of treated tumors showed extensive tumor necrosis, decreased tumor cell proliferation, increased tumor cell apoptosis, and a marked decrease in tumor vasculature. These results suggest that EGFR blockade by IMC-C225 combined with topoisomerase I inhibitors may be an effective therapy against chemorefractory colorectal carcinoma tumors.

Introduction

Colorectal cancer is the third most common cancer in men and women in the United States with an estimated 130,200 new cases diagnosed in 2000, including 93,800 patients with colon cancer and 36,400 with rectal cancer (1). Metastatic colorectal cancer is the second-leading cause of death from cancer in North America, largely because of the poor clinical response of colorectal tumors to conventional chemotherapeutics. The topoisomerase I inhibitor irinotecan (CPT-11) is a water soluble, semisynthetic derivative of camptothecin that has shown activity against a number of different tumor types in preclinical models and in clinical trials of patients with various human cancers (2). The active metabolite of CPT-11, SN-38, stabilizes a ternary complex between the nuclear enzyme topoisomerase I and double-stranded DNA, which results in replication fork arrest and chromatid breaks (3). CPT-11 is active clinically in the second-line treatment of patients with colorectal cancer failing or refractory to first-line 5-FU[2] and leucovorin therapy (4). More recently, CPT-11 administered as a three drug regimen along with 5-FU and leucovorin has been shown to prolong survival in patients with colorectal cancer that is refractory to treatment with 5-FU/leucovorin (5). Despite clinical improvements attributed to the addition of CPT-11 therapy for metastatic colorectal cancer, nearly all patients will eventually become refractory to CPT-11. Thus, new treatment options are needed to improve survival in patients with CPT-11 refractory colorectal cancer.

The EGFR and its ligands EGF and TGF-α play an important role in the growth and survival of human colorectal tumors (reviewed in Refs. 6 and 7). EGF and/or TGFα mRNA and protein are found in a majority of human colorectal tumors and cell lines, and coexpression of these ligands and EGFR has been reported, suggesting the potential existence of an autocrine loop. In addition, the expression of EGFR in colorectal cancer correlates with more aggressive disease and poor prognosis. Hence, blockade of the EGFR receptor may be a useful strategy for treatment of adenocarcinoma of the colon. IMC-C225 binds the EGFR with high affinity, competes for ligand binding, and

Received 9/26/01; revised 1/22/02; accepted 2/16/02.
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[2] The abbreviations used are: 5-FU, 5-fluorouracil; EGFR, epidermal growth factor receptor; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (irinotecan); EGF, epidermal growth factor; TGF, transforming growth factor; MAb, monoclonal antibody; MTD, maximum tolerated dose; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; DAB, diaminobenzidine.

down-regulates receptor expression on the cell surface (reviewed in Ref. 8). Several studies have shown that IMC-C225 is capable of inhibiting growth of EGFR-expressing tumor cells *in vitro*, and treatment with IMC-C225 results in marked inhibition of tumor growth in nude mice bearing xenografts of human cancer cell lines (8–10). Moreover, treatment with IMC-C225 in combination with chemotherapeutic drugs or radiotherapy is effective in eradicating well-established tumors in nude mice (8–11). IMC-C225 is currently in Phases II and III clinical testing in a number of EGFR+ malignancies (9). The objective of this study was to determine the potential therapeutic utility of the MAb IMC-C225 when combined with CPT-11 therapy to inhibit growth of human colon carcinoma tumor xenografts in a nude mouse model.

Materials and Methods

Cell Lines

The colon carcinoma cell lines DLD-1 and HT-29 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (DLD-1) or McCoy's 5A (HT-29) media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Lenexa, KY) and 2 mM GlutaMAX (Invitrogen). Cells were cultured at 37°C in a 5% CO_2 atmosphere and passaged routinely by Trypsin-EDTA (Invitrogen) treatment.

Anti-EGFR MAb IMC-C225

Clinical grade anti-EGFR MAb IMC-C225 (ERBITUX) was produced by the ImClone Systems, Inc. manufacturing facility (Somerville, NJ).

Flow Cytometry

Subconfluent cultures of colon carcinoma cell lines, grown in 100-mm^2 plates, were washed in ice-cold HBSS and detached by incubating the monolayers in HBSS with EDTA. Aliquots of 10^6 cells were incubated for 1 h on ice with primary antibody (IMC-C225 or control IgG), diluted in PBS with 1% BSA and 0.02% sodium azide (flow buffer). Cells were washed twice with flow buffer and then incubated for 30 min on ice with FITC-labeled goat antihuman IgG Fc-specific secondary antibody (KPL, Gaithersburg, MD) diluted in flow buffer. Cells were washed as above and analyzed for FITC emission intensity on an Epics XL (Beckman-Coulter, Hialeah, FL) flow cytometer. Dead cells and debris were eliminated from the analysis on the basis of forward and sideways light scatter.

Phosphorylation Assay

Phosphorylation assays were done by seeding DLD-1 or HT-29 cells at 5×10^6/150-mm^2 tissue culture plate in media containing 0.5% fetal bovine serum in 5 μg/ml IMC-C225 or normal human IgG (Jackson Immunoresearch, West Grove, PA). After culturing for 24 h, cells were then stimulated with 5 ng/ml EGF or 5 ng/ml TGF-α (Sigma Chemical Co., St. Louis, MO) for 20 min at 37°C. Monolayers were than washed with ice-cold PBS containing 1 mM sodium orthovanadate. Cells were lysed, immunoprecipitated with IMC-C225, and subjected to Western blot analysis. The phosphorylation patterns were determined by probing the blots with a horseradish peroxidase-conjugated antiphosphotyrosine MAb (UBI) followed by detection using the Lumi-GLO method (KPL). Loading equivalence of EGFR in each lane was verified by stripping and reprobing blots with a polyclonal anti-EGFR antibody (Calbiochem, San Diego, CA).

Animals

Female athymic (*nu/nu*) mice (5–6 weeks old) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were housed under pathogen-free conditions in microisolator cages with laboratory chow and water available *ad libitum*. All experiments and procedures were performed in accordance with the United States Department of Agriculture, Department of Health and Human Services, and NIH policies regarding the humane care and use of laboratory animals.

Treatment of s.c. Colorectal Carcinoma Xenografts

Subcutaneous colon carcinoma tumors were established by injecting 5×10^6 DLD-1 or HT-29 cells mixed in Matrigel (Collaborative Research Biochemicals, Bedford, MA) s.c. into the left flank of athymic mice. Three separate study designs were used to examine the activity of IMC-C225, irinotecan (CPT-11; Pharmacia & Upjohn, Kalamazoo, MI), and combination therapy.

Study Design I. Tumors were allowed to reach 150–300 mm^3 in size, and then mice were randomized into groups of five animals each. Mice were treated with saline q3d, IMC-C225 at 1 mg/dose (q3d), IMC-C225 at 0.5 mg/dose (q3d), CPT-11 at 100 mg/kg (q7d), combination treatment with IMC-C225 at 1 mg/dose (q3d) + CPT-11 at 100 mg/kg (q7d), and IMC-C225 at 0.5 mg/dose (q3d) + CPT-11 at 100 mg/kg (q7d). Treatment of animals was continued for the duration of the study.

Study Design II. Tumors were allowed to reach 150–300 mm^3 in size, and then mice were randomized into groups of 10 animals each. Mice were treated with saline q3d, IMC-C225 at 1 mg/dose (q3d), CPT-11 at 150 mg/kg (q7d), or combination treatment with IMC-C225 at 1 mg/dose (q3d) + CPT-11 at 150 mg/kg (q7d). Treatment of animals was continued for the duration of the study.

Study Design III. Tumors were allowed to reach 150 mm^3 in size, and then mice were treated with two cycles of CPT-11 (100 mg/kg; days 0 and 7). After two cycles of CPT-11 treatment (day 12), those CPT-11-treated mice with tumor volumes > 2-fold the volume on day 0 ("refractory" mice) were randomized into three groups of seven to eight mice per group. The frequency of CPT-11 refractory mice was 50–60% of the total CPT-11-treated group. CPT-11 refractory mice were treated with IMC-C225 at 1 mg/dose (q3d), CPT-11 at 100 mg/kg (q7d), or combination treatment with IMC-C225 at 1 mg/dose (q3d) + CPT-11 at 100 mg/kg (q7d). Treatment of animals was continued for the duration of the study.

The MTD for CPT-11 therapy was defined as the maximum dose that did not result in drug-related morbidity and/or weight loss of >20% of body weight at the beginning of therapy. Mice were monitored daily for signs of CPT-11-related toxicity (body weight, diarrhea, and lethality).

All drug treatments were administered by i.p. injection. Tumors were measured twice each week with calipers, and

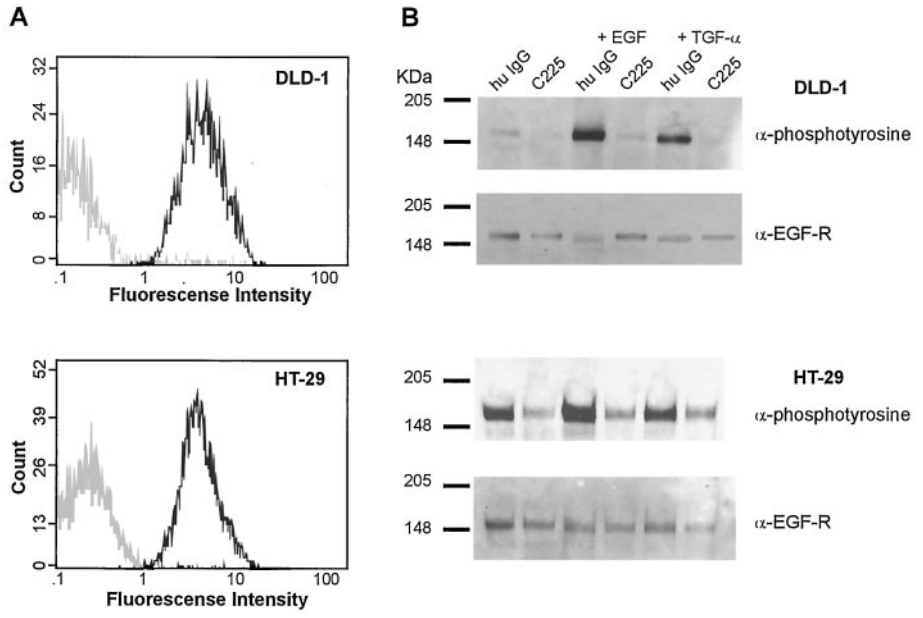

Fig. 1 A, EGFR expression on DLD-1 and HT-29 colon carcinoma cells. Flow cytometry analysis histograms of IMC-C225 binding (*black*) or a control human antibody (*gray*). *B,* phosphorylation assay showing IMC-C225 inhibition of EGFR phosphorylation. DLD-1 or HT-29 cultures were incubated with IMC-C225 or normal human IgG followed by stimulation with exogenous EGF or TGF-α. Western blotting with antiphosphotyrosine antibody (*top panels*). Blots were stripped and reprobed with anti-EGFR antibody (*bottom panels*).

tumor volumes were calculated by the formula [π/6 (w1 × w2 × w2)], where "w1" represents the largest tumor diameter, and "w2" represents the smallest tumor diameter.

Histology

Tumors were harvested from animals at multiple time points after treatment and snap frozen in liquid nitrogen for anti-CD31 immunohistochemistry and TUNEL staining. For anti-CD31 immunohistochemistry, 6-μm cryostat sections were fixed at room temperature in acetone for 5 min and were postfixed in 10% neutral buffered formalin for 10 s before staining. Endogenous peroxidase activity was blocked by a 30-min incubation with 0.3% H_2O_2 in H_20. After blocking with 5% BSA, 10% normal goat serum + 0.02% Tween 20, FITC anti-CD31 (clone Mec13.3, BD PharMingen, Franklin Lakes, NJ), and FITC rat IgG isotype control (PharMingen) were diluted to a final concentration of 1 μg/ml in blocking buffer and were incubated with the tissue sections for 60 min at room temperature. Slides were then washed in PBS to remove excess antibody. Slides were incubated with 1 μg/ml horseradish peroxidase-conjugated anti-FITC (Roche Molecular Biochemicals, Indianapolis, IN) for 30 min at room temperature and were washed subsequently in PBS. Immunostaining was developed for 4 min using a liquid DAB substrate kit (Zymed, San Francisco, CA) per manufacturer's instructions. The reactions were stopped by successive washes in tap and distilled water and were lightly counterstained with Mayer's hematoxylin. Sections were coverslipped using a permanent mounting medium. TUNEL staining was performed using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI). Briefly, 6-μm cryostat sections were fixed in 4% paraformaldehyde for 10 min at room temperature and rinsed in PBS with 0.1% Triton X-100. Sections were then incubated in Equilibration Buffer for 5 min at room temperature followed by incubation in TUNEL Mix, prepared according to kit instructions, for 1 h at 37°C. After successive washes in PBS, sections were coverslipped using an antifade reagent.

For H&E and Ki-67 staining, resected tumors were fixed in zinc-buffered formalin (Shandon Lipshaw, Pittsburgh, PA) overnight at 4°C. After paraffin embedding and sectioning at 6 μm, formalin-fixed sections were stained with Mayer's H&E (Richard Allen, Kalamazoo, MI). For Ki-67 immunohistochemistry, slides were heated in a water bath at 95°C-99°C in Target Retrieval Solution (DAKO, Carpinteria, CA) for 20 min, followed by a 20-min cool down period at room temperature. After heat retrieval, sections were rinsed well in PBS and stained with rabbit antihuman Ki-67 antigen (DAKO N-series, ready to use) per manufacturer's instructions and were lightly counterstained with Mayer's hematoxylin.

The Proliferation Index was determined by Ki-67 immunostaining and calculating the number of DAB-positive pixels per total number of nuclear pixels (hematoxylin-positive pixels plus DAB-positive pixels × 100) in five fields at ×200. The Apoptosis Index, determined by TUNEL staining, is calculated from the number of TUNEL-positive pixels per total number of Hoechst-positive pixels × 100 in five fields at ×200. The Apoptosis:Proliferation Ratio equals the Apoptosis Index/Proliferation Index × 1000.

Statistical Analysis

Tumor volumes and histological quantitations were analyzed using Student's *t* test. Antitumor activity of combination therapy with IMC-C225 and CPT-11 *in vivo* was analyzed by the fractional product method as described (12). Analyses were computed using the SigmaStat statistical package (v. 2.03; Jandel Scientific, San Rafael, CA).

Results

The *in vivo* effects of IMC-C225 and CPT-11 therapy alone or in combination were tested in a DLD-1 and HT-29 s.c.

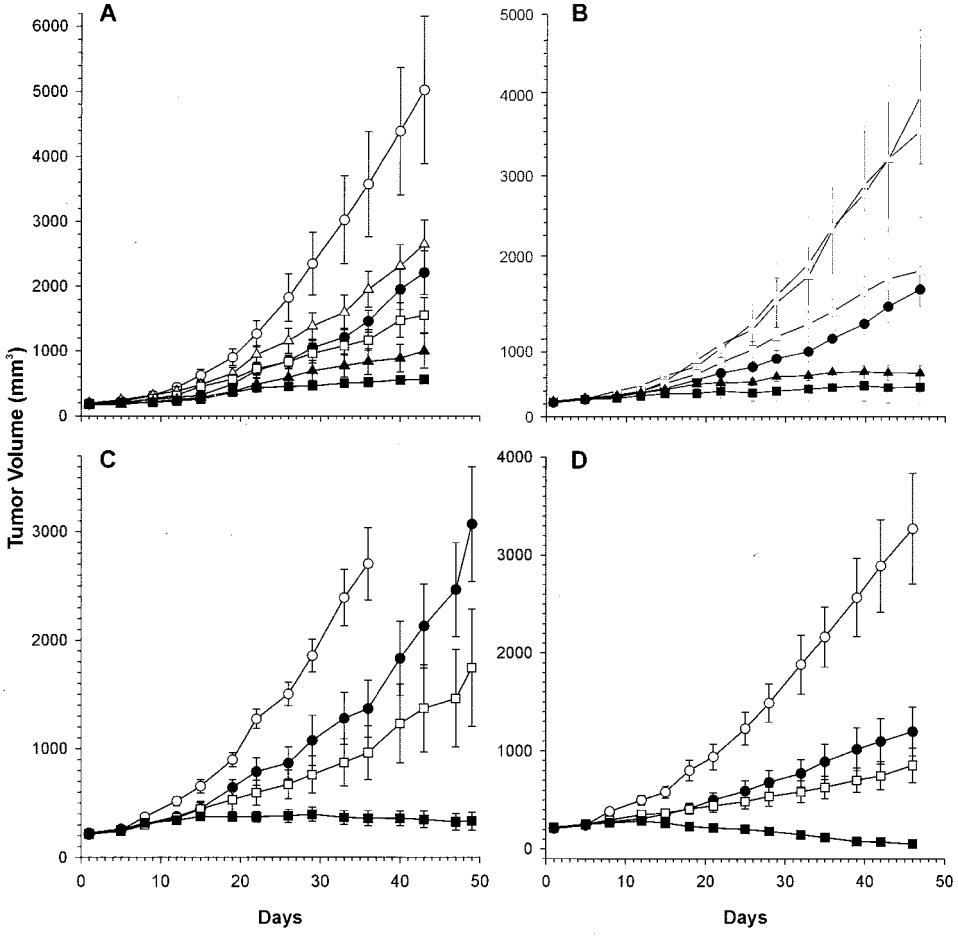

Fig. 2 Growth inhibition of DLD-1 and HT-29 colorectal tumor xenografts in nude mice. *A* and *B*, dose-dependent effects are shown for treatment of established DLD-1 (*A*) and HT-29 (*B*) tumors treated with saline (○), IMC-C225 at 1 mg (●) or 0.5 mg (△) per dose/q3d, CPT-11 at 100 mg/kg/week (□), CPT-11 (100 mg/kg/week) plus IMC-C225 at 1 mg/dose/q3d (■), and CPT-11 (100 mg/kg/week) plus IMC-C225 at 0.5 mg/dose/q3d (▲). *C* and *D*, combination therapy on established DLD-1 (*C*) and HT-29 (*D*) tumors treated with saline (○), IMC-C225 at 1 mg (●), CPT-11 at 150 mg/kg/week (□), and CPT-11 (150 mg/kg/week) plus IMC-C225 at 1 mg/dose/q3d (■). *Bars*, ±SE.

xenograft model in athymic nude mice. DLD-1 and HT-29 cells express moderate levels of EGFR, and IMC-C225 inhibits both EGF and TGF-α-stimulated EGFR tyrosine phosphorylation in both cell lines (Fig. 1, *A* and *B*). Three different therapy designs were tested in the DLD-1 and HT-29 tumor models. In therapy schedule I, mice with well-established (~200 mm^3) DLD-1 or HT-29 tumors were treated by i.p. administration of two different doses of IMC-C225 (1 or 0.5 mg; q3d) with or without CPT-11 (100 mg/kg; q7d). A total of 16 cycles of IMC-C225 and 7 cycles of CPT-11 was administered. Treatment with IMC-C225, CPT-11, or the combination was well tolerated for the duration of therapy. IMC-C225 treatment alone at 1- or 0.5-mg doses did not significantly inhibit growth of DLD-1 or HT-29 tumors ($P > 0.05$; Fig. 2, *A* and *B*). Although CPT-11 treatment alone (100 mg/kg/week) was active against DLD-1 ($P < 0.02$) tumors, the treatment did not significantly inhibit growth of HT-29 tumors ($P > 0.05$). In contrast, enhanced antitumor activity using combination therapy with IMC-C225 and CPT-11 was observed in both DLD-1 and HT-29 tumors compared with mice treated with control, IMC-C225, or CPT-11 alone.

In the second study design, we attempted to achieve a maximal therapeutic effect by increasing the dose of CPT-11 to the MTD. Pilot studies determined that the MTD of CPT-11 in mice was 150 mg/kg when given q7d (data not shown). The MTD of CPT-11 was then used in combination therapy with IMC-C225 in the DLD-1 and HT-29 xenograft models (Fig. 2, *C* and *D*). A total of 17 cycles of IMC-C225 and 7 cycles of CPT-11 was administered. Combination therapy with 1 mg of IMC-C225 and 150 mg/kg CPT-11 resulted in a significant inhibition of tumor growth compared with single-agent IMC-C225 ($P < 0.002$) or compared with single-agent CPT-11 ($P < 0.03$). In the DLD-1 model, complete inhibition of tumor growth was observed in the majority of animals. In addition, regression of established DLD-1 tumors was observed in 60% of animals treated with the combination of IMC-C225 and CPT-11. The activity of IMC-C225 and CPT-11 treatment was even more pronounced in the HT-29 model. Regression of established HT-29 tumors was observed in 100% of the animals treated with IMC-C225 and CPT-11 combination therapy. The indices for combination therapy are >1, indicating an enhanced interaction between IMC-C225 and CPT-11. Table 1 summarizes the analysis of the enhanced combination effect at different time points during therapy.

The results from studies described above demonstrated that DLD-1 or HT-29 tumors are poorly responsive to IMC-C225 or CPT-11 therapy alone. To further examine the effect of IMC-C225/CPT-11 combination therapy against CPT-11 refractory

Table 1 Combination therapy with IMC-C225 and CPT-11

	Fractional tumor volume (FTV)[a] relative to control animals					
		Combination Therapy				
	Day[b]	IMC-C225	CPT-11	Expected[c]	Observed	Ratio of expected: observed FTV[d]
DLD-1	26	0.577	0.447	0.258	0.254	1.015
	29	0.579	0.410	0.237	0.213	1.111
	33	0.534	0.365	0.195	0.153	1.272
	36	0.506	0.356	0.180	0.133	1.349
HT-29	25	0.481	0.393	0.189	0.162	1.166
	28	0.456	0.360	0.164	0.120	1.364
	32	0.409	0.310	0.127	0.078	1.638
	35	0.411	0.291	0.120	0.054	2.232

[a] FTV, calculated as mean tumor volume experimental/mean tumor volume control.
[b] Day after start of treatment. Tumor volumes were measured twice weekly ($n = 8$ for each group of mice).
[c] (Mean FTV of IMC-C225) $\times$ (mean FTV of CPT-11).
[d] Obtained by dividing the expected FTV by the observed FTV. A ratio of >1 indicates a more than additive effect, and a ratio of <1 indicates a less than additive effect.

colorectal tumors, a refractory model was designed and tested. Animals with established DLD-1 or HT-29 tumors were treated with two cycles of CPT-11 (100 mg/kg; days 0 and 7). At day 12, tumor volumes were measured, and animals with tumors that had increased $>2\times$ the original tumor volume at the start of CPT-11 therapy were considered CPT-11 refractory. These mice were selected and randomized into treatment groups receiving continued CPT-11 (100 mg/kg; q7d), switched to IMC-C225 therapy (1 mg; q3d) or combination therapy on the same schedule. DLD-1 or HT-29 tumors in animals that continued to receive CPT-11 grew at similar kinetics before randomization, *i.e.*, these tumors did not respond to additional CPT-11 therapy (Fig. 3). IMC-C225 therapy alone did not have a significant antitumor effect on CPT-11 refractory DLD-1 or HT-29 tumors and grew at similar kinetics to those tumors treated with CPT-11 ($P > 0.05$ compared with CPT-11-treated mice). In contrast, combination therapy with IMC-C225 and CPT-11 significantly inhibited the growth of CPT-11 refractory DLD-1 or HT-29 tumors ($P < 0.01$ and <0.01, respectively) with enhanced antitumor activity compared with either agent alone (Table 2).

Histological examination of HT-29 xenografts at day 47 after treatment showed marked differences in tumors from animals administered the combined IMC-C225/CPT-11 therapy compared with tumors from animals receiving control or single agent therapy. Extensive tumor necrosis was observed in DLD-1 and HT-29 tumors treated with a combination of IMC-C225 and CPT-11 (Fig. 4*A*). A reduction in tumor cell proliferation (Ki-67 staining), a reduction in tumor vasculature (CD31 staining), and an increase in tumor apoptosis (TUNEL staining) was observed in tumors treated with IMC-C225 and CPT-11. Quantitative analysis of the tumor cell proliferation, apoptosis, and vessel staining showed a significant difference between combination therapy *versus* control and between combination therapy *versus* single-agent therapy ($P < 0.001$ for all comparisons; Fig. 4*B*). In HT-29 tumors, combination treatment resulted in a $\geq$1.4-fold decrease in tumor cell proliferation, a $\geq$2.9-fold increase in tumor cell apoptosis, and a $\geq$4.3-fold decrease in vessel staining. The Apoptosis:Proliferation Ratio was increased $\geq$4.5-fold in IMC-C225/CPT-11-treated tumors compared with either agent alone. Similar findings were observed in DLD-1 tumors. These findings show that the antitumor activity of combined treatment with IMC-C225 and CPT-11 is a result of increased tumor cell apoptosis and necrosis compared with treatment with either agent alone.

Discussion

EGFR and its ligands EGF and TGF-α are important factors in the normal physiology of the human gastrointestinal tract (for review, see Ref. 13). EGF is a potent epithelial mitogen that is produced in the Paneth cells of the small intestine and the Brunner's glands of the duodenum (14). EGF stimulates intestinal crypt stem cell proliferation in both the small and large intestine and is an important survival factor for the intestinal mucosa (14, 15). EGFR and TGF-α expression can be detected in normal epithelial mucosa throughout the gastrointestinal tract (16). The EGFR and its ligands also play an important role in the growth and survival of human colorectal tumors (6, 7). EGFR is expressed at varying levels in human colorectal tumors and colorectal carcinoma cell lines. Moreover, coexpression of EGF and/or TGFα and EGFR has been reported in several human colon tumor cell lines, suggesting the potential existence of an autocrine stimulatory loop (17–19). In addition, studies have demonstrated that the EGFR expression level is related to the progression of the colon cancer disease, as well as survival after tumor resection (20, 21). The relationship between EGFR expression and the hepatic metastasis of colon adenocarcinoma has been reported in a limited number of studies (22). These studies suggest that expression of EGFR in colon carcinoma correlates with the ability to produce liver metastases. Hence, the role of the EGFR signaling pathway in colorectal cancer has led to considerable interest in the potential therapeutic utility of agents that block EGFR function.

Studies herein were performed to determine the activity of the anti-EGFR MAb IMC-C225 in combination with the topoisomerase I inhibitor CPT-11 in colorectal tumor models and to provide a rationale for clinical development of this combination. We hypothesized that combining IMC-C225

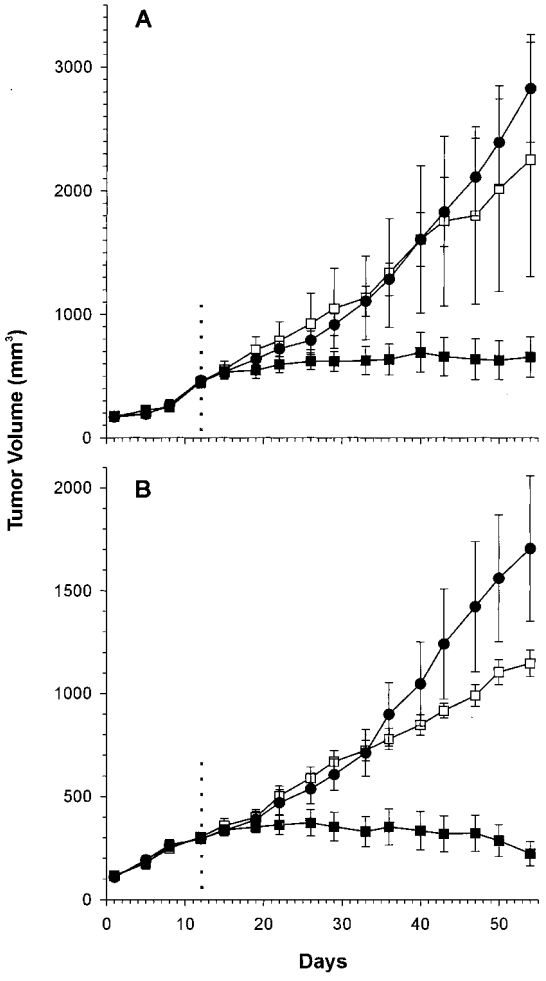

Fig. 3 Growth inhibition of CPT-11 refractory colorectal tumor xenografts in nude mice. Mice with established DLD-1 (*A*) or HT-29 (*B*) tumors were treated with two cycles of CPT-11 therapy (100 mg/kg) on days 0 and 7. Mice with tumors that did not respond to CPT-11 therapy ($>2 \times$ initial tumor volume at day 12; shown as *dotted vertical line*) were selected, randomized, and then treated with IMC-C225 at 1 mg/dose/q3d (●), continued CPT-11 at 100 mg/kg/week (□), or combination therapy (■). *Bars*, ±SE.

with CPT-11 would enhance the antitumor activity of these drugs against tumors that are poorly responsive to either drug alone. Our findings show that functional inhibition of EGFR with IMC-C225 combined with CPT-11 therapy is highly effective in two different colorectal tumor models. Importantly, HT-29 and DLD-1 colorectal tumor xenografts were found to be poorly sensitive to CPT-11 or IMC-C225 monotherapy. However, when IMC-C225 treatment was added to CPT-11 therapy, enhanced tumor growth inhibition was observed. To further evaluate the enhanced effects of combining IMC-C225 with CPT-11, we studied the combination of these two agents in a more rigorous CPT-11 refractory model. In these experiments, mice with established HT-29 or DLD-1 tumors that did not respond to CPT-11 therapy were selected and subsequently treated with CPT-11, IMC-C225, or the combination. Remarkably, IMC-C225 treatment was able to reverse the CPT-11 refractory nature of both HT-29 and DLD-1 tumors. These results are consistent with a number of studies demonstrating the same principle; namely, combined therapy with IMC-C225 and antineoplastic drugs results in a marked potentiation of antitumor activity compared with monotherapy. IMC-C225 or its parent murine antibody 225 has been shown to augment the antitumor activity of several anticancer agents, including cisplatin, doxorubicin, 5-FU, gemcitabine, and paclitaxel in preclinical models (23–27). Similar enhancement of activity has been observed in studies where IMC-C225 is used in combination with irradiation (11, 28, 29). Importantly, the combination of IMC-C225 with cytotoxic drugs/radiation often results in tumor regression and, in many cases, eradication of established tumors. It should be noted that overexpression of EGFR by tumor cells is not required for the enhanced activity observed when combining IMC-C225 with chemotherapy and particular in these studies when combining IMC-C225 with CPT-11. The two colorectal tumor lines used in our studies express moderate levels of EGFR ($\sim 1 \times 10^5$) and respond to ligand stimulation, and receptor activation can be inhibited by treatment with IMC-C225. These findings suggest that the biology of the EGFR pathway in tumor cells, rather than the overall receptor level, is important for the activity of IMC-C225 when combined with chemotherapeutic agents. The effectiveness of combining IMC-C225 and chemotherapy or radiation therapy observed in preclinical studies has been extended to clinical trials in patients with squamous cell carcinoma of the head and neck (30, 31).

The activity of IMC-C225 or its parental murine antibody 225 has been demonstrated previously in a number of *in vitro* and *in vivo* preclinical models of human colon adenocarcinoma (32–34). In these studies, treatment with IMC-C225 inhibited the growth of colorectal tumor cells *in vitro* and growth of s.c. tumor xenografts. Combination treatment with IMC-C225 and other antineoplastic agents or radiation has been shown to enhance the antitumor effect against established colorectal tumor xenografts (34–36). Ciardiello *et al.* (35) tested IMC-C225 in combination with the topoisomerase I inhibitor topotecan in the human GEO colon adenocarcinoma xenograft model. Treatment of mice bearing established GEO colon tumors with either IMC-C225 or topotecan alone was ineffective at controlling the growth of GEO tumors. However, combined treatment resulted in a significant suppression of tumor growth and almost complete tumor regression and a significant survival advantage compared with animals receiving monotherapy. These results are consistent with those obtained in the present study with CPT-11. Interestingly, Ciardiello *et al.* (35) found that sequential treatment with topotecan followed by IMC-C225 therapy resulted in the most effective antitumor response. In our studies, CPT-11 and IMC-C225 was administered to mice simultaneously during the initial treatment cycle followed by weekly CPT-11 treatment with sustained serum levels of IMC-C225 administered on a q3d schedule. We found no dependency for sequential treatment with CPT-11 followed by IMC-C225. Moreover, we have found optimal therapeutic effects in animal models when CPT-11 and IMC-C225 are administered together

Table 2 Response of CPT-11 refractory xenografts to IMC-C225 and CPT-11

	Fractional tumor volume (FTV)[a] relative to control animals					
		Combination therapy				
	Day[b]	Switch to IMC-C225	Continued CPT-11	Expected[c]	Observed	Ratio of expected: observed FTV[d]
DLD-1	36	0.566	0.544	0.308	0.269	1.144
	40	0.495	0.495	0.245	0.213	1.149
	43	0.456	0.475	0.217	0.171	1.268
	47	0.404	0.474	0.191	0.143	1.335
	50	0.393	0.467	0.184	0.123	1.498
HT-29	36	0.420	0.485	0.204	0.190	1.073
	40	0.355	0.439	0.156	0.140	1.115
	43	0.345	0.467	0.161	0.120	1.343
	47	0.295	0.423	0.125	0.096	1.307
	50	0.287	0.405	0.116	0.074	1.562

[a] FTV, calculated as mean tumor volume experimental/mean tumor volume control.
[b] Day after start of treatment. Tumor volumes were measured twice weekly ($n = 8$ for each group of mice).
[c] (Mean FTV of IMC-C225) $\times$ (mean FTV of CPT-11).
[d] Obtained by dividing the expected FTV by the observed FTV. A ratio of >1 indicates a more than additive effect, and a ratio of <1 indicates a less than additive effect.

rather than sequentially.[3] The discrepancy between our results and that of Ciardiello *et al.* (35) are unclear. These differences may be related to drug treatment schedule, pharmacodynamics of CPT-11 *versus* topotecan, mechanisms of interaction between IMC-C225 and CPT-11 *versus* topotecan, or differences in the tumor models.

Histological examination of HT-29 and DLD-1 tumors treated with IMC-C225 and CPT-11 therapy showed marked changes in several markers that can be attributed to the antitumor effect of combination therapy. These comprise a decrease in tumor cell proliferation, an increase in tumor cell apoptosis, and a reduction in tumor vasculature. The reduction in tumor cell proliferation and tumor vasculature and increase in apoptosis led to extensive tumor necrosis in IMC-C225/CPT-11-treated colorectal tumors. The incidence of tumor cell apoptosis suggests that the effects of IMC-C225/CPT-11 treatment on colon carcinoma tumors *in vivo* were not merely cytostatic but rather affect the growth and survival of these tumors. These findings are consistent with those of previous studies that have demonstrated IMC-C225-induced tumor cell apoptosis in other EGFR+ human tumor models in nude mice (25, 27, 37). The decreased CD31 vessel staining observed in HT-29 and DLD-1 xenografts are also consistent with results of other studies and suggest that antiangiogenic mechanisms may be associated with IMC-C225 treatment (25, 37, 38). However, given the dramatic antitumor effect of combination therapy observed in these studies, it is difficult to determine to what extent generalized tumor necrosis contributed to the reduction of microvessels in treated tumors. We speculate that reduced vasculature in HT-29 and DLD-1 tumors treated with IMC-C225 and CPT-11 is because of both generalized tumor necrosis and a direct effect on neoangiogenesis.

A number of mechanisms have been identified that characterize the effect of IMC-C225 on the growth and survival of EGFR+ tumors in preclinical models (reviewed in Ref. 8). The direct mechanism responsible for IMC-C225's activity is inhibition of EGFR tyrosine kinase activation. The outcome of this blockade is reflected in the disruption of any number of processes regulated by the EGFR pathway in a given tumor cell. These processes include: (*a*) regulation of cell cycle progression; (*b*) cell survival pathways; (*c*) tumor cell invasion; and (*d*) angiogenesis. Several mechanisms appear to contribute to the enhanced cytotoxic response observed with combinations of IMC-C225 and chemo/radiation. Genotoxic damage induced by chemotherapy or radiation leads to induction of arrest at the G_1-S cell cycle checkpoint or activation of apoptosis; both are regulated by the EGFR signaling pathway. Disruption of EGFR-mediated survival signals and an increase in programmed cell death appear to be major mechanisms whereby IMC-C225 synergizes with cytotoxic agents (8, 11). Changes in the expression or activation of a number of molecules after IMC-C225 treatment contribute to this response; these include p27 (Kip1), cdk-2, Rb, Bcl-2, and Bax. Studies of sublethal DNA damage repair and potentially lethal damage repair analyses in cultured tumor cells have demonstrated a strong inhibitory effect of IMC-C225 on postradiation DNA damage repair (11). These studies have also shown that exposure of tumor cells to IMC-C225 induces a redistribution of DNA-dependent protein kinase from the nucleus to the cytosol and reduction in proliferating cell nuclear antigen expression, which is known to play a role in DNA repair (39). Hence, IMC-C225 inhibits both survival pathways and DNA repair mechanisms and, thus, enhances the apoptotic potential of chemotherapeutic drugs or radiation that induce cellular damage and programmed cell death. Finally, IMC-C225 may also enhance the response to cytotoxic therapy through mechanisms involving reduced angiogenesis and tumor cell invasion. The mechanisms responsible for the enhanced antitumor activity we observed in the present studies have not been fully defined. Our findings from analysis of HT-29 and DLD-1 tumors suggest that the enhanced antitumor effects IMC-C225/CPT-11 therapy are attributable to cell cycle, apoptotic, and possibly angiogenic mechanisms of tumor growth.

[3] M. C. Prewett, unpublished data.

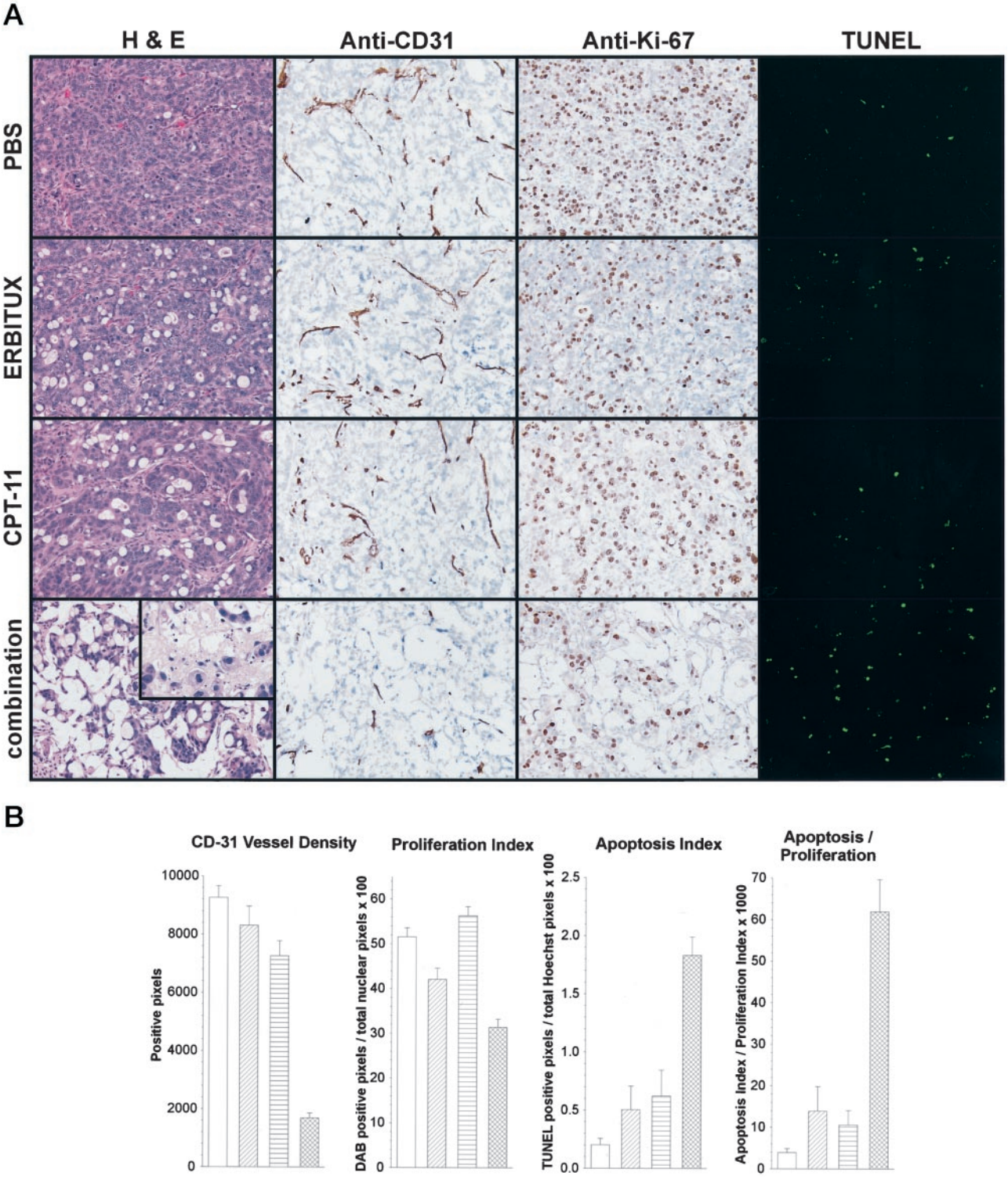

Fig. 4 *A*, histological examination of HT-29 tumor xenografts (day 47) stained with H&E, anti-CD31 vessel staining (*brown*), anti-Ki-67 nuclear antigen (*black*), and apoptosis by FITC-labeled TUNEL assay (*green*). (magnification: ×200). A higher magnification of combination therapy-treated HT-29 tumors stained with H&E is shown (*inset*) to illustrate necrosis in these tumors (×400). *B*, quantitation of CD31 vessel staining, Ki-67 Proliferation Index, Apoptosis Index, and Apoptosis:Proliferation Ratio. *Columns*, mean number positive pixels in 10 fields. *Bars*, ±SD. Tumors from mice treated with vehicle (*white*), IMC-C225 (*diagonal hatched*), CPT-11 (*horizontal hatched*), or IMC-C225 plus CPT-11 (*cross-hatched*).

However, additional tumorigenic regulatory processes, such as DNA repair and unknown mechanisms of CTP-11 resistance, are likely to play a contributing role. Additional studies will be necessary to fully elucidate the mechanisms contributing to the IMC-C225/CPT-11 antitumor response and determine which molecular pathways are involved in this response.

In conclusion, these results demonstrate enhanced antitumor activity of IMC-C225 combined with CPT-11 therapy compared with CPT-11 or IMC-C225 treatment alone. This combination was highly effective against established, CPT-11 refractory colorectal tumors. The precise mechanisms responsible for the combined effects of IMC-C225 and CPT-11 in these colorectal tumor models are still unclear and will require additional studies. Nevertheless, the ability of combination therapy to inhibit growth of chemoresistant colorectal carcinoma tumors suggests that EGFR blockade by IMC-C225 combined with CPT-11 has potential as a promising therapeutic strategy for clinical testing in CPT-11 refractory colorectal cancer.

Acknowledgments

We thank Dr. Peter Bohlen for critical review of the manuscript and Sarah Michaud for assistance in preparation of the manuscript. We dedicate this work to the memory of our good friend and colleague, Angel Santiago.

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