Antitumor Activity of Sequential Treatment with Topotecan and Anti-Epidermal Growth Factor Receptor Monoclonal Antibody C225[1]

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ABSTRACT

Epidermal growth factor (EGF)-related proteins such as transforming growth factor α (TGF-α) control cancer cell growth through autocrine and paracrine pathways. Overexpression of TGF-α and/or its receptor (EGFR) has been associated with a more aggressive disease and a poor prognosis. The blockade of EGFR activation has been proposed as a target for anticancer therapy. Monoclonal antibody (MAb) C225 is an anti-EGFR humanized chimeric mouse MAb that is presently in Phase II clinical trials in cancer patients. Previous studies have suggested the potentiation of the antitumor activity of certain cytotoxic drugs, such as cisplatin and doxorubicin, in human cancer cell lines by treatment with anti-EGFR antibodies. We have evaluated in human ovarian, breast, and colon cancer cell lines, which express functional EGFR, the antiproliferative activity of MAb C225 in combination with topotecan, a cytotoxic drug that specifically inhibits topoisomerase I and that has shown antitumor activity in these malignancies. A dose-dependent supraadditive increase of growth inhibition *in vitro* was observed when cancer cells were treated with topotecan and MAb C225 in a sequential schedule. In this respect, the cooperativity quotient, defined as the ratio between the actual growth inhibition obtained by treatment with topotecan followed by MAb C225 and the sum of the growth inhibition achieved by each agent, ranged from 1.2 to 3, depending on drug concentration and cancer cell line. Treatment with MAb C225 also markedly enhanced apoptotic cell death induced by topotecan. For example, in GEO colon cancer cells, 5 nM topotecan, followed by 0.5 µg/ml MAb C225, induced apoptosis in 45% cells as compared with untreated cells (6%) or to 5 nM topotecan-treated cells (22%). Treatment of mice bearing established human GEO colon cancer xenografts with topotecan or with MAb C225 determined a transient inhibition of tumor growth because GEO tumors resumed the growth rate of untreated tumors at the end of the treatment period. In contrast, an almost complete tumor regression was observed in all mice treated with the two agents in combination. This determined a prolonged life span of the mice that was significantly different as compared with controls ($P < 0.001$), to MAb C225-treated group ($P < 0.001$), or to the topotecan-treated group ($P < 0.001$). All mice of the topotecan plus MAb C225 group were the only animals alive 14 weeks after tumor cell injection. Furthermore, 20% of mice in this group were still alive after 19 weeks. The combined treatment with MAb C225 and topotecan was well tolerated by mice with no signs of acute or delayed toxicity. These results provide a rationale for the evaluation of the anticancer activity of the combination of topoisomerase I inhibitors and anti-EGFR blocking MAbs in clinical trials.

INTRODUCTION

Medical treatment of cancer is presently based on cytotoxic drugs that determine cell killing by acting on cellular targets that are generally common to both cancer and normal cells. Therapeutic regimens generally consist of a combination of two or more non-cross-resistant drugs. More recently, the increase in dose of various cytotoxic drugs to supertoxic levels obtained with the use of hematopoietic cell rescue has been tested and shown effective in some malignancies. However, several human cancer types are relatively insensitive or become resistant to the treatment with cytotoxic drugs.

The increasing understanding of the molecular mechanisms of neoplastic transformation and progression has prompted the search for biological agents interfering with the intracellular targets involved in this process. Several of these agents have recently entered clinical evaluation in cancer patients. In this respect, peptide growth factors are involved in regulating normal and cancer epithelial cell proliferation and differentiation (1, 2). EGF[3]-related growth factors, such as TGF-α, are potent

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[3] The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor α; MAb, monoclonal antibody.

mitogens for several human epithelial cell types including breast, colon, ovary, kidney, prostate, and lung and have been implicated in human cancer development and progression through autocrine and paracrine pathways (3). TGF-α binds to the extracellular domain of the EGFR and activates its intracellular tyrosine kinase domain (3). Enhanced expression of TGF-α and/or EGFR has been detected in the majority of human carcinomas (3) and has been associated with a poor prognosis in several human tumor types, such as breast cancer (4). For these reasons, the blockade of the TGF-α-EGFR autocrine pathway by using anti-EGFR blocking MAbs, recombinant proteins containing TGF-α or EGF fused to toxins, or EGFR-specific tyrosine-kinase inhibitors has been proposed as a potential therapeutic modality (5–12). Several blocking anti-EGFR MAbs that inhibit the *in vitro* and *in vivo* growth of human cancer cell lines that express TGF-α and EGFR have been generated (13–16). Among these, MAb 528 and MAb 225 are two mouse MAbs that have been extensively characterized for their biological and pharmacological properties and represent the first anti-EGFR blocking agents that have entered clinical evaluation in cancer patients (5, 6). MAb 528 and MAb 225 bind to the EGFR with affinity similar to TGF-α, compete with ligand for receptor binding, and block ligand-induced activation of EGFR tyrosine kinase (13, 14). To avoid human anti-mouse antibody production that could interfere with the therapeutic efficacy of repeated administrations of mouse MAbs, a chimeric human-mouse MAb 225 (MAb C225), which contains the human IgG1 constant region, has recently been developed and purified for clinical use and is in Phase II trials in patients with advanced cancer (17, 18).

In recent years, there has been a growing interest in combining conventional chemotherapeutic agents with biological agents that selectively inhibit key intracellular targets involved in the process of neoplastic transformation. In this respect, it has been shown that the combined treatment of mice bearing well-established human tumor xenografts with blocking anti-EGFR MAbs and with some cytotoxic drugs, such as doxorubicin and cisplatin, significantly increases the antitumor activity of these drugs (6, 19, 20). In the present study, we have investigated whether MAb C225 has any cooperative effect with topotecan in a series of human cancer cell lines *in vitro* and in nude mice bearing human GEO colon cancer xenografts. We have selected the cytotoxic agent topotecan, a novel promising chemotherapeutic agent that selectively inhibits the nuclear enzyme topoisomerase I, because it has shown antitumor activity in a variety of human malignancies, including ovarian cancer, small cell lung cancer, breast cancer, and colorectal cancer, in which EGFR is generally overexpressed (21).

MATERIALS AND METHODS

Materials. MAb C225 is a human-mouse chimeric anti-EGFR IgG1 class MAb, the biochemical and biological characteristics of which have been described previously (17). MAb C225 was kindly provided by Dr. H. Waksal (ImClone Systems, New York, NY). The topoisomerase I inhibitor topotecan was a generous gift of SmithKline Beecham Italia (Milan, Italy).

Cell Lines. GEO human colon cancer, OVCAR-3 human ovarian cancer, and ZR-75-1 human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). 1A9 and their paclitaxel-resistant derivative PTX-10 and PTX-22 human ovarian cancer cell lines (22) were kindly provided by Dr. A. Fojo (National Cancer Institute, Bethesda, MD). The p53 status of the cancer cell lines is the following: wild-type gene, GEO,[4] ZR-75-1 (23); point mutated gene, OVCAR-3, G to A in codon 248 (23); and unknown, 1A9, PTX-10, PTX-22. All cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine (ICN, Irvine, UK) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Growth in Soft Agar. Cells (10^4 cells/well) were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24-multi-well cluster dishes (Becton Dickinson, Lincoln Park, NJ) and treated with different concentrations of MAb C225 and/or topotecan. After 10–14 days, the cells were stained with nitro blue tetrazolium (Sigma Chemical Co., St. Louis, MO), and colonies larger than 0.05 mm were counted as described previously (24).

Apoptosis. To evaluate the induction of apoptosis, 10^5 cells were plated in complete medium in 60-mm tissue culture dishes (Becton Dickinson) and treated every day for a total of 3 days with different concentrations of MAb C225 and/or topotecan. For the combined treatment, cells were treated every day for 3 days with the indicated concentration of topotecan and then every day for 3 days with the indicated concentration of MAb C225. After 7 days, both adherent and detached cells were harvested. Flow cytometric analysis of apoptotic cell death was performed on cell pellet fixed in 70% ethanol, washed in PBS, and mixed with RNase (Sigma) and propidium iodide (Sigma) solution as reported previously (25). DNA content was analyzed by a FACScan flow-cytometer (Becton Dickinson, San Jose, CA) coupled with a Hewlett Packard computer, and the percentage of apoptotic cells was calculated by gating the hypodiploid region on the DNA content histogram using the LYSYS software (Becton Dickinson) as reported previously (25).

GEO Xenografts in Nude Mice. Five- to 6-week-old female Balb/cAnNCrlBR athymic ($nu+/nu+$) mice were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance with institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimated to the University of Naples Medical School Animal Facility for one week before being injected with cancer cells. Mice were injected s.c. with 10^7 GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA). After 7 days, when well-established tumors of ~0.2 cm^3 in diameter were detected, 10 mice/group were treated i.p. with topotecan alone (2 mg/kg/dose, twice weekly on days 1 and 2 for 2 weeks), with MAb C225 alone (0.25 mg/dose, twice weekly on days 3 and 6 for 5 weeks), or with both drugs. Tumor size was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter) (2), as reported previously (26). The exper-
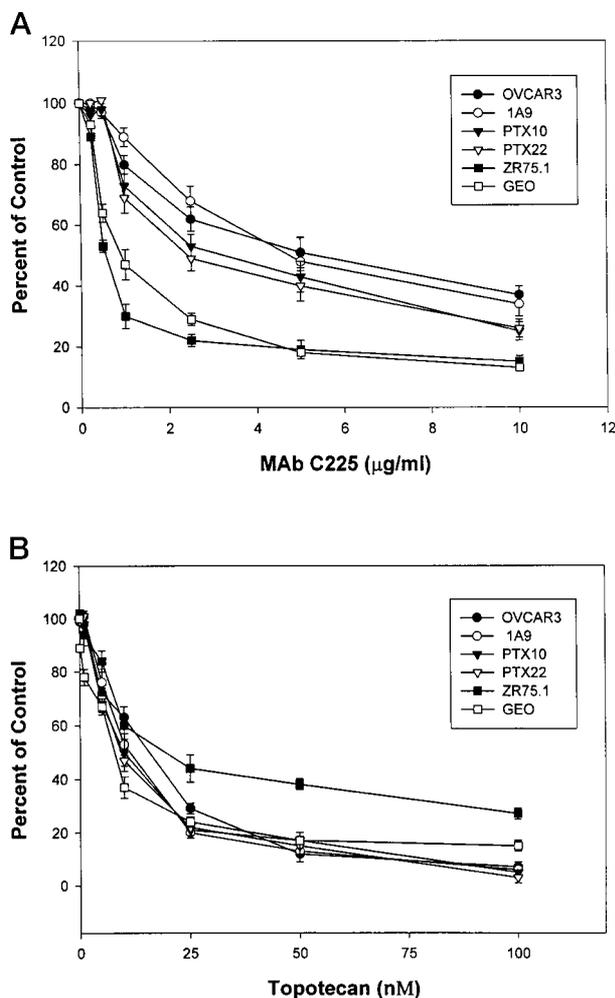
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[4] Unpublished observations.

Fig. 1 Dose-dependent growth inhibitory effects of MAb C225 (*A*) or of topotecan (*B*) on the soft agar growth of human cancer cell lines. Data represent the average of three different experiments, each performed in triplicate; *bars,* SD.

iment was repeated three times with a total of 30 mice for each group.

Statistical Analysis. The Student's *t* test (27) and the Mantel-Cox log-rank test (28) were used to evaluate the statistical significance of the results. All *P*s represent two-sided tests of statistical significance. All analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

RESULTS

As shown in Fig. 1, we first evaluated the effect of topotecan or MAb C225 treatment on the soft agar growth of various human epithelial cancer cell lines. For this purpose, we selected GEO colon cancer, ZR-75-1 breast cancer, and OVCAR-3 and 1A9 ovarian cancer cell lines. We also tested two paclitaxel-resistant derivatives of 1A9 cells that possess specific point mutations in the β-*tubulin* gene (22). All these human cancer cell lines have functional EGFR, ranging from ~20,000 (ZR-75-1) to 40,000 (GEO), 100,000 (1A9, PTX-10, and PTX-22), 150,000 (OVCAR-3) EGF binding sites/cell (24, 29).[4] Treatment with the anti-EGFR blocking MAb C225 determined a dose-dependent inhibition of colony formation in soft agar at doses ranging between 0.25 and 10 µg/ml in all cancer cell lines tested (Fig. 1*A*). Similarly, a dose-dependent, growth-inhibitory effect was observed when the different cancer cell lines were treated with the topoisomerase I-selective drug topotecan at doses between 1 and 100 nM (Fig. 1*B*). To determine whether the combined treatment with topotecan and MAb C225 could enhance the antiproliferative effect of single-agent treatment, the six cancer cell lines were treated with different combinations of the two compounds in a sequential schedule of treatment with topotecan followed by MAb C225. As shown in Figs. 2 and 3, a supraadditive growth-inhibitory effect was observed at all doses of MAb C225 and topotecan tested. Furthermore, when lower doses of these agents were used, the antiproliferative effect was clearly cooperative in all cell lines examined. For example, in GEO cells, treatment with 5 nM topotecan or with 0.25 µg/ml MAb C225 determined ~10% growth inhibition, whereas the sequential treatment caused a 50% inhibition of colony formation in soft agar (Fig. 2). The cooperativity quotient of this treatment, defined as the ratio between the actual growth inhibition obtained with topotecan followed by MAb C225 and the sum of the growth inhibition achieved by each agent, was approximately 2.5.

The supraadditive antiproliferative effects of the combination of topotecan and MAb C225 described above could be dependent on the sequence of treatment. We, therefore, tested an inverted treatment schedule in which cancer cells were treated first with MAb C225, then with the cytotoxic drug topotecan. In ZR-75-1, OVCAR-3, and GEO cancer cells pretreatment with either 0.25 or 0.5 µg/ml MAb C225 reduced the growth-inhibitory effect of topotecan at all doses tested (data not shown). Similar interference in growth inhibition was observed when treatment with topotecan and MAb C225 was concomitant (data not shown). These results suggest that treatment with the anti-EGFR blocking MAb should follow the cytotoxic drug in the sequence of treatment.

We next determined whether the cooperative growth-inhibitory effect of topotecan and MAb C225 could involve induction of programmed cell death in cancer cells. GEO and OVCAR-3 cells were treated with different concentrations of topotecan or MAb C225 for 3 days or with 5 nM topotecan for 3 days followed by 0.5 µg/ml MAb C225 for 3 days. The percentage of apoptotic cells was determined on day 7 from the beginning of treatment (Fig. 4). MAb C225 treatment alone did not induce apoptosis in either GEO or OVCAR-3 cells. Topotecan treatment increased in a dose-dependent manner apoptotic cell death both in GEO and OVCAR-3 cells. The sequential treatment with the two agents determined a marked induction of apoptosis in both cell lines. In fact, 5 nM topotecan, followed by 0.5 µg/ml MAb C225, induced apoptosis in 45% of GEO cells compared with untreated cells (6%) or to 5 nM topotecan-treated cells (22%; Fig. 4*A*). A similar potentiation of apoptosis was observed in OVCAR-3 cells (Fig. 4*B*). These results suggest that the cooperative antiproliferative activity of topotecan and MAb C225 is accompanied by induction of programmed cell death.
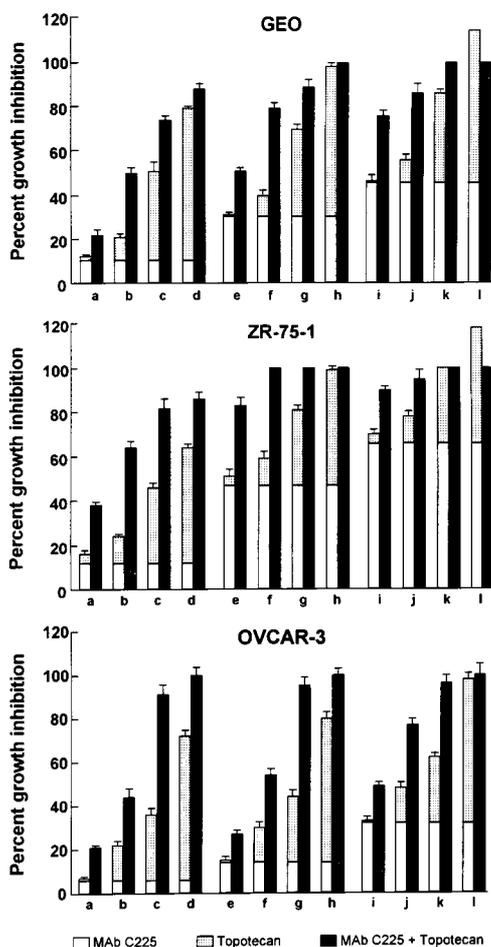
Fig. 2 Growth-inhibitory effects of the sequential treatment with topotecan and MAb C225 on the soft agar growth of GEO, ZR-75-1, and OVCAR-3 cancer cells. GEO: topotecan, 1 nM (*a, e,* and *i*), 5 nM (*b, f,* and *j*), 10 nM (*c, g,* and *k*), 25 nM (*d, h,* and *l*). MAb C225: 0.25 μg/ml (*a–d*), 0.5 μg/ml (*e–h*), 1 μg/ml (*i–l*). ZR-75-1: topotecan, 1 nM (*a, e,* and *i*), 5 nM (*b, f,* and *j*), 10 nM (*c, g,* and *k*), 25 nM (*d, h,* and *l*). MAb C225: 0.25 μg/ml (*a–d*), 0.5 μg/ml (*e–h*), 1 μg/ml (*i–l*). OVCAR-3: topotecan, 1 nM (*a, e,* and *i*), 5 nM (*b, f,* and *j*), 10 nM (*c, g,* and *k*), 25 nM (*d, h,* and *l*). MAb C225: 0.25 μg/ml (*a–d*), 0.5 μg/ml (*e–h*), 1 μg/ml (*i–l*). Cells were plated in soft agar as described in "Materials and Methods" and treated sequentially with topotecan on days 1 and 2 and with MAb C225 on days 3 and 5 or with each agent alone. Colonies were counted after 10–14 days. Data are expressed as the percentage of growth inhibition as compared with the growth of untreated control cells. For each pair of *columns,* the height of the *columns* on the *left* represents the sum of the effect of each agent and, therefore, the expected growth inhibition if their effect is additive when used in combination. The total height of the *columns* on the *right* indicates the observed growth inhibition when the agents are used in combination. The difference between the heights of the paired *columns* reflects the magnitude of cooperativity on growth inhibition. Data represent the average of three different experiments, each performed in duplicate; *bars,* SD.

We next evaluated whether the cooperative effect observed *in vitro* could be obtained also *in vivo.* We have previously studied the effect of different novel therapeutic agents including MAb C225 in nude mice bearing GEO colon cancer xenografts (26). GEO cells form moderately differentiated adenocarcino-
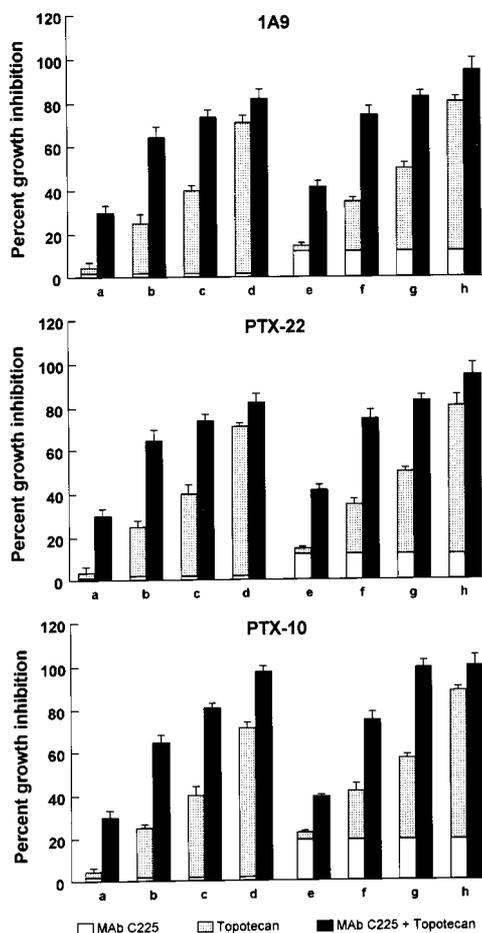
Fig. 3 Growth-inhibitory effects of the sequential treatment with topotecan and MAb C225 on the soft agar growth of 1A9, PTX-10, and PTX-22 cancer cells. Topotecan: 1 nM (*a* and *e*), 5 nM (*b* and *f*), 10 nM (*c* and *g*), 25 nM (*d* and *h*). MAb C225: 0.5 μg/ml (*a–d*), 1 μg/ml (*e–h*). Cells were plated in soft agar as described in "Materials and Methods" and treated sequentially with topotecan on days 1 and 2 and with MAb C225 on days 3 and 5 or with each agent alone. Colonies were counted after 10–14 days. Data are expressed as percentage of growth inhibition as compared with the growth of untreated control cells. For each pair of *columns,* the height of the *columns* on the *left* represents the sum of the effect of each agent and, therefore, the expected growth inhibition if their effect is additive when used in combination. The total height of the *columns* on the *right* indicates the observed growth inhibition when the agents are used in combination. The difference between the heights of the paired *columns* reflects the magnitude of cooperativity on growth inhibition. Data represent the average of three different experiments, each performed in duplicate; *bars,* SD.
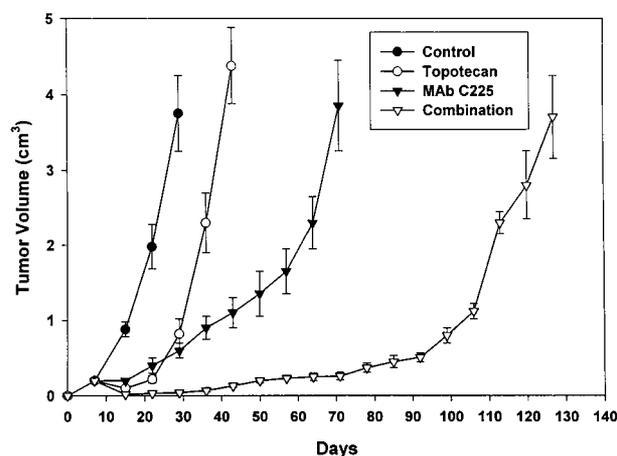
mas that express the EGFR and various specific ligands when injected s.c. in immunodeficient mice. Therefore, we used this model to investigate the antitumor activity of MAb C225 and topotecan combination. GEO cells were injected s.c. into the dorsal flank of nude mice. When established GEO tumors of ~0.2 cm^3 were detectable, mice were treated i.p. with topotecan, 2 mg/kg/dose, on days 1 and 2 of each week for a total of 2 weeks or with 0.25 mg/dose MAb C225, twice weekly on days 3 and 6 of each week for a total of 5 weeks, or with both compounds. Tumor size was measured weekly, and mice sur-
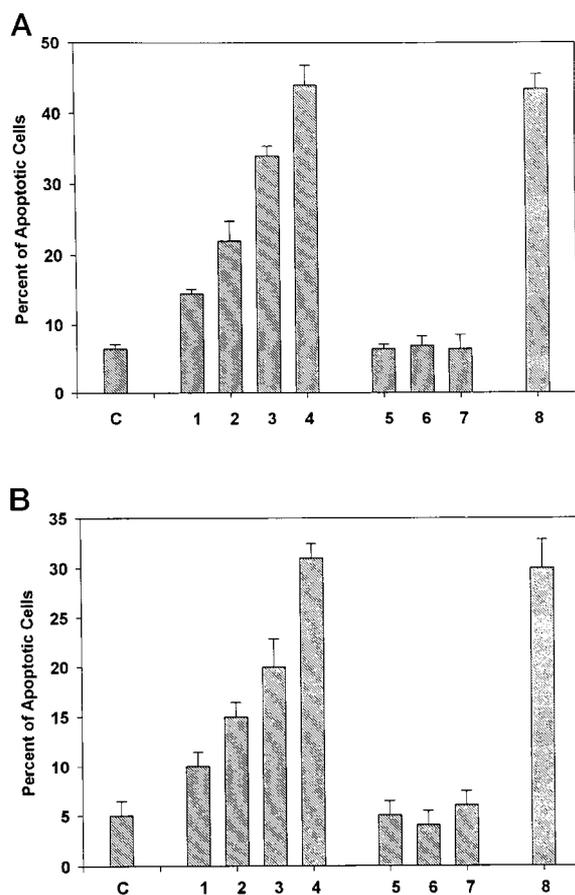
Fig. 4 Induction of apoptosis by treatment with MAb C225 and/or topotecan in GEO (*A*) or OVCAR-3 (*B*) cancer cells. Cells were treated with: topotecan, 1 (*column 1*), 5 (*column 2*), 10 (*column 3*), and 25 (*column 4*) nM each day for 3 days; MAb C225, 0.25 (*column 5*), 0.5 (*column 6*), 1 (*column 7*) μg/ml each day for 3 days; topotecan, 5 nM each day for 3 days followed by 0.5 μg/ml MAb C225, each day for 3 days (*column 8*). *C,* untreated control cells. Flow cytometric analysis of DNA fragmentation was performed 7 days after the beginning of treatment. Data represent the average of triplicate determinations; *bars,* SD.


Fig. 5 Antitumor activity of topotecan and MAb C225 on established GEO human colon carcinoma xenografts. Mice were injected s.c. in the dorsal flank with 10^7 GEO cells as described in "Materials and Methods." After 7 days (average tumor size, 0.2 cm³), mice were treated i.p. with topotecan alone (2 mg/kg/dose, twice weekly on days 1 and 2 of each week for 2 weeks), or with MAb C225 alone (0.25 mg/dose, twice weekly on days 3 and 6 of each week for 5 weeks), or with both drugs with the same sequential schedule. Each group consisted of 10 mice. The experiment was repeated for three times. Data represent the average on a total of 30 mice for each group; *bars,* SD. Student's *t* test was used to compare tumor sizes among different treatment groups at day 29 after GEO cell injection. MAb C225 *versus* control ($P < 0.001$); topotecan *versus* control ($P < 0.001$); topotecan followed by MAb C225 *versus* control ($P < 0.001$); topotecan followed by MAb C225 *versus* MAb C225 ($P < 0.001$); topotecan followed by MAb C225 *versus* topotecan ($P < 0.001$).

vival was monitored. As illustrated in Fig. 5, mice treatment with each agent inhibited tumor growth *in vivo* as compared with control untreated mice. However, shortly after the end of the 2 weeks of treatment with topotecan or of the 5 weeks of treatment with MAb C225, GEO tumors resumed the growth rate of untreated tumors. When the two agents were used in combination, an almost complete regression of tumors in all mice was observed. Furthermore, GEO tumor growth was significantly delayed in mice that received MAb C225 plus topotecan treatment. In fact, tumors grew very slowly for ~50–70 days following the end of treatment when they resumed a growth rate similar to controls (Fig. 5).

As shown in Fig. 6, GEO tumors reached a size not compatible with normal life in all untreated mice within 4–5 weeks. A small but significant increase in mice survival was observed in the group treated with topotecan alone ($P < 0.02$). MAb C225 treatment determined a longer survival as compared with the control group ($P < 0.001$) and to the topotecan treated-

group ($P < 0.001$). The delayed GEO tumor growth in the MAb C225 plus topotecan-treated group was accompanied by a prolonged life span of the mice that was significantly different as compared with controls ($P < 0.001$), to MAb C225-treated group ($P < 0.001$), or to topotecan-treated group ($P < 0.001$). In fact, all mice of this group were the only animals alive 14 weeks after tumor cell injection (Fig. 6). Furthermore, 20% of mice in this group were still alive after 19 weeks. The combined treatment with MAb C225 and topotecan was well tolerated by mice, because no weight loss or other signs of acute or delayed toxicity were observed.

DISCUSSION

The possibility of combining conventional cytotoxic drugs with novel agents that specifically interfere with key pathways controlling cancer cell survival, proliferation, invasion, and/or metastatic spreading has generated a wide interest in the past few years. In fact, this could be a promising therapeutic approach for several reasons: (*a*) because the cellular targets for these agents and their mechanism(s) of action are different from those of cytotoxic drugs, it is possible their combination with chemotherapy without cross-resistance; and (*b*) alterations in the expression and/or the activity of genes that regulate mitogenic signals not only can directly cause perturbation of cell growth but also may affect the sensitivity of cancer cells to conventional chemotherapy and radiation therapy (30). EGFR overexpression has been generally found in human cancer cell lines that are
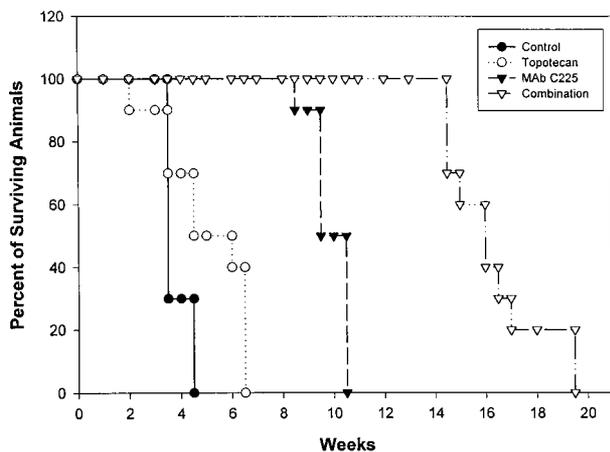
Fig. 6 Effects of topotecan and/or MAb C225 treatment on the survival of GEO tumor-bearing mice. The experimental design is described in the legend to Fig. 5. ●, Control; ○, topotecan; ▼, MAb C225; ▽, topotecan followed by MAb C225. Thirty mice/group were monitored for survival. Differences in animal survival among groups were evaluated using the Mantel-Cox log-rank test. Mice survival was significantly different between the MAb C225-treated group and the control group ($P < 0.001$); the MAb C225-treated group and the topotecan-treated group ($P < 0.001$); the topotecan-treated group and the control group ($P < 0.02$); the topotecan plus MAb C225-treated group and the control group ($P < 0.001$); the topotecan plus MAb C225-treated group and the MAb C225-treated group, ($P < 0.001$); and the topotecan plus MAb C225-treated group and the topotecan-treated group, ($P < 0.001$).

resistant to different cytotoxic drugs (31–33). In this respect, it has been postulated that EGFR overexpression would serve as a cell survival response to counteract apoptotic signaling in cancer cells exposed to a cytotoxic drug (33).

The results of the present study demonstrate that treatment with the anti-EGFR MAb C225 increases the topoisomerase I inhibitor topotecan-induced antiproliferative effects in several human epithelial cancer cell lines that express functional EGFR. In fact, blockade of EGFR after treatment with topotecan determines growth inhibition *in vitro* and *in vivo* that is not simply additive. In established GEO human colon carcinoma xenografts, treatment with each agent transiently inhibits tumor growth because tumors resume the growth rate of untreated control after cessation of therapy. In contrast, the sequential treatment with topotecan and MAb C225 determines tumor regression in all mice. In this group of mice, tumors grow very slowly for approximately 8–9 weeks following the end of treatment when they finally resume a growth rate similar to controls. This effect is accompanied by a significant benefit in animal survival in the group treated with both agents as compared with the groups treated with a single agent. In fact, 100% of mice treated with topotecan and MAb C225 are still alive 14 weeks after cancer cell injection, whereas all mice died within 6 or 10 weeks in the groups treated with topotecan or with MAb C225, respectively. This study also shows that the combined treatment with topotecan and MAb C225 is well tolerated by mice without signs of toxicity, suggesting that additional cycles of topotecan followed by MAb C225 could be given in an attempt to increase mice survival and possibly to obtain eradication of established tumors.

The growth-inhibitory effect of the combined treatment with topotecan and MAb C225 is schedule dependent because a clear cooperative antiproliferative activity is observed only when cancer cells are first treated with topotecan and then with MAb C225. This can be due to the effects on cell cycle determined by treatment with each agent. Topotecan is effective in killing cells mostly during the S and G_2 phases of the cell cycle (21), whereas the anti-EGFR blocking antibody MAb C225 determines a cytostatic growth-inhibitory effect with G_1 arrest (34, 35). Therefore, pretreatment with MAb C225 could render cells less responsive to topotecan. On the other hand, a possible mechanism by which MAb C225 exerts a supraadditive antiproliferative effect when administered following topotecan could be by increasing programmed cell death. We have observed a significant potentiation of apoptosis in both GEO and OVCAR-3 cells treated with topotecan and MAb C225 in a sequential schedule. In this context, the blockade of the EGFR-mediated signaling could render cancer cells unable to overcome the genotoxic damage induced by topotecan inhibition of topoisomerase I and, therefore, could render them more susceptible to enter programmed cell death.

The results of the present study extend those of previous reports that have shown a cooperative antitumor activity of some cytotoxic drugs such as cisplatin, doxorubicin, or paclitaxel with various blocking antibodies generated against the EGFR or the closely related type I tyrosine kinase receptor c-*erb*B-2 (15, 19, 20, 36–40). Therefore, treatment with agents that are able to selectively inhibit biochemical pathways controlling mitogenic signaling, DNA integrity, and/or cell cycle progression may cooperate with cytotoxic drugs by rendering cancer cells more susceptible to irreparable damage. In fact, an increase in apoptosis and growth inhibition induced by several drugs such as topoisomerase II inhibitors, taxanes, and platinum derivatives has been demonstrated in a large series of human cancer cell lines for agents that selectively inhibit type I protein kinase A, an intracellular serine-threonine kinase that acts down-stream to various mitogenic signals including the activated EGFR (41–43). Similarly, inhibition of c-*raf* expression by an antisense oligonucleotide enhances the antitumor activity of cisplatin, doxorubicin, or mitomycin C in human cancer tumor xenografts in nude mice (44). Taken together, these studies support the hypothesis that it is possible to enhance antitumor activity by treatment with maximum tolerated doses of cytotoxic drugs and specific inhibitors of signal transduction pathways instead of increasing chemotherapy doses to supertoxic levels that require complex medical support, including hematopoietic rescue (30). Because Phase I studies have shown that MAb C225 can be given to cancer patients with weekly repeated i.v. injections at doses that are well tolerated and that saturate the EGFR (45), our study provides a rationale for translating the combination of topotecan and MAb C225 into clinical trials. In this respect, preliminary data have shown the feasibility of a treatment with cisplatin and MAb C225 in patients with advanced head and neck or lung carcinomas (18). Similarly, a Phase II study has shown recently that recombinant humanized anti-c-*erb*B-2 MAb (Herceptin) could increase the antitumor activity of cisplatin in metastatic breast cancer patients overexpressing c-*erb*B-2 (46). Finally, a preliminary report of a randomized Phase III trial has demonstrated recently

that addition of Herceptin to first-line chemotherapy of advanced breast cancer patients with paclitaxel or doxorubicin increases the anticancer activity of chemotherapy alone (47).

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