Antitumor Effect of Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies plus cis-Diamminedichloroplatinum on Well Established A431 Cell Xenografts

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ABSTRACT

We have explored the therapeutic effects of anti-epidermal growth factor receptor monoclonal antibodies (MAbs) 225 and 528 on well established A431 epidermoid carcinoma xenografts, approximately 400 mm³ (1 cm in diameter) at the start of treatment. In previous reports we demonstrated that MAb 225 and 528 prevented the growth of A431 cell xenografts in nude mice when treatment was begun on the day of tumor cell inoculation. Since anti-epidermal growth factor receptor MAb therapy of well established tumors was unable to retard growth, we explored combination therapy with MAb plus the chemotherapeutic agent cis-diamminedichloroplatinum (cis-DDP). Additive and concentration-dependent growth-inhibitory effects of MAb with cis-DDP were observed in cultures of A431 cells. Neither intensive treatment with 225 MAb (1 mg/mouse, i.p. on day 8 after tumor inoculation, and twice weekly for 4 weeks) nor a maximally tolerated single dose of cis-DDP [150 μg/25 g (6 mg/kg) mouse weight, i.p. on day 8] had significant effects on tumor growth. However, the two treatments in combination resulted in substantial xenograft growth inhibition, compared with both an untreated control group and animals treated with a single modality. When a second dose of cis-DDP (150 μg/25 g) was added after 10 days, combination therapy with 225 MAb produced striking antitumor effects. At the end of 1 month tumor xenografts had disappeared in all but one mouse, and no tumor relapses occurred during 6 months of observation. Identical results were obtained with anti-epidermal growth factor receptor MAb 528 in combination with cis-DDP. The results of these studies provide a novel approach to the treatment of well established xenografts, which may have application in the therapy of human malignancies.

INTRODUCTION

High expression of EGF receptors and the presence of a potential TGF-α mediated autocrine stimulation pathway in many human cancers have stimulated investigation of therapy with MAbs that block binding of ligand to EGF receptors (1–10). We have produced and characterized two MAbs against the EGF receptor, 225 IgG1 and 528 IgG2a, which bind to the receptor with affinity similar to EGF and TGF-α, compete with these ligands for receptor binding, and block EGF receptor tyrosine phosphorylation (11–14). These MAbs inhibit proliferation of a variety of cultured malignant human cell lines which express EGF receptors and TGF-α, including with vulva (11–13), breast (15, 16), colon (17, 18), lung (19), renal (20), and prostate cancers (21). MAb 455, which binds to EGF receptors without inhibiting the binding of ligand, had no effect on proliferation of cultured cell lines (11, 22).

Additional experiments demonstrated the capacity of these anti-EGF receptor MAbs to produce concentration-dependent inhibition of xenografts of human tumor cell lines, which appeared to depend upon autocrine EGF receptor stimulation for growth in culture. A dose of 1 mg 225 or 528 MAb twice weekly, by i.p. injection, could sustain serum levels adequate to saturate EGF receptors (23). Anti-EGF receptor MAb therapy successfully inhibited the growth of xenografted tumor cell lines from squamous cancers of the vulva and lung, as well as adenocarcinomas of the breast and colon (18, 19, 23–27). Inhibitory activity against xenografts was also observed with the F(ab’)² fragment of 225 MAb, indicating that pharmacological blockade of EGF receptor by antibody without the capacity for an immune response can also mediate the antitumor effect in vivo (28). In these studies, treatment with MAb was begun the day of tumor cell inoculation.

To initiate investigation of the clinical application of our anti-EGF receptor MAbs, we performed a phase I clinical trial of a single dose of 111In-labeled 225 MAb (29). At MAb doses of 40 mg or higher, imaging studies on patients with advanced squamous cell carcinoma of the lung visualized each primary tumor and presumed sites of metastatic disease with diameter >1 cm. The serum concentration of 225 MAb could be maintained at >40 nM (receptor saturating levels) for more than 3 days without toxicity. Collectively, these preclinical and early clinical studies make a strong case for exploring the potential therapeutic role of anti-EGF receptor MAbs as pharmacological agents that block tyrosine kinase mediated signal transduction.

The premise for our approach is that anti-EGF receptor MAbs block the access of EGF or TGF-α to their receptors, resulting in the inhibition of tumor growth. Studies with cell cultures suggest that blockade of EGF receptors by MAbs results primarily in cytostatic rather than cytotoxic effects (11–14). Furthermore, treatment with MAb has not successfully eliminated well established xenografts (23). An exception is the DiFi colon adenocarcinoma cell line which is extremely sensitive to MAb treatment, both in culture and in xenografts (18). Therefore, for the treatment of most well established tumors, it was important to explore methods for enhancing the antitumor effects of anti-EGF receptor MAbs.

There has been interest in combining cytotoxic chemotherapeutic agents with growth-inhibitory biological agents, including MAbs against the EGF receptor (30, 31) and against HER2/c-erbB-2 protein (p185HER2) (32, 33). There is an excellent rationale for exploring these combinations. The antiproliferative effect of MAbs targeted against receptors on plasma membranes may be limited by competition with growth factors produced and released in the cellular environment. Chemotherapy can act to reduce the number of malignant cells, thereby reducing the concentration of essential autocrine growth factors produced in the immediate environment of residual viable cells. The potential of cell membrane receptors and signal transduction pathways to serve as opportune targets for cancer chemotherapy also has received considerable attention (34, 35). Furthermore, recent studies indicate that both chemotherapy-induced cytotoxicity and death of cells deprived of essential growth factors may involve programmed cell death or apoptosis (36–39), suggesting a common cytotoxic pathway that might be augmented by combination therapy.

cis-DDP is an alkylating agent which produces interstrand and intrastrand base cross-linking in DNA and is one of the most active drugs against the human epithelial tumors that express high levels of |cancerres.aacjournals.org on April 14, 2017. © 1993 American Association for Cancer Research.
whether the antitumor effect of anti-EGF receptor MAb against well-established A431 cell xenografts can be enhanced by combination treatment with cis-DDP.

MATERIALS AND METHODS

Materials. Human epidermoid carcinoma cell line A431 and anti-EGF receptor MAbs 225, 528, and 455 have been previously described (11, 12). BALB/c nude mice used in this study were bred and maintained in the animal facility at Memorial Sloan-Kettering Cancer Center as previously described (41). cis-DDP (platinol) was a gift from the Bristol Myers-Squibb Company.

Cell Culture and Growth Assay. A431 cells were maintained in 1:1 Dulbecco's modified Eagle's medium/Ham's F-12 mixture (v/v) supplemented with 10% fetal calf serum. Cells were distributed into 6-well plates, and treatment was started the next day. cis-DDP was freshly dissolved in phosphate-buffered saline and added to appropriate wells with or without anti-EGF receptor MAb at concentrations indicated in the figure legends. After 24 h, the drug was removed by washing the cells twice, followed by addition of cell culture medium and MAb. The medium and MAb were replenished every 2–3 days. After 6 days of culture, cells were harvested by trypsinization and counted with a Coulter counter.

A431 Cell Xenografts. BALB/c nude mice were implanted s.c. with 10⁷ A431 cells/mouse. Tumors were measured every 3–4 days with vernier calipers. Tumor size was calculated by the formula:

\[ \text{tumor size} = \frac{\pi}{6} ab^2 \]

where \( a \) is the length and \( b \) is the width, and \( a \geq b \). Animals with established tumor xenografts were divided into groups with comparable tumor size and treated, as described in the text and figure legends. Briefly, for anti-EGF receptor MAb treatment, mice received 1 mg of MAb i.p. in phosphate-buffered saline twice a week, starting on day 3, 5, or 8 after tumor cell inoculation and ending on day 32. Previous studies have established this as the dose of MAb required for prevention of tumor growth (23). Various doses and schedules of i.p. cis-DDP were explored in tumor-bearing nude mice, beginning 8 days after tumor cell inoculation. The mice were followed for the observation of xenograft growth rate, body weight changes, and life span.

RESULTS

Effects of Anti-EGF Receptor MAb 225 upon Well Established Tumor Xenografts. The data in Fig. 1 show that inhibition of A431 cell xenografts by 225 MAb is dependent upon tumor size. We demonstrated previously that anti-EGF receptor MAbs 225 and 528 prevented the formation of s.c. tumor xenografts when the nude mice were treated with 1 mg MAb i.p. beginning on the day of tumor cell inoculation and that treatment with an irrelevant IgG antibody had no inhibitory effect (23, 24). Treatment of nude mice with 225 MAb beginning 3 days after tumor cell inoculation, when the mean tumor size was about 150 mm³, also was able to inhibit the formation of A431 cell xenografts (Fig. 1). However, if MAb treatment was delayed until 5 days after tumor cell inoculation, when tumors reached a mean size of about 200 mm³, 225 MAb could only retard tumor xenograft growth. If the treatment was started 8 days after tumor cell inoculation, when the tumor was well established with a mean size of about 400 mm³ (approximately 1 cm in diameter), 225 MAb had almost no effect on xenograft growth. The experiment in Fig. 1 is representative of three independent studies showing limited response to MAb therapy at this and higher doses. Similar results also were observed with 528 MAb treatment (data not shown).

Additive Cytotoxicity of cis-DDP and Anti-EGF Receptor MAb in Cell Culture. Experiments were carried out to determine the effect of cis-DDP at subtoxic dose, both alone and combined with 225 or 528 MAb, on the growth of A431 cell cultures. Fig. 2A shows the response to increasing concentrations cis-DDP. To more closely mimic in vivo treatment conditions, the exposure time of A431 cells to cis-DDP was only for the first 24 h, and the cells were maintained in culture for an additional 5 days after removal of the drug. cis-DDP inhibited A431 cell proliferation in a dose-dependent fashion. The continuous presence of 20 nM 225 MAb (a saturating concentration) for 6 days produced additive inhibitory effects on cell growth. Conversely, this additive effect was also observed when the concentration of 225 MAb varied from 0.2 to 200 nM, in cultures treated for 24 h with 0.1 μg/ml (0.33 μM) cis-DDP (Fig. 2B). Identical results were obtained when 225 MAb was replaced by 528 MAb, which also blocks binding of EGF/TGF-α; however, no additive effect was found when 225 MAb was replaced by 455 MAb, which binds to EGF receptors but does not block binding of EGF/TGF-α (data not shown).

Toxicity and Antitumor Activity of cis-DDP on Xenografted Nude Mice. In preliminary studies, we found that nude mice bearing tumor xenografts were more vulnerable to toxicity from chemotherapy than non-tumor-bearing mice. Therefore, we explored the dose of...
cis-DDP tolerated by nude mice bearing well-established A431 cell xenografts. On the basis of the pilot data, a dose of cis-DDP at either 100 or 300 μg/25 g (4 or 12 mg/kg) mouse weight was selected for i.p. administration on day 8 after A431 cell inoculation. Fig. 3 presents the dose-dependent toxicity of cis-DDP on the xenografted nude mice. Mice receiving the higher dose (300 μg/25 g) of cis-DDP lost 10–15% of body weight and reduced their physical activities 1 week after chemotherapy, and all of these animals died within 44 days (Fig. 3B), in spite of a marked reduction in xenograft growth rate (Fig. 3A). The deaths observed in this higher cis-DDP dose group were attributed primarily to toxicity from cis-DDP. Treatment with the lower dose (100 μg/25 g) of cis-DDP had little effect on the size of the xenografts, and there was no detectable toxicity on the nude mice. Therefore, the deaths of mice treated with the lower dose of cis-DDP were mainly attributed to the tumor burden, as with the control mice (Fig. 3, A and B).

Combination Treatment of Xenografts with cis-DDP and Anti-EGF Receptor MAb. To explore whether cis-DDP could augment the weak antiproliferative effect of MAb against well-established A431 cell xenografts, combination treatment with cis-DDP and 225 MAb was administered. The therapy was started 8 days after A431 cells were inoculated and had reached a mean size of approximately 400 mm³ (1 cm in diameter). Based on the results of the toxicity studies, a single cis-DDP dose at 150 μg/25 g (6 mg/kg) mouse weight was administered. To examine other possible drug schedules, we also explored splitting the single cis-DDP dose (150 μg/25 g) into three injections of 50 μg/25 g at 1-week intervals in the same experiment. Fig. 4 shows the results of these experiments. As expected, 225 MAb (1 mg/mouse, twice weekly for 4 weeks) was unable to inhibit A431 cell xenograft growth. The single dose of cis-DDP (150 μg/25 g) on day 8 had a modest effect initially, but the tumors started to grow as rapidly as controls after day 15. However, xenograft growth was significantly inhibited by the combination of cis-DDP plus MAb treatment: the tumors remained at their initial size until day 22 and then began to resume growth at a reduced rate (Fig. 4A). The parallel study in Fig. 4B shows that the split dose of cis-DDP (50 μg/25 g) given every 7 days produced inhibition of xenograft growth that may have been modestly more effective than the single higher dose of cis-DDP (150 μg/25 g), with no significant change in the response to combination therapy.

For optimal combination therapy, these results suggest that a maximally tolerated dose of cis-DDP should be administered initially and that multiple doses of cis-DDP might be required. Therefore, an experimental protocol was designed to administer a second full dose of cis-DDP 10 days after the initial dose of cis-DDP (150 μg/25 g), when it could be tolerated. A striking antitumor response was observed. In the experiment shown in Fig. 5, the growth of A431 cell xenografts was completely suppressed, and the tumors gradually shrank. By day 32, after 8 injections of 225 MAb and two treatments with cis-DDP, tumors had been eliminated in 6 out of 7 treated mice. One mouse had a residual tumor less than 100 mm³. All of the mice in the 225 MAb alone, cis-DDP alone and control groups died or had to be sacrificed due to bulky tumor burdens. However, in the combination treatment group, mice with regressed tumors were observed for over 6 months and remained tumor-free. The single mouse treated with combination therapy which had a residual tumor lump also survived for over 6 months with a slowly growing tumor. These results were duplicated in subsequent experiments.

The treatment protocol in Fig. 5 was repeated using anti-EGF receptor MAb 528, which shares many properties with 225 MAb except that they differ in their IgG isotype. Fig. 6 shows the results obtained with combination therapy. There was complete tumor regression in each of the 7 mice in the 528 MAb plus cis-DDP combination treatment group, and these mice remained tumor free for over 6 months.

DISCUSSION

The present studies demonstrate curative therapy of well-established tumor xenografts (approximately 400 mm³) by treatment with an anti-EGF receptor MAb. This required combination therapy with the chemotherapeutic agent, cis-DDP. Successful tumor eradication depended upon preliminary experiments to determine maximum tolerated doses of chemotherapy (Fig. 3) as well as optimal dose and scheduling of MAb (23). Most importantly, we treated under conditions in which either MAb alone or cis-DDP alone had almost no capacity to inhibit tumor xenograft growth, thereby establishing that strong antitumor interactions result from combination therapy. We also have observed similar results with another drug, Adriamycin (31).

Combination treatment with anti-EGF receptor MAb 108 IgG₂a plus a single dose of cis-DDP has been shown previously to prevent the formation of human epidermoid carcinoma KB cell xenografts (30). Treatment of these xenografts was performed only 1 day after tumor cell inoculation. Another study examined treatment with anti-p185HER2 MAb TAB 250 in combination with cis-DDP against human SKOV3 ovarian tumor xenografts (32). Combination therapy resulted in the elimination of tumor xenografts less than 50 mm³ in size, but for larger tumors (150–200 mm³) this response to combined therapy was not observed.

We have demonstrated that A431 cell proliferation in culture is dependent upon autocrine stimulation of EGF receptors by growth factor (11–14, 42). What are the explanations for the apparent resis-
tance to MAb-mediated EGF receptor blockade when A431 cell xenografts become well established? First, we have observed that the inhibition of A431 cell proliferation by MAb was more prominent in low cell density cultures than in high cell density cultures (data not shown). A similar observation was also made with our anti-EGF receptor MAbs in cultures of colon and breast cells (22, 43). It was suggested that this may be attributed to low local concentrations of autocrine-derived TGF-α in the low density cell cultures, creating enhanced vulnerability to EGF receptor blockade by MAb. A comparable situation may occur when small numbers of tumor cells are injected into nude mice, because low concentrations of autocrine-derived TGF-α would be produced at the local site. Since administration of exogenous EGF stimulates the growth of A431 cell xenografts in nude mice (44), it is likely that autocrine-derived levels of TGF-α are less than optimal for A431 cell growth in vivo, and the local concentration of growth factor could be rate limiting when there are small numbers of tumor cells.

Second, the poor antitumor response to treatment of well established A431 cell xenografts with anti-EGF receptor MAb might be due to inadequate delivery of MAb to the tumor because of limiting blood supply or poor diffusion into tumor tissues. However, two lines of evidence suggest otherwise. We have examined the localization of anti-EGF receptor MAb in A431 cell xenografts after 2 weeks of therapy and find it well distributed in central portions of the tumor (data not shown). Furthermore, while A431 cell xenografts are resistant to MAb 225 therapy initiated on day 8, well established xenografts of other tumor cells can respond to anti-EGF receptor MAb treatment by arresting growth (23), or, in the case of DiFi cells, by undergoing total regression (18). These observations suggest that individual characteristics of particular tumor cells may account, in part, for the response to delayed initiation of anti-EGF receptor MAb therapy.

The mechanism(s) accounting for enhanced antitumor effects by combination treatment with anti-EGF receptor MAb and cis-DDP are under exploration. We have considered a number of possibilities. It is possible that two methods for inhibiting cell growth have produced additive effects by two independent pathways, and the apparent additive effects of anti-receptor MAb and cis-DDP in cell culture would support this explanation. However, the combination of MAb and cis-DDP clearly produced a dose- and schedule-dependent antitumor activity that was more than additive in the in vivo situation: under conditions in which single agent therapy had little effect, combined treatment completely eradicated well established tumors. The factors relating to increased efficacy of MAb-mediated receptor blockade when the tumor burden is small (see above), suggest that cytoreduction produced by cis-DDP could greatly enhance the antitumor activity of MAb. In our xenograft experiments, treatment with cis-DDP alone did not reduce the tumor burden to levels which were shown to respond to MAb alone. However, it is possible that a modest cytotoxic effect of chemotherapy caused changes in the microenvironment (e.g., increased vascular permeability) that could enhance the capacity of MAb to reach tumor cells.

It is interesting to speculate that the mechanism of cell death resulting from combined therapy involves a common pathway. Damage to cellular DNA by agents such as cis-DDP is followed by arrest of cell cycle traversal accompanied by active DNA repair. If this is
unsatisfactorily, programmed cell death is activated, leading to apoptosis (36-39). Cells deprived of essential growth factors also arrest cell cycle traversal, typically at a restriction point in G1 phase, and there is evidence that malignant cell cultures have reduced capacity to growth arrest when deprived of serum or growth factors (45). Recent studies have demonstrated that when some hematopoietic cell lines are deprived of essential growth factors they undergo apoptosis in cell culture (39). These observations suggest that the interactions of cis-DDP chemotherapy and EGF receptor blockade may have induced cell death by acting upon common biochemical pathways involving apoptosis.

Reduction in protein phosphorylation that results from blockade of EGF receptor-mediated kinase activity might also explain the efficacy of combination therapy. The possible roles of phosphorylation via this signal transduction pathway upon uptake of cis-DDP and upon repair of cis-DDP-mediated DNA damage are presently under exploration. The biochemical pathways that mediate cell cycle arrest are known to involve phosphorylation of a number of key mediators including, for example, p53 and cdc2 kinase. These phosphorylations are many steps removed from EGF receptor activation but might be influenced by blockade of receptor kinase activity.

Experiments are underway testing these possible mechanisms. We have demonstrated previously that treatment with a nonspecific polyclonal murine IgG can not substitute for anti-EGF receptor MABs in inhibiting the growth of xenografts alone (23) or combined with chemotherapeutic agent, doxorubicin (31). In addition, others have demonstrated that treatment of human tumor xenografts with tumor-inhibiting the growth of xenografts alone (23) or combined with anti-EGF receptor MAbs can not substitute for anti-EGF receptor MAbs inhibiting the growth of xenografts. Based on these observations, clinical trials of combination therapy with human chimerized MABs combined with cis-DDP chemotherapy, well established tumor xenografts can be successfully eliminated. Based on these observations, clinical trials of combination therapy with human chimerized MABs combined with cis-DDP chemotherapy will be carried out. This may be a novel form of therapy for the many types of human tumors which appear to depend upon EGF receptor stimulation for proliferation (11-21).

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REFERENCES


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