Antitumor Activity of Combined Treatment of Human Cancer Cells with Ionizing Radiation and Anti-Epidermal Growth Factor Receptor Monoclonal Antibody C225 plus Type I Protein Kinase A Antisense Oligonucleotide

Cataldo Bianco, Roberto Bianco, Giampaolo Tortora, Vincenzo Damiano, Patrizia Guerrieri, Paolo Montemaggi, John Mendelsohn, Sabino De Placido, A. Raffaele Bianco, and Fortunato Ciardiello


ABSTRACT

Recent studies have suggested that selective inhibition of mitogenic pathways may improve the antitumor activity of ionizing radiation. The epidermal growth factor receptor (EGFR) is overexpressed and is involved in autocrine growth control in the majority of human carcinomas. Protein kinase A type I (PKAI) plays a key role in neoplastic transformation and is overexpressed in cancer cells in which an EGFR autocrine pathway is activated. We used two specific inhibitors of EGFR and PKAI that are under clinical evaluation in cancer patients: C225, an anti-EGFR chimeric human-mouse monoclonal antibody (MAb); and a mixed-backbone antisense oligonucleotide targeting the PKAI Rα subunit (PKAI AS). We tested in human colon cancer (GEO) and ovarian cancer (OVCAR-3) cell lines the antiproliferative activity of MAb C225 and/or PKAI AS in combination with ionizing radiation. In vivo antitumor activity was evaluated in nude mice bearing established GEO xenografts. Dose-dependent inhibition of soft agar growth was observed in both cancer cell lines with ionizing radiation, C225, or PKAI AS oligonucleotide. A cooperative antiproliferative effect was obtained when cancer cells were treated with ionizing radiation followed by MAb C225 or PKAI AS oligonucleotide. This effect was observed at all doses tested in both GEO and OVCAR-3 cancer cell lines. A combination of the three treatments at the lowest doses produced an even greater effect than that observed when two modalities were combined. Treatment of mice bearing established human GEO colon cancer xenografts with radiotherapy (RT), MAb C225, or PKAI AS oligonucleotide produced dose-dependent tumor growth inhibition that was reversible upon treatment cessation. A potentiation of the antitumor activity was observed in all mice treated with RT in combination with MAb C225 or PKAI AS oligonucleotide. Long-term GEO tumor growth regression was obtained following treatment with ionizing radiation in combination with MAb C225 plus PKAI AS oligonucleotide, which produced a significant improvement in survival compared with controls (P < 0.001), the RT-treated group (P < 0.001), or the group treated with MAb C225 plus PKAI AS oligonucleotide (P < 0.001). All mice of the RT + MAb C225 + PKAI AS group were alive 26 weeks after tumor cell injection. Furthermore, 50% of mice in this group were alive and tumor-free after 35 weeks. This study provides a rationale for evaluating in cancer patients the combination of ionizing radiation and selective drugs that block EGFR and PKAI pathways.

INTRODUCTION

Treatment with ionizing radiation induces different biochemical effects in cancer cells, with activation of multiple signaling pathways that lead to either programmed cell death or cell proliferation. The latter effect is probably the result of activation of various mitogenic pathways (1, 2). It has recently been demonstrated that ionizing radiation induces the EGFR3/raf/raf/MAPK pathways through the direct activation of the EGFR tyrosine kinase and the release of TGFα, a specific ligand for EGFR (1, 2). This may be clinically relevant because it could represent a mechanism by which cancer cells become able to escape radiation-induced cell death. In this respect, EGF-related growth factors, such as TGFα, have been implicated in human cancer development and progression through autocrine and paracrine pathways (3). TGFα binds to the extracellular domain

Received 5/31/00; revised 8/17/00; accepted 8/22/0.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) and the Consiglio Nazionale delle Ricerche (CNR) Target Project on Biotechnologies.

2 To whom requests for reprints should be addressed, at Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli “Federico II,” Via S. Pansini 5, 80131 Naples, Italy. Phone: 39-081-7462061; Fax: 39-081-7462066; E-mail: fortunatociardiello@yahoo.com.

3 The abbreviations used are: EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; TGFα, transforming growth factor α; MAb, monoclonal antibody; PKAI, protein kinase A type I; PS, phosphorothioate; MBO, mixed-backbone oligonucleotide; RT, radiotherapy.
of EGFR and activates its intracellular tyrosine kinase domain (3). Ligand binding induces dimerization of EGFR and its autophosphorylation on several tyrosine residues in the intracellular domain, creating a series of high-affinity binding sites for various transducing molecules that are involved in transmitting the mitogenic signal through the rasraf/MAPK pathway (3).

Enhanced expression of TGFα and/or EGFR has been detected in the majority of human carcinomas and has been associated with poor prognosis (3). EGFR overexpression has also been found in human cancer cell lines that are resistant to different cytotoxic drugs (4, 5). For these reasons, blocking of the TGFα-EGFR autocrine pathway has been proposed as a therapeutic target (6). Several pharmacological and biological approaches have been developed for blocking EGFR activation and/or function in cancer cells. Anti-EGFR blocking MAbs, recombinant proteins containing TGFα or EGF fused to toxins, and EGFR-selective tyrosine kinase inhibitors have been characterized for their biological and potentially therapeutic properties (7–15). One of these agents, MAb C225, a chimeric human-mouse IgG1 MAb, has recently entered phase II and III clinical evaluation in cancer patients (16–19).

The cAMP-dependent PKA is an intracellular enzyme involved in controlling cell growth and differentiation (20). The PKAI isomor is overexpressed in human cancer and is directly involved in EGFR mitogenic signaling (21). We have shown that PKAI, through interaction of its RI subunit with Grb2 adapter protein, has structural interaction with the ligand-activated EGFR, cooperating in the propagation to MAPK of the mitogenic signal (22). Different PKAI inhibitors are under clinical development. Down-regulation of PKAI by unmodified or PS-modified antisense oligonucleotides targeting its RI subunit causes cell growth inhibition in a variety of human cancer cell lines and has antitumor activity in nude mice (23–25). Modified oligonucleotides of a novel class, defined as MBOs, have been synthesized recently and have significantly improved pharmacokinetic and toxicological properties in vivo compared with PS oligonucleotides (26, 27). In this respect, an antisense RI MBO with hybrid DNA/RNA structure containing 2′-O-methyl-ribonucleosides at the 5′ and 3′ ends (PKSI AS), has been synthesized (27). This MBO, named GEM231, has completed phase I clinical trials and has shown an improved safety profile and metabolic stability compared with first-generation PS oligonucleotides (28).

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. Previous studies have shown that treatment with MAb C225 or PKAI AS oligonucleotide potentiates the antitumor activity of several cytotoxic drugs in human cancer cells (25, 29–31). In this study, we evaluated whether a similar cooperative effect could be obtained when two human cancer cell lines (GEO and OVCAR-3) were treated with MAb C225 and PKAI AS oligonucleotide in combination with RT.

MATERIALS AND METHODS

Materials. MAb C225 is a human-mouse chimeric anti-EGFR IgG1 MAb, whose biochemical and biological character-
RESULTS

As shown in Fig. 1, we first evaluated the effects of ionizing radiation and/or MAb C225 treatment on the cloning efficiency of two human epithelial cancer cell lines in soft agar. We selected GEO colon cancer and OVCAR-3 ovarian cancer cell lines because they have functional EGFRs that have 

\[
\text{tumor size} = \frac{4}{3} \pi \text{g/ml} \times \text{diameter}^3
\]

as described in “Materials and Methods.” Data represent the average of three different experiments, each performed in triplicate; bars, SD.

Statistical Analysis. The Student’s *t* test and the Mantel-Cox log-rank test were used to evaluate the statistical significance of the results. All *P* values 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) as reported previously (31).

To determine whether combined treatment with ionizing radiation and MAb C225 could enhance the antiproliferative effect of single treatment, the two cancer cell lines were treated in a sequential schedule with ionizing radiation followed by MAb C225. A supra-additive growth inhibitory effect was observed at all doses of MAb C225 and ionizing radiation tested. When lower doses were used in combination, the antiproliferative effect was clearly cooperative in both GEO and OVCAR-3 cells. As an example, in GEO cells, treatment with 25 cGy of ionizing radiation or with 0.25 μg/ml MAb C225 produced ~10 or 22% growth inhibition, respectively, whereas sequential treatment caused 80% inhibition of colony formation in soft agar (Fig. 1A). The cooperativity quotient of this treatment, defined as the ratio between the actual growth inhibition obtained with ionizing radiation followed by MAb C225 and the sum of the growth inhibition achieved by each treatment, was ~2.5. We next evaluated whether a similar cooperative antiproliferative effect could be achieved by combining RT with the blockage of PKAI function by a specific PKAI AS oligonucleotide. As indicated in Fig. 2, in both GEO and OVCAR-3 cells, supra-additive inhibition of cloning efficiency in soft agar was obtained following sequential treatment with ionizing radiation and PKAI AS oligonucleotide. This effect was specific for the combined treatment with the PKAI AS oligonucleotide because treatment with a scramble control oligonucleotide at doses up to 1 μM did not enhance the antiproliferative effect of ionizing radiation in either GEO or OVCAR-3 cancer cells (data not shown).

On the basis of functional and biological interactions between EGFR and PKAI, we previously showed that a concomitant blockade of these two mitogenic pathways may represent a therapeutic strategy (21, 32–34). Therefore, we studied whether any cooperative antiproliferative effect could be obtained when the anti-EGFR blocking antibody MAb C225 and the PKAI AS oligonucleotide were used in combination with RT. Following ionizing radiation, a combination of these two agents caused an even greater inhibitory effect than that observed when a single agent was combined with RT in both GEO and OVCAR-3 cells (Fig. 3). In fact, at single-treatment doses that produced only 5–10% inhibition of colony formation in soft agar, the combination of the three treatments caused 75–85% inhibition of soft agar growth of GEO and OVCAR-3 cells.

We next evaluated whether the cooperative growth inhibitory effect of MAb C225 and PKAI AS following ionizing radiation treatment could also be obtained in vivo. GEO cells were injected s.c. into the dorsal flanks of nude mice. When established GEO tumors of ~0.2–0.3 cm³ were detectable, mice were given RT or were treated i.p. with PKAI AS oligonucleotide or with the anti-EGFR MAb C225. Fig. 4 shows that each treatment significantly inhibited GEO tumor growth in vivo in a dose-dependent fashion. However, this effect was reversible

---

**Fig. 1** Dose-dependent growth-inhibitory effects of the combined treatment of ionizing radiation and/or MAb C225 on the soft agar growth of GEO (A) and OVCAR-3 (B) cells. Cells were treated with different doses of ionizing radiation and/or the indicated concentrations of MAb C225 (μg/ml) as described in “Materials and Methods.” Data represent the average of three different experiments, each performed in triplicate; bars, SD.
because shortly after the end of the treatment with RT, MAb C225, or PKAI AS oligonucleotide, GEO tumors resumed a growth rate similar to that of untreated controls (data not shown). We next evaluated the effects of RT in combination with MAb C225 or with PKAI AS oligonucleotide on mice bearing GEO xenografts. As illustrated in Fig. 5, treatment with MAb C225 (1 mg/dose twice weekly for 3 weeks) after ionizing radiation (10 Gy/dose daily, days 1–4) suppressed tumor growth in all mice. In mice that received MAb C225 plus RT, GEO tumors grew very slowly for 50–60 days following the end of treatment; tumors then resumed a growth rate similar to controls (Fig. 5A). The delayed GEO tumor growth in the MAb C225 + RT-treated group of mice was accompanied by a prolonged life span that was significantly different from controls (P < 0.001), the MAb C225-treated group (P < 0.001), or the RT-treated group (P < 0.001). Furthermore, 20% of mice in this group were still alive without any evidence of tumor 35 weeks after the GEO cancer cells were injected (Fig. 5B). A similar but less pronounced potentiation of the antitumor activity of RT was observed when mice were treated with PKAI AS oligonucleotide (10 mg/kg/dose, injected on days 1–5 of each week for 3 weeks; Fig. 6).

We also evaluated the antitumor activity of the triple combination of ionizing radiation treatment and EGFR plus PKAI blockade. Treatment with MAb C225 + PKAI AS oligonucleotide after ionizing radiation produced complete tumor regression in all mice; this regression was maintained for >100 days after the end of treatment (Fig. 7A). This effect was reflected in both a significant increase in survival and in a high proportion of cures in the mice receiving the triple-combination treatment. As shown in Fig. 7B, GEO tumors reached a size not compatible with normal life in all untreated mice within 6 weeks. A small increase in mouse survival was observed in the group treated with RT alone (P < 0.05). Mice treated with MAb C225 + PKAI AS oligonucleotide survived longer than those in the control
Survival was markedly increased in the group of mice receiving the triple treatment ($P<0.001$). In fact, all mice of this group were alive 26 weeks after injection of GEO tumor cells.

Fig. 5  A, antitumor activity of ionizing radiation and MAb C225 on established GEO human colon carcinoma xenografts. GEO cells ($10^7$ suspended in 200 µl of Matrigel) were injected s.c. into the dorsal flanks of mice. After 7 days (average tumor size, 0.2–0.3 cm$^3$), mice were treated with ionizing radiation (RT; 10 Gy/dose daily, days 1–4 for a total of 40 Gy) alone, MAb C225 (1 mg/dose i.p., twice weekly on days 1 and 4 for 3 weeks) alone, or with a combination of both. Each group consisted of 10 mice. Data represent the average; bars, SD. Student's $t$ test was used to compare tumor sizes among different treatment groups at day 28 following GEO cell injection: $P<0.001$ for MAb C225 versus control; $P<0.001$ for RT versus control; $P<0.001$ for RT followed by MAb C225 versus control. $B$, effects of ionizing radiation and/or MAb C225 treatment on the survival of mice bearing GEO tumors. Ten mice per group were monitored for survival. Differences in survival among groups were evaluated using the Mantel-Cox log-rank test. Survival was significantly different between the MAb C225-treated group and the control group ($P<0.001$), the MAb C225-treated group and the RT-treated group ($P<0.01$), the RT-treated group and the control group ($P<0.02$), the RT plus MAb C225-treated group and the control group ($P<0.001$), the RT plus MAb C225-treated group and the MAb C225-treated group ($P<0.001$); and the RT plus MAb C225-treated group and the RT-treated group ($P<0.001$).
more, no histological evidence of GEO tumors was observed in 50% of the mice in this group 35 weeks after tumor cell injection.

**DISCUSSION**

The possibility of combining conventional anticancer treatments, such as cytotoxic drugs or RT, with novel drugs that selectively interfere with important pathways controlling cancer cell survival, proliferation, invasion, and metastasis has generated a wide clinical interest. This could be a promising therapeutic approach for several reasons. First, because the cellular targets for these agents and their mechanism(s) of action are...
different from those of cytotoxic drugs and ionizing radiation, their combination without potential cross-resistance is conceivable. Second, 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 chemotherapy and RT (35). In this respect, EGFR overexpression has generally been found in human cancer cell lines that are resistant to different cytotoxic drugs (3–5). Furthermore, ionizing radiation induces the activation of the EGFR tyrosine kinase and the release of its specific ligand, TGF-α (2). For these reasons, it has been postulated that EGFR overexpression and activation could be a survival response to counteract apoptotic signals in cancer cells exposed to ionizing radiation or to cytotoxic drugs (2, 35). In fact, it has been proposed that is possible to enhance the anticancer activity by treatment with maximum tolerated doses of cytotoxic drugs or of RT in combination with selective inhibitors of signal transduction pathways instead of increasing chemotherapy or ionizing radiation doses to supertoxic levels that require complex medical support for the cancer patient, such as hematopoietic cell rescue (35).

In the present study, we have shown that treatment with the anti-EGFR blocking chimeric human-mouse antibody MAb C225 potentiates the cytotoxic effects of ionizing radiation in human colon and ovarian cancer cell lines that express functional EGFR. The growth-inhibitory effect in vitro is accompanied by a marked increase in antitumor activity in vivo, suggesting that the EGFR blockade is able to overcome cancer cell survival signals induced by ionizing radiation treatment. These data are in agreement with and extend those of recent studies by Huang et al. (36), who evaluated the effects of MAB C225 on the radiosensitivity of human head-and-neck squamous carcinoma cell lines in vitro, and Milas et al. (37), who showed in vivo enhancement of tumor radioresponse by MAB C225 treatment in nude mice bearing A431 human epidermoid carcinoma xenografts. Furthermore, our study is the first report of a cooperative antiproliferative effect of blocking of PKAI, a serine-threonine kinase acting downstream to EGFR, in combination with RT. The growth-inhibitory effects of MAB C225 and/or PKAI AS treatment in combination with RT seems to be p53-independent because similar results have been obtained in human cancer cells bearing either a normal wild-type or a mutated p53 gene.

In this study, we also demonstrated that the combined blocking of EGFR and PKAI function and signaling by treatment with MAB C225 and a PKAI AS oligonucleotide following ionizing radiation results in even more efficient cytotoxic activity. In fact, established GEO tumors were eradicated in 50% of mice receiving a relatively short-term treatment with one course of ionizing radiation followed by MAB C225 plus PKAI AS oligonucleotide for 3 weeks.

The results of this study are of potential clinical interest. In fact, they provide a rationale for the combination of MAB C225 and PKAI AS oligonucleotide in the treatment of human epithelial cancers after RT. MAB C225 is in phases II-III clinical development, both alone and in combination with cytotoxic drugs or with RT. In this respect, a pilot phase I study has suggested high antitumor activity of MAB C225 in combination with RT in stage III-IV head-and-neck cancer patients that is maintained as a complete response in 13 of 15 treated patients, with the response lasting 12–27 months (19, 38). Furthermore, the PKAI AS oligonucleotide that we used in the present study has completed phase I evaluation in cancer patients and is in phase II trials (28).

ACKNOWLEDGMENTS

We thank Dr. S. Agrawal for the gift of the MBO oligonucleotides. We also acknowledge the excellent technical assistance of G. Borriello.

REFERENCES


Antitumor Activity of Combined Treatment of Human Cancer Cells with Ionizing Radiation and Anti-Epidermal Growth Factor Receptor Monoclonal Antibody C225 plus Type I Protein Kinase A Antisense Oligonucleotide

Cataldo Bianco, Roberto Bianco, Giampaolo Tortora, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/6/11/4343

Cited articles
This article cites 38 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/6/11/4343.full#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/6/11/4343.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.