Radiosensitization of Tumor-targeted Radioimmunotherapy with Prolonged Topotecan Infusion in Human Breast Cancer Xenografts

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ABSTRACT

Clinical radioimmunotherapy (RIT) of solid tumors holds great promise, but as yet has been unable to deliver tumoricidal radiation doses without unacceptable toxicity. Our experimental approach aims to potentiate the therapeutic action of radioimmunoconjugates at the tumor site and thus improve the efficacy of RIT by combination with other treatment modalities. The topoisomerase I inhibitors are a unique class of chemotherapeutic agents that interfere with DNA breakage-reunion by inhibiting the action of topoisomerase I. Preclinical studies suggest that prolonged infusion of topoisomerase I inhibitors enhances cell toxicity due to ionizing radiation. We evaluated the efficacy of combined treatment with continuous administration of topotecan and 90Y-MX-DPTA BrE3 monoclonal antibody (which recognizes an epitope of breast epithelial mucin expressed in most breast cancers) on human mammary carcinoma xenografts in nude mice. Topotecan or 90Y-BrE3 treatment alone delayed overall tumor growth rate transiently but did not affect survival. The combination of RIT with topotecan substantially reduced growth of relatively large established tumors and caused complete tumor regressions and prolonged tumor-free survival in a substantial proportion of treated animals. In vitro studies demonstrated an increase in apoptotic rate and a decrease in cell proliferation of tumor cell lines treated with this combination. We combined the radiosensitization property of topotecan and the specificity of systemic RIT to establish a novel therapy for solid tumors in an experimental tumor xenograft model.

INTRODUCTION

Breast cancer continues to be the second leading cause of cancer in women in the United States, with more than 45,000 deaths/year (1). Early detection, improved surgical techniques, and more aggressive systemic therapy have improved quality of life and survival for patients with less advanced disease. Unfortunately, patients who have metastatic tumors continue to have a very grim prognosis with a median survival of <2 years. Although chemotherapy and hormonal therapy are effective in shrinking tumors in patients with metastases, median time to treatment failure is ~9 months, and survival prolongation is usually <6 months. The prevalence of the disease and its refractory nature have been the impetus for novel therapeutic approaches to metastatic and recurrent breast cancer.

RIT\(^2\) is a promising therapeutic modality for the treatment of a wide variety of malignancies. Tumor responses have been reported from multiple clinical trials using this type of therapy in patients with advanced lymphoma, leukemia, and breast cancer (2, 3). The antitumor effect of RIT is primarily due to the radioactivity targeted to the tumor by an antibody, which emits continuous exponentially decreasing low-dose-rate irradiation with heterogeneous dose deposition. Although tumor regressions are probably the result of the targeted radiation alone, the extent of the response often exceeds that expected for the estimated radiation dose and dose rate. In some situations, the antibody itself may thus contribute to tumor killing as well.

However, the potential for RIT of solid tumors has not been realized mainly due to the relatively small amounts of radiolabeled antibody that can be targeted to tumors and the dose-limiting toxicity associated with systemic administration of large levels of radioactivity. A variety of methods have been investigated to improve the tumor targeting of radioimmunoconjugates including the use of antibody fragments (4), targeting with recombinant single-chain antibodies (5), and administration of agents to modulate tumor vasculature (6). An alternative approach is to potentiate the therapeutic index of radioimmunoconjugates at the tumor site and thus improve the efficacy of RIT.

Many studies have demonstrated that topoisomerase I inhibitors can potentiate the lethal effects of ionizing radiation on tumor cells (7, 8). Type I topoisomerases generate single-strand DNA breaks and relieve torsional stress by unwinding duplex DNA. During the relaxation reaction, a covalent bond is formed between a tyrosine group of topoisomerase I inhibitor and the 3'-phosphoryl end of the broken DNA strand, creating transient protein-DNA cross-links. Topotecan stabilizes this complex, preventing the religation of the DNA strand. Interaction between the stabilized tertiary complex and the replication fork is thought to convert single-strand breaks into double-strand breaks and cause cell death (9, 10). Initial studies done by Giovanella et al. (11) demonstrated improved treatment efficiencies of several active camptothecin analogues with prolonged exposure in a xenograft model. This concept of prolonged exposure of topoisomerase I inhibitor was translated into improved efficacy and tolerance clinically in Phase I and subsequent Phase II studies with topotecan in humans (12, 13).

The purpose of this study was to develop an effective strategy for the treatment of breast cancer by capitalizing on the potential synergy between a chemotherapeutic agent, which selects for the enhanced DNA damage in tumor cells, and RIT. We investigated the therapeutic efficacy and the tumor-killing mechanism of this novel combined modality of continuous administration of topotecan and 90Y-BrE3 RIT for human mammary carcinoma in a nude mouse xenograft model.

MATERIALS AND METHODS

BrE3 Antibody. BrE3 antibody is a murine IgG1 MAb that reacts with a polypepticic 400-kDa moiety of breast epithelial mucin (14). The antibody was developed at the Cancer Research Institute of Contra Costa and has been shown to react with 90% of breast carcinomas tested by immunopathology as well as pancreatic and ovarian carcinomas. It shows minimal cross-reactivity with normal breast tissue. The murine antibody was provided by Coulter Immunology (Division of Coulter Corp., Hialeah, FL) as a sterile, low pyrogen solution in both its unconjugated form and as BrE3 antibody conjugated with

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3 The abbreviations used are: RIT, radioimmunotherapy; MAb, monoclonal antibody; MX-DPTA, 1,4-methylbenzyl isothiocyanate-diethylenetriamine pentaacetic acid; MTT, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide.

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MX-DPTA for labeling with $^{90}$Y. Humanized BrE3 MAb is a human IgG1 isotype with greater than 90% homology with human IgG1 (provided by Cancer Research Institute of Contra Costa, Walnut Creek, CA). The humanized construct has almost 3 times the affinity for the epitope as the murine version (15).

$^{90}$Y Radiolabeling of MX-DPTA-BrE3. Pharmaceutical grade $^{90}$Y (Pacific Northwest Laboratory) was buffered in 0.05 M acetate, incubated with MX-DPTA-BrE3 for 1 h at room temperature and equilibrated with 5 mM EDTA. Purification was performed on a Bio-Gel P6 column eluted with 1% HSA. Instant TLC was performed to determine percent radioactivity present as $^{90}$Y-MX-DPTA-BrE3. Unlabeled antibody was mixed with radiolabeled antibody with a specific activity that ranged from 4.2 to 7.1 $\mu$Ci/µg so that the administered protein dose was 50 $\mu$g and the $^{90}$Y dose ranged between 180 and 200 $\mu$Ci of 100 $\mu$g. This dose was chosen to exceed the 90% MTD (200 $\mu$Ci of $^{90}$Y-BrE3, as determined in preliminary studies, data not shown). Immunoreactivity was assessed using BrE3 antigen-coated beads (15).

**In Vitro Measurement of Cell Proportionation.** $^{90}$Y-BrE3, topotecan and the combination were tested for in vitro effect on cell proliferation. The tumor cell lines that we used for in vitro studies were MDA-MB-435 and MDA-MB-157 human mammary carcinomas. MDA-MB-157 cell line expresses the BrE3 epitope, whereas MDA-MB-435 stains negative for the BrE3 epitope (16). The colorimetric (MTT) assay was used to determine cell survival and proliferation; $1 \times 10^4$ cells were found to be within the linear range for MTT absorbance readings for both of these cell types. Briefly, $1 \times 10^4$ cells were plated in 96-well plates overnight in DMEM with 10% FBS. $^{90}$Y-BrE3 (2–8 $\mu$Ci) was added to one-half of the wells and incubated for 1 h. Cells were washed three times to remove unbound antibodies. Escalating concentrations of topotecan (0–10 $\mu$m) were then added to the appropriate wells. Cells incubated in media only served as the control. Plates were incubated for 3 days, and MTT was then added and allowed to incubate for an additional 4 h. Cells were lysed with HCI-isopropyl alcohol, and absorbance was measured on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentage of cell growth inhibition was calculated by $\frac{[A_{control} - A_{treatment}] / A_{control} \times 100]}{100}$.

**In Vitro Measurement of Apoptosis.** MDA-MB-435 and MDA-MB-157 human mammary carcinoma cells were plated in 96-well plates at $1 \times 10^4$ cells/well and incubated overnight in DMEM with 10% FBS. $^{90}$Y-BrE3 (1–8 $\mu$Ci) was added to one-half of the wells and incubated for 1 h. The cells were washed three times to remove the unbound antibody. Escalating concentrations of topotecan (0–10 $\mu$m) were then added to the appropriate wells. At the end of 3 days of incubation, cells were lysed, and the supernatant collected for determining the level of apoptosis using an ELISA. (Cell Death Detection ELISA; Boehringer Mannheim, Indianapolis, IN). The results of this assay are presented as the increase in apoptotic ratio as compared with untreated controls. For each cell line and $^{90}$Y-BrE3-topotecan combination, the corrected absorbance measurement of apoptosis $A_{apoptotic}$ of treated samples was normalized by the respective untreated cellular control, and this was plotted as a function of the $^{90}$Y-BrE3 concentration.

**Mice.** Athymic female nude mice. 8–10 weeks old, were obtained from Taconic Animal Laboratory (Germantown, NY). Animals were treated in accordance with the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals published by the NIH. All experiments were approved by the Institutional Animal Care and Use Committee of New York University. Mice were kept under sterile conditions in a laminar flow room in a Petri dish, and incubated in a 5% CO2 humidified incubator for 14 days. The number of animals received only 2 mg/m$^2$/day topotecan only. Previously in our laboratory, −200 $\mu$Ci of $^{90}$Y-BrE3 were determined to be the maximum tolerated dose in this experimental mouse model. We chose to adopt a dosing schedule in mice that in humans has proved to be better tolerated and more active than bolus administration (13, 17). Each group consisted of five animals. Bio weights and tumor dimensions were measured every 3–4 days. Animals were followed until death or terminated as soon as the tumor size exceeded 750 mg.

After the optimal combined treatment was determined, treatment efficacy was evaluated initially in nude mouse with moderate tumor burden. Athymic female nude Swiss NIH mice were implanted with human mammary carcinoma (MX-1) on day 0. On day 14 when tumors were −100 mg, mice were randomized into four treatment groups: control (no treatment); BrE3 alone (i.p. 200 $\mu$Ci of $^{90}$Y-BrE3); Topo alone (topotecan 1 mg/m$^2$/day for 14 days via s.c. Alzet pump); BrE3 and Topo (i.p. 200 $\mu$Ci of $^{90}$Y-BrE3 and topotecan 1 mg/m$^2$/day for 14 days). Each group consisted of five to seven animals. Body weights and tumor weights were measured every 3–4 days. Animals were followed until death or terminated as soon as tumor size exceeded 750 mg.

In Vitro Combined Therapy for Large Existing Tumor Xenografts. Athymic female nude Swiss NIH mice were implanted with human mammary carcinoma (MX-1) on day 0. On day 21 post-tumor implantation when tumors were −200–250 mg, mice were randomized into six treatment groups: control (no treatment); BrE3 alone (i.p. 180 $\mu$Ci of $^{90}$Y-BrE3); Topo alone (topotecan 1 mg/m$^2$/day for 14 days via s.c. Alzet pump); BrE3 and Topo (i.p. 180 $\mu$Ci of $^{90}$Y-MOPC-21, a murine nonspecific isotype-matched MAb; Sigma Chemical Co., St. Louis, MO); MOPC-21 and Topo. Body weights and tumor weights were measured every 3–4 days. Animals were followed until death or designated as a death and terminated as soon as tumor size exceeded 750 mg. Experiments were performed twice with 5–7 animals per group; the total number of animals per group ranged from 10 to 13 animals.

**Isolation of Bone Marrow Cells and Toxicity Assessments.** Nude mice bearing MX-1 tumors were randomized to receive 200 $\mu$Ci of $^{90}$Y-BrE3 with or without continuous infusion of topotecan (1 mg/m$^2$/day for 14 days). Three animals from each group were sacrificed, and blood and bone marrow were collected at 24 h, 48 h, 72 h, 7 days, and 14 days after administration of respective treatments. At sacrifice, the animal pelt was wet thoroughly with 70% isopropyl alcohol and peeled back to expose hind limbs. Using sterile scissors to separate the femur by cutting the hip and knee joints, ends of the long bone were trimmed to expose the interior of the marrow shaft. A 26-gauge hypodermic needle was inserted into the open shaft to flush bone marrow cells with sterile saline into a Petri dish. Bone marrow cells were aspirated through a 25-gauge hypodermic syringe several times to break up cell clumps. Bone marrow cells were counted on a hemocytometer, and WBC, platelet counts, and hematocrits were measured on heparinized capillary tubes, and WBC, platelet counts, and hematocrits were assessed.

**Data Analysis.** All comparisons were calculated with the StatView statistical program (SAS Institute Inc., Cary, NC) using ANOVA and where significance was found; individual groups were compared by the unpaired t test. Synergy of combined therapies effects were calculated with Calcusyn Statistical package. Significance was considered as $P < 0.05$. Data are presented as mean ± SD, unless otherwise indicated.
RESULTS

Effect of RIT on Cell Proliferation and Apoptotic Rate in MDA-MB-157 and MDA-MB-435 Cells Treated with Topotecan.

Fig. 1 illustrates the tumor growth inhibition in cells treated with topotecan alone, 90Y-BrE3 alone, or the combined therapy. In both cell lines, topotecan exerted a dose-dependent reduction of cell proliferation. However, in the presence of 90Y-BrE3 MAbs, only the MDA-MB-157 cell line, which retained 90Y-BrE3 MAbs, exhibited a further increase in cell growth inhibition. The combination of antibody and topotecan caused an additive suppression of cell growth only in the MDA-MB-157 cell line.

Fig. 2 depicts the enhancement of apoptosis in treated cells. Topotecan induced a dose-dependent increase of apoptosis in both MDA-MB-157 and MDA-MB-435 cell lines. 90Y-BrE3 caused increased apoptosis only in MDA-MB-157 cells. However, no increase was seen in MDA-MB-435 cells treated with both topotecan and 90Y-BrE3 MAbs. For MDA-MB-157 cells treated with the combination, an additive effect on apoptosis was observed.

In Vivo Dosage Escalation of Topotecan in Combination with 90Y-MX-DTPA BrE3 MAb.

Fig. 3 shows the effect of escalating doses of topotecan in combination with 90Y-BrE3 on tumor growth. 90Y-BrE3 treatment or 2 mg/m2/day topotecan alone had only a slight effect on tumor growth as compared with the untreated tumor-bearing control (P = 0.06). There were no statistically significant differences in tumor responses in groups treated with 0.2 mg/m2 (325 ± 23 mg, P = 0.1) and 0.5 mg/m2 (289 ± 24 mg, P = 0.09) topotecan in combination with 90Y-BrE3 as compared with the group treated with 90Y-BrE3 alone (321 ± 21 mg). All animals that received 2 mg/m2/day...
day topotecan in combination with $^{90}$Y-BrE3 died within 8 days of treatment. However, there was a substantial reduction in tumor growth and frank tumor regression in animals treated with 1 mg/m$^2$/day topotecan in combination with $^{90}$Y-BrE3 ($86\pm15$ mg, $P<0.05$). No mortality or weight loss was noted in this treatment group.

Fig. 4 depicts the effect of 1 mg/m$^2$/day topotecan administered continuously for 14 days in combination with 200 $\mu$Ci of $^{90}$Y-BrE3 on MX-1 tumor growth. Treatments were initiated 14 days after tumor implantation (mean tumor weights, 65 $\pm$ 10 mg). On day 34 post-tumor implantation, there was no significant difference between the untreated mice and those treated with 1 mg/m$^2$/day topotecan alone; however, mice treated with 200 $\mu$Ci of $^{90}$Y-BrE3 had a significant reduction in tumor growth ($187\pm23$ mg, $P<0.05$) as compared with untreated mice ($413\pm54$ mg). More dramatically at day 34, the mice treated with the combined therapy of 1 mg/m$^2$/day topotecan and 200 $\mu$Ci of $^{90}$Y-BrE3 had a significant regression of tumor ($20\pm4$ mg, $P<0.001$), with complete tumor eradication in six of seven mice.

In Vivo Combined Therapy for Nude Mice with Large Existing Tumor Xenografts. Fig. 5 shows the effect of 1 mg/m$^2$/day topotecan administered continuously for 14 day in combination with 180 $\mu$Ci of $^{90}$Y-BrE3 on the growth of large established MX-1 tumors. Treatments were initiated 14 days after tumor implantation (mean tumor weights, 223 $\pm$ 22 mg). All untreated tumor-bearing mice were dead by 69 days after tumor implantation with mean tumor weights of 737 $\pm$ 17 mg of $^{90}$Y-BrE3 or topotecan treatment alone only reduced tumor growth transiently; eventually, tumors in these two groups resumed growth at a rate similar to controls and grew to sizes comparable with those of untreated tumor-bearing mice ($675\pm39$ and $719\pm59$ mg, respectively) at day 89 post-tumor implantation. Animals that received combined treatment of 1 mg/m$^2$ topotecan and 180 $\mu$Ci of $^{90}$Y-MX-DTPA-BrE3 had a substantial reduction in tumor weight ($43\pm26$ mg, $P<0.001$), which resulted in complete tumor regression in 10 of 13 mice ($P<0.001$, compared with untreated controls).

Treatment with isotype-matched, nonspecific MAbs ($^{90}$Y-MOPC-12) alone did not have any significant effect on tumor growth; mean tumor weight was 749 $\pm$ 25 mg at day 75 post-tumor implantation. The synergistic effect of combined treatment with $^{90}$Y-BrE3 and topotecan was not observed when $^{90}$Y-MOPC-12 was substituted for $^{90}$Y-BrE3. There was only a transient inhibition of tumor growth and prolonged survival similar to those treated with topotecan alone.
Bone Marrow Toxicity. Table 1 depicts the bone marrow toxicity associated with combined RIT and continuous topotecan infusion. There was a significant decline in colony-forming units and WBC at 48 h, 72 h, and 7 days after 90 Y-BrE3 treatment with or without topotecan (P < 0.01). No difference in bone marrow toxicity was observed between topotecan- and non-topotecan-treated animals. Recovery back to normal values was observed at 14 days.

DISCUSSION

This study demonstrates that topoisomerase I inhibitors potentiated the therapeutic index of 90 Y-based RIT in treatment of human mammary carcinoma in a nude mouse xenograft model. Several observations were made: (a) topotecan or 90 Y-BrE3 treatment alone similarly delayed the overall tumor growth transiently, but did not affect overall survival; (b) topotecan enhanced the specific tumor killing induced by the 90 Y-BrE3 in established human tumor xenografts in a dose-dependent manner; (c) the combination of RIT with topotecan substantially reduced tumor growth and caused prolonged tumor-free survival in a synergistic manner; (d) treatment-associated bone marrow toxicities reached nadir at 3 days and recovered by 14 days after therapy; (e) apoptosis, measured in vitro, was correlated with the effects of topotecan and/or antibody on in vivo tumor growth.

Chemotherapeutic agents can enhance the antitumor effect on radioreistant cells, and agents such as cisplatin, Taxol, and 5-fluorouracil may act as radiosensitizers. Various clinical trials have examined the efficacy of combined RIT and chemotherapy in different tumor types. Improved therapeutic index and tumor regression with the combined modality have been shown in mouse xenograft models (18, 19) and human clinical trials (20, 21).

Recently, enhancement of the cytotoxic effect of radiation by topoisomerase I inhibitors has been investigated. Experimental models using topoisomerase I inhibitors in combination with external beam radiation therapy have shown enhancement of cell kill in cell culture and in vivo (8, 22). It has been postulated that the synergism between the topoisomerase I inhibitors and ionizing radiation is due to the ability of these inhibitors to interfere with repair of radiation-induced DNA damage (23). Also, ionizing radiation sensitizes cells to topoisomerase I inhibitors by slowing the cell progression through S phase, thus increasing the number of cells in S phase where they are most sensitive to topoisomerase I inhibitors (7). While external beam irradiation of locoregional disease is possible in some diseases, this is a less viable approach with respect to widely disseminated, metastatic solid tumors. Radioimmunoconjugates provide a vehicle for targeting therapeutic doses of radiation to dispersed tumor throughout the body.

The results obtained with the radioconjugated MAb are similar to those of many other studies when RIT has been administered to larger, established xenografts (19, 24, 25); generally, transient tumor growth-inhibitory responses are seen with few complete regressions and long-term, tumor-free survivors. Similarly, although topotecan alone exhibited some tumor-inhibitory effects that were transient and tumors eventually regrew at the same rate as controls, in all treated animals.

In contrast, the combination of a continuous infusion of topotecan and 90 Y-BrE3, in a regimen that caused no observable toxicity, substantially reduced tumor growth, caused complete tumor regressions, and resulted in prolonged tumor-free survival in a substantial portion of the animals. Isotype-matched radioconjugated MAB did not have this effect, suggesting that local accumulation of radioactivity, other than any effect of systemic radiation, is responsible. It is particularly noteworthy, we believe, that these results were obtained in an experimental system in which therapy was administered at 14 or 21 days after tumor implantation, when the target tumors are fully established, vascularized, and representative of a relatively large tumor burden. Moreover, the dose and mode of administration of topotecan, as a continuous infusion over 14 days, were meant to emulate an effective clinical approach to use of this agent in patients with cancer (13).

Recently, apoptosis has been recognized as a critical process not only in turnover of embryonal and replicating normal adult tissue but also in the response of cells to a variety of factors, including radiation and chemotherapy. Apoptosis can be induced by radiation (26), DNA-damaging agents (27), binding to specialized receptors (28, 29), and growth factor withdrawal (30). Understanding the mechanism of tumor killing induced by RIT could lead to selection of combined therapies that enhance tumor responses. Our in vitro data demonstrate a decrease in cell proliferation and increase in apoptosis in cells treated with the combined therapy as compared with untreated cells, 90 Y-BrE3 treated alone, and topotecan treated alone. In both MDA-MB435 and MDA-MB157, topotecan exerted a dose-dependent cytotoxicity, as demonstrated in cell proliferation and apoptosis assays. Dose-escalating cytotoxicity of 90 Y-BrE3 MABs was observed only in the MDA-MB157 (BrE3-positive) cell line; whereas in the MDA-MB435 (BrE3 negative) cell line, no significant degree of tumor killing was observed. When combining topotecan with 90 Y-BrE3 MABs, additive cytotoxicity was seen only in MDA-MB157 cells. These findings suggest that topotecan and 90 Y-BrE3 MABs given together can enhance the tumoricidal effect. Although the results of these mechanistic studies are at present only correlative, the close association between the in vitro and in vivo findings suggests that enhanced apoptosis could be related to the much greater effectiveness of the combination therapy.

In summary, this report provides in vitro and in vivo evidence that topotecan can potentiate the therapeutic efficacy of RIT. Apoptosis may contribute to tumor killing, and understanding the mechanism

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^a^ CFU, colony-forming unit; Topo, topotecan.

^a^–^d^ P < 0.01 versus untreated control.
will help to enhance the current treatment modality for cancer. It is suggested that the combination of topoisoenzyme I inhibitors with radioimmunoconjugates may help overcome the limitations of low antibody uptake in tumors and increase the usefulness of RIT in clinical settings.

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REFERENCES

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