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Radiosensitizing Effect of Electrochemotherapy in a Fractionated Radiation Regimen in Radiosensitive Murine Sarcoma and Radioresistant Adenocarcinoma Tumor Model

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Electrochemotherapy can potentiate the radiosensitizing effect of bleomycin, as shown in our previous studies. To bring this treatment closer to use in clinical practice, we evaluated the interaction between electrochemotherapy with bleomycin and single-dose or fractionated radiation in two murine tumor models with different histology and radiosensitivity. Radiosensitive sarcoma SA-1 and radioresistant adenocarcinoma CaNT subcutaneous tumors grown in A/J and CBA mice, respectively, were used. The anti-tumor effect and skin damage around the treated tumors were evaluated after electrochemotherapy with bleomycin alone or combined with single-dose radiation or a fractionated radiation regimen. The anti-tumor effectiveness of electrochemotherapy was more pronounced in SA-1 than CaNT tumors. In both tumor models, the tumor response to radiation was not significantly influenced by bleomycin alone or by electroporation alone. However, electrochemotherapy before the first tumor irradiation potentiated the response to a single-dose or fractionated radiation regimen in both tumors. For the fractionated radiation regimen, normal skin around the treated tumors was damaged fourfold less than for the single-dose regimen. Electrochemotherapy prior to single-dose irradiation induced more damage to the skin around the treated tumors and greater loss of body weight compared to other irradiated groups, whereas electrochemotherapy combined with the fractionated radiation regimen did not. Electrochemotherapy with low doses of bleomycin can also be used safely for radiosensitization of different types of tumors in a fractionated radiation regimen, resulting in a good anti-tumor effect and no major potentiating effect on radiation-induced skin damage. © 2009 by Radiation Research Society

INTRODUCTION

Many different combinations treatments have been tested to potentiate the effect of radiotherapy, including the combination of radiation with molecular-targeted agents (e.g. antiangiogenic or vascular-targeted approaches, hypoxic cell sensitizers or selective hypoxic cell cytotoxins), immunotherapy and chemotherapeutic drugs. Many of these improve anti-tumor effectiveness in combination with radiation therapy and have been translated successfully into clinical treatment of different tumors (*1–7*).

Electrochemotherapy is a treatment in which the anti-tumor effectiveness is potentiated by increased delivery of chemotherapeutic drugs into tumors by means of electroporation. The principal mechanism of this therapy is increased permeabilization of cell membranes, resulting in increased binding of the chemotherapeutic drugs to DNA; binding to DNA was increased fourfold for bleomycin and twofold for cisplatin with appropriate electric-field distribution in the tumors (*8–13*). Electrochemotherapy is already being used in clinical settings, and the results of published clinical trials have demonstrated that electrochemotherapy has excellent anti-tumor effects in different cutaneous and subcutaneous tumors, predominantly in palliative care, resulting in approximately 80% objective responses of the treated tumor nodules (*14–18*).

Radiosensitization of tumors by bleomycin is an established approach, predominantly in the treatment of locally advanced esophageal cancer and of head and neck tumors (*3, 19–23*). In addition, our previous studies have shown that electrochemotherapy with bleomycin preceding single-dose irradiation has a 1.9-fold radiopotentiating effect, whereas electrochemotherapy with cisplatin has a 1.6-fold radiopotentiating effect. The effect was demonstrated in two tumor models, sarcoma LPB and Ehrlich-Lettre ascites carcinoma tumors (EAT), as well as in a clinical case report (*24–27*). Furthermore, the anti-tumor effect of a moderate dose of γ radiation and a low concentration of doxorubicin hydrochloride can be

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enhanced significantly by combination with electroporation (*28*). *In vitro* studies have also demonstrated enhancement of cisplatin-induced radiosensitization of cells by electroporation could be for intrinsically less chemo- and radiosensitive tumor cells (*29*). To bring this treatment closer to clinical use, we evaluated the interaction between electrochemotherapy with bleomycin and fractionated radiation in two murine tumor models with different histology and radiosensitivity and compared it to single-dose irradiation. Skin damage in the irradiated area was also evaluated.

MATERIALS AND METHODS

Animals and Tumors

Inbred CBA and A/J mice were purchased from the Institute of Pathology, Faculty of Medicine, University of Ljubljana (Slovenia). Mice were maintained at 21°C with a controlled 12 h light/dark cycle in a specific-pathogen-free animal colony. Mice of both sexes that were 12–14 weeks old and weighed 20–25 g were included in the experiments.

The radioresistant poorly differentiated murine mammary adenocarcinoma CaNT (*30*) syngeneic to CBA mice and the radiosensitive sarcoma SA-1 (Jackson Laboratory, Bar Harbor, ME) (*31*) syngeneic to A/J mice were used. SA-1 cells were obtained from ascitic tumors since the cells cannot be grown *in vitro*. The CaNT cells were obtained from solid tumors, which were homogenized mechanically without enzymes and filtered (35 μm nylon mesh, BD Biosciences Europe, Erembodegem, Belgium) to yield a single-cell suspension. Cells were prepared in 0.9% NaCl solution at final concentrations of 9×10^7 cells/ml in the case of the CaNT cells and of 5×10^6 cells/ml in the case of the SA-1 cells due to the different plating efficiencies of tumor cells. Solid subcutaneous tumors were induced in the rear dorsum by the injection of 100 μl of cell suspension. When the tumors reached approximately 40 mm^3 in volume (7–12 days), the mice were marked, divided randomly into the different experimental groups, and subjected to a specific protocol. The protocols were approved by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission No. 323-02-170/2004/6).

Irradiation of Tumors

Non-anesthetized mice were irradiated using a Darpac 2000 X-ray unit (Gulmay Medical Ltd., Shepperton, UK), operated at 220 kV, 10 mA, with 1.8-mm aluminum filtration. Tumors were irradiated at a dose rate of 2.2 Gy/min with single doses or with 5–10 2-Gy fractions given daily 5 days per week. Highly radioresistant CaNT adenocarcinoma (TCD_{50} = 70 Gy) tumors were irradiated with either a single dose of 20 Gy or the same cumulative dose on a fractionated schedule (Fig. 1) (*32*). The more radiosensitive SA-1 sarcoma tumors (TCD_{50} = 25 Gy, our unpublished data) were irradiated with either a single dose of 10 Gy or the same cumulative dose on a fractionated schedule. The lower radiation dose was chosen to compare the antitumor effects on both tumor models at the same level of tumor radioresponse. Mice were immobilized in containers designed to hold the tumor-bearing portion of the rear dorsum in the radiation field. The containers were placed on a holder for six mice with apertures for the irradiation of the tumors and mounted on the X-ray unit. To ensure uniform doses throughout the tumor volume, the mice were turned 180° halfway through each irradiation.

Electrochemotherapy

Electrochemotherapy was performed as described previously (*4*). A stock solution (3 mg/ml) of bleomycin (Heinrich Marck Nachf. GmbH, Germany) was prepared in phosphate-buffered saline. The experiments were performed using 0.5 mg of bleomycin per kg mouse weight, which was prepared daily in a 0.9% NaCl solution. Three minutes after intravenous injection of bleomycin (injection volume 100 μl), electric pulses were applied to the tumors (Fig. 1). Eight square-wave electric pulses, delivered in two sets of four pulses in two mutually perpendicular directions, of 1040 V (at voltage-to-distance ratio 1300 V/cm), with pulse duration of 100 μs and a repetition frequency of 1 Hz were delivered by two flat, parallel stainless-steel electrodes 8 mm apart (two stainless-steel strips: length 15 mm, width 7 mm, with rounded corners) that were placed percutaneously at the opposite margins of the tumor. Good contact between the electrodes and the skin was ensured by means of a conductive gel. Electric pulses were generated by a Jouan GHT 1287 electroporator (Saint Herblain, France).

Combined-Treatment Protocol and Treatment Evaluation

To determine whether electric pulses increase the radiosensitizing effect of bleomycin, electrochemotherapy was combined with local tumor irradiation with a 20-min interval between the single-dose irradiation or the first dose of the fractionated radiation. Fractionated irradiation was given daily 5 days per week (Fig. 1).

Tumor growth was followed by measuring three mutually orthogonal tumor diameters (e_1, e_2 and e_3) with a Vernier caliper three times per week. Tumor volumes were calculated according to the formula for the volume of an ellipsoid, $V = \pi \times e_1 \times e_2 \times e_3/6$. The arithmetic mean of tumor volumes and standard errors of the mean (SEM) were calculated for each experimental group. The tumor doubling time was determined for each individual tumor from the growth curves on the day when the tumor reached twice the initial volume. The tumor growth delay was calculated for each individual tumor by subtracting the doubling time of that tumor from the mean doubling time of the control group and then averaged for each experimental group. Each experimental group consisted of at least eight mice. The radiosensitization effects were compared using potentiation factors that were calculated from the means of the tumor doubling times.

Acute Skin Response and Animal Weight

Skin reaction in the irradiated field around the tumor was scored once a week from 10 to 46 days after the first radiation dose in the same animals used for the tumor growth delay assay. For each mouse, a score was estimated on the basis of a scale rating from 0 to 5 (0, no reaction; 1, edema, mild erythema; 2, edema, moderate erythema, dry skin desquamation less than 20% of irradiated skin; 3, edema, severe erythema, dry skin desquamation with increasing diameter of the plaques more than 21% of irradiated skin; 4, edema, severe erythema, moderate moist desquamation with ulceration 21–49% of irradiated skin; 5, edema, severe erythema, severe moist desquamation with ulceration more than 50% of irradiated skin) [adapted from refs. (*33, 34*)]. The average skin reactions were calculated for each animal over a 46-day period (this period covers the appearance and disappearance of the reactions in the combined treatment groups). The median values of the skin reactions for the mice in each dose group were plotted. To determine whether single or combined therapies had an effect on body weight, animals were weighed before the first treatment and thereafter every second day until the end of the experiments. Weight was calculated as a percentage of the initial weight of the animal. The maximum weight losses in different groups were compared.

Statistical Analysis

Statistical analysis was carried out using the program SigmaPlot 11 (Systat Software GmbH., Erkrath, Germany). All data were tested for normality of distribution. The differences between the mean values of

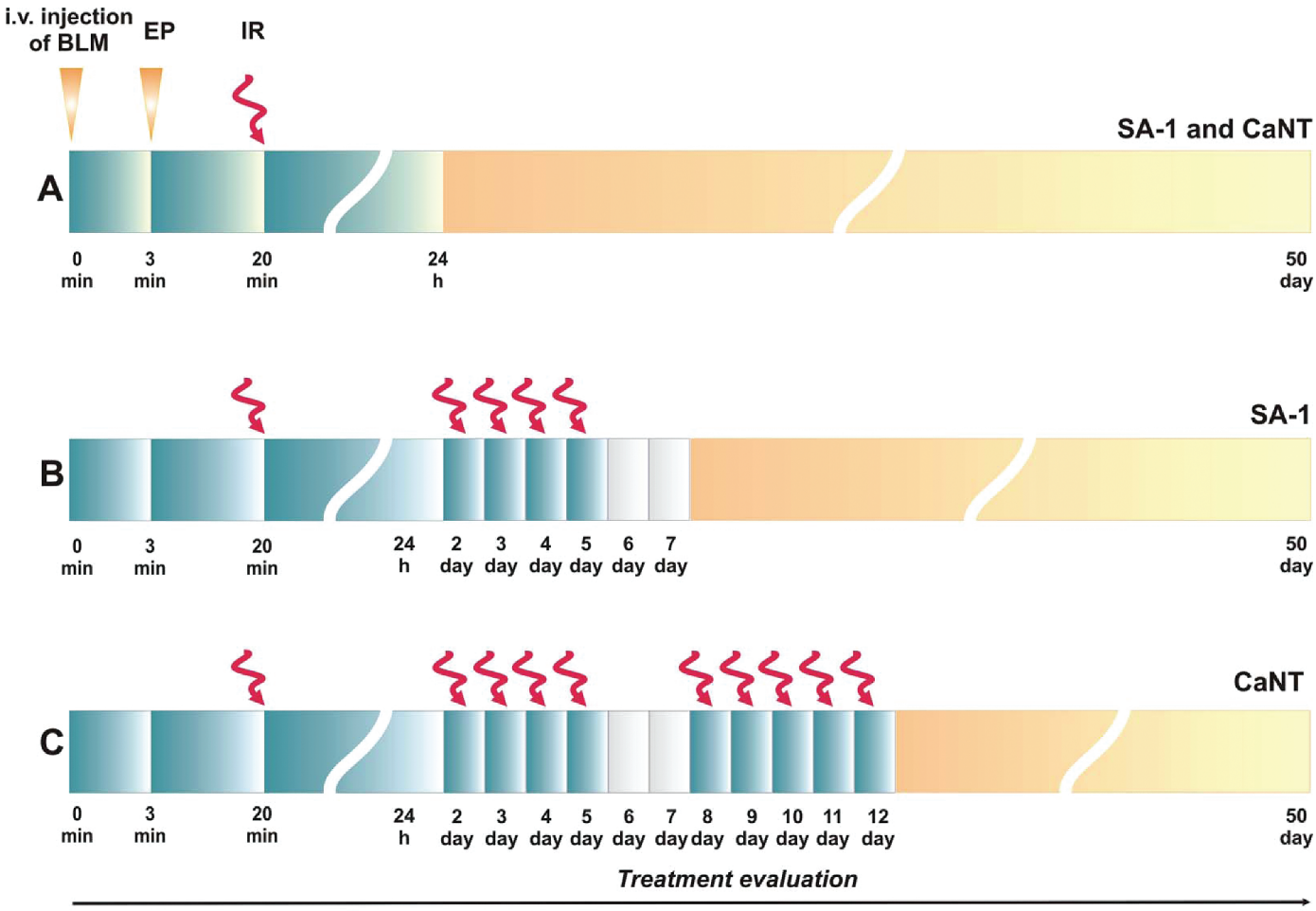

FIG. 1. Treatment protocol of combined electrochemotherapy and tumor irradiation (IR). A: SA-1 and CaNT tumors were treated with single-dose irradiation 20 min after electrochemotherapy [an i.v. bleomycin (BLM) injection and electric pulses (EP)]. B: SA-1 tumors were irradiated five times with 2 Gy per fraction after electrochemotherapy. C: CaNT tumors were irradiated 10 times with 2 Gy per fraction for 2 consecutive weeks after ECT.

groups were tested for significance by a *t* test after one-way ANOVA was performed and fulfilled. Statistically significant differences between the maximum weight losses were analyzed by the Holm-Sidak method after one-way ANOVA. Data for skin damage were evaluated by Kruskal-Wallis ANOVA.

RESULTS

Electrochemotherapy Response in Sarcoma and Adenocarcinoma Tumors

The anti-tumor effectiveness of electrochemotherapy was evaluated in SA-1 sarcoma and CaNT adenocarcinoma. The treatment parameters were the same for both tumor models. The initial tumor volumes at the time of the treatment and the tumor doubling times of the nontreated tumors were also the same (Fig. 2, Table 1). Application of only electric pulses to the tumors, and systemic treatment with 0.5 mg of bleomycin/kg had no significant effect on tumor growth. Electrochemotherapy was highly effective on both tumors. The response of SA-1 sarcoma tumors to electrochemotherapy was significantly greater than the response of the CaNT adenocarcinoma tumors based on a comparison of tumor growth delays ($P = 0.014$).

Radiosensitization of the Tumors

The radiosensitizing effect of electrochemotherapy in combination with single or fractionated radiation was tested in the same two tumor models. Electrochemotherapy was performed once, 20 min before single-dose tumor irradiation or 20 min before the first dose of fractionated radiation. SA-1 and CaNT tumors were irradiated with different total doses because of their different radiosensitivities. Radiosensitive SA-1 tumors (TCD_{50} = 25 Gy) were irradiated with a total dose of 10 Gy and radioresistant CaNT tumors (TCD_{50} = 70 Gy) with 20 Gy; in the fractionated regimen, the same total dose was split in fractions of 2 Gy/day given 5 days per week (Fig. 3, Table 1).

In the study, the bleomycin dose was 0.5 mg/kg, a dose that had no radiosensitizing effect by itself in either

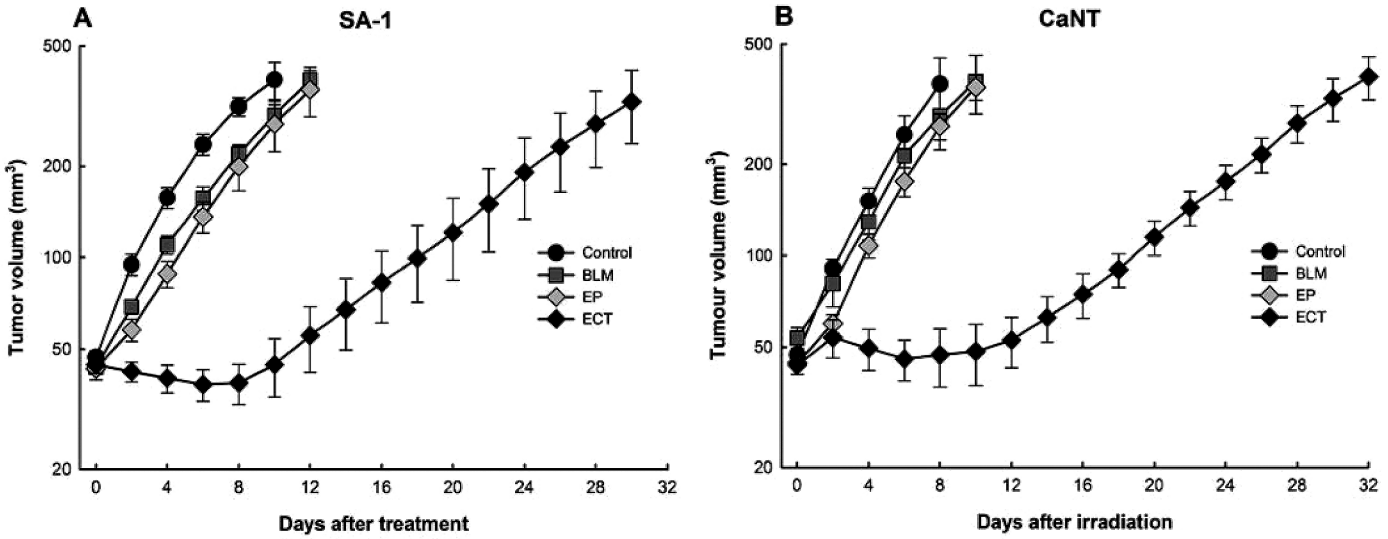

FIG. 2. Tumor growth curves for SA-1 sarcoma (panel A) and CaNT adenocarcinoma (panel B) tumors treated with electrochemotherapy (ECT), bleomycin (BLM) and electric pulses (EP). Points are means ± SEM. Data are from at least eight animals per treatment group.

tumor models. Application of electric pulses to the tumors had no radiosensitizing effect.

Electrochemotherapy sensitized both tumor (tumors) to single-dose radiation. The resulting growth delays were greater than the sum of the delays for electrochemotherapy irradiation alone (Table 1).

In the fractionated radiation regimen, the doses were split into five fractions of 2 Gy/day for the radiosensitive SA-1 sarcoma and 10 fractions for the radioresistant CaNT adenocarcinoma. The growth delays were slightly smaller than for the single-dose irradiation; however, they were not significantly different (Fig. 3, Table 1). The effect of fractionated tumor irradiation was not potentiated significantly by systemic administration of bleomycin or electroporation.

Electrochemotherapy produced radiosensitization to fractionated-dose regimens for both tumors. The resulting growth delays were greater than the sum of the delays for electrochemotherapy and fractionated radiation (Table 1).

The potentiating effect of electrochemotherapy was more pronounced in combination with the fractionated

TABLE 1
Anti-tumor Effectiveness of Electrochemotherapy alone or Combined with Tumor Irradiation in a Single-Dose or Fractionated Regimen

	SA-1 tumor				CaNT tumor			
Group	*N*	Doubling time[a] (days)	Growth delay[b]	*P*	*N*	Doubling time (days)	Growth delay	*P*
Electrochemotherapy								
Control	9	2.1 ± 0.2[a]			10	2.2 ± 0.2		
Electroporation[c]	8	3.8 ± 0.3	1.7	0.57[b]	8	3.6 ± 0.4	1.4	0.66[b]
BLM[d]	8	3.2 ± 0.1	1.1	0.72[b]	10	2.6 ± 0.2	0.4	0.90[b]
Electrochemotherapy[e]	8	22.3 ± 1.2	20.2	<0.001[b]	8	17.8 ± 1.0	15.6	<0.001[b]
Single-dose irradiation		10 Gy				20 Gy		
Radiation	11	15.0 ± 2.9	12.9		15	17.1 ± 1.3	14.9	
BLM + radiation	12	15.1 ± 4.2	13.0	0.91[c]	10	17.7 ± 2.1	15.5	0.831[c]
Electroporation + radiation	12	15.4 ± 1.7	13.3	0.884[c]	8	17.3 ± 2.8	15.1	0.953[c]
Electrochemotherapy + radiation	11	40.1 ± 1.7	38.0	<0.001[c]	11	39.5 ± 4.8	37.3	<0.001[c]
Fractionated irradiation		5 × 2 Gy/day				10 × 2 Gy/day		
Radiation	10	7.0 ± 1.0	4.9		15	10.3 ± 1.7	8.1	
BLM + radiation	12	7.4 ± 0.6	5.3	0.881[d]	10	11.6 ± 1.6	9.4	0.621[d]
Electroporation + radiation	11	8.5 ± 0.6	6.4	0.585[d]	8	10.8 ± 1.4	8.6	0.848[d]
Electrochemotherapy + radiation	12	32.5 ± 1.0	30.4	<0.001[d]	10	32.2 ± 0.6	30.0	<0.001[d]

[a] Mean ± SE.
[b] Compared to control group.
[c] Compared to a single dose of radiation.
[d] Compared to a fractionated dose of radiation.

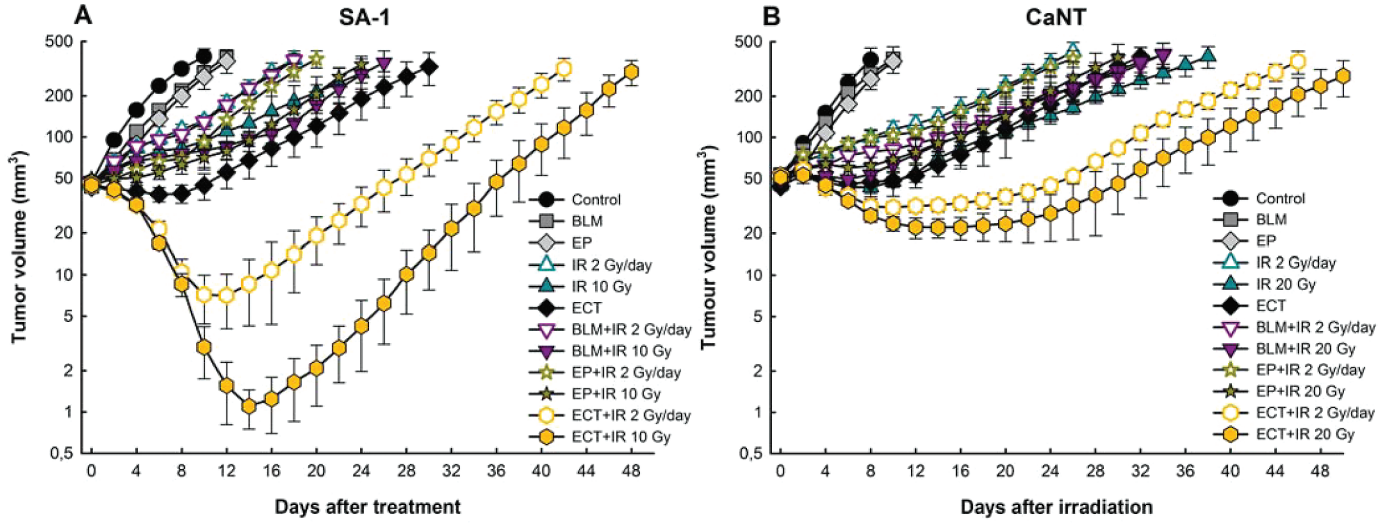

FIG. 3. Tumor growth curves of SA-1 sarcoma (panel A) and CaNT adenocarcinoma (panel B) tumors treated with electrochemotherapy (ECT) with bleomycin (BLM) combined with tumor irradiation (IR) with either a single-dose or a fractionated radiation regimen. Points are means ± SEM. Data are from at least eight animals per treatment group.

irradiation than with the single-dose irradiation. Electrochemotherapy potentiated the single-dose radiation response by a factor of 2.7 and the fractionated dose response by a factor of 4.6 in SA-1 tumors (calculated from tumor doubling times for combined electrochemotherapy and radiation compared to radiation only). In CaNT tumors, the effect of single-dose irradiation was potentiated by a factor of 2.3 and of that of fractionated irradiation by a factor of 3.1. Although the effect was evident in both tumor models, the potentiation of the radiation response was more pronounced in the radiosensitive SA-1 sarcoma than in the radioresistant CaNT adenocarcinoma (Fig. 4).

Side Effects

Irradiation of tumors with either the single-dose or fractionated regimen alone or in combination induced weight loss up to 10%; however, all animals were in good physical condition. While the animals lost weight during the first 10–12 days after tumor irradiation, their weight stabilized thereafter. Electrochemotherapy plus radiation induced significantly greater weight loss compared to other irradiated groups. A/J mice with SA-1 tumors were affected more than CBA mice with CaNT tumors. For SA-1 tumors the fractionated regimen combined with electrochemotherapy induced significantly less animal body weight loss than single-dose radiation; no difference was observed in CaNT tumors (Fig. 5).

The effect of tumor irradiation alone and in combination with bleomycin, electric pulses and electrochemotherapy on the skin in the irradiated field around the tumor was examined. Single-dose irradiation alone or in combination with other therapies induced much more

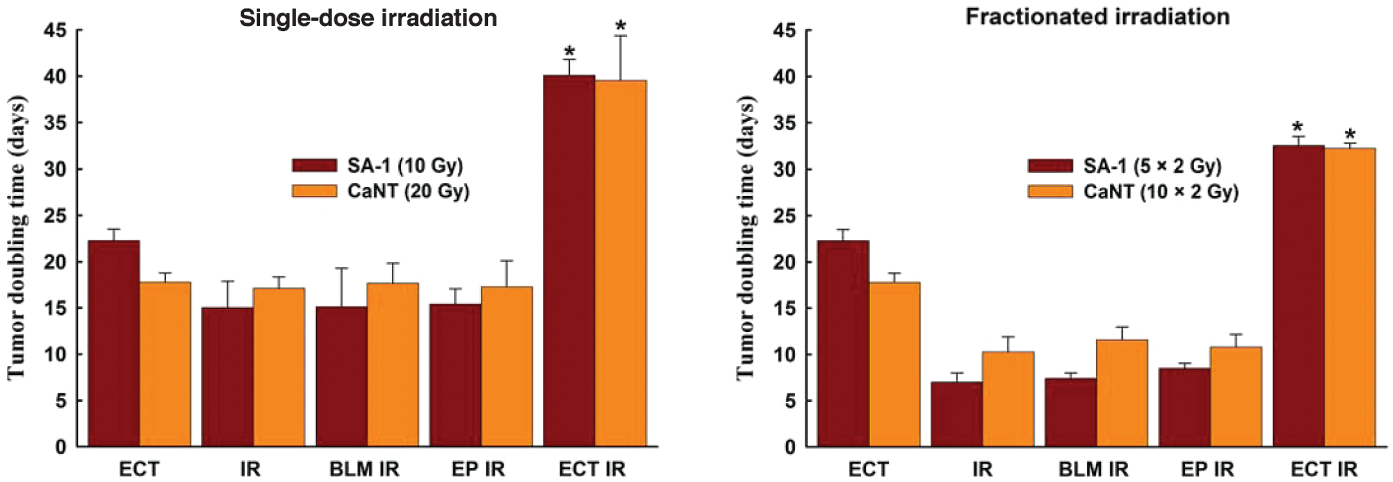

FIG. 4. Anti-tumor effectiveness of electrochemotherapy (ECT) with bleomycin (BLM) combined with tumor irradiation (IR) using either the single-dose or fractionated radiation regimen. Points are means ± SEM. Data are from at least eight animals per treatment group.*The response to combined treatment was significantly greater than in the other treatment groups.

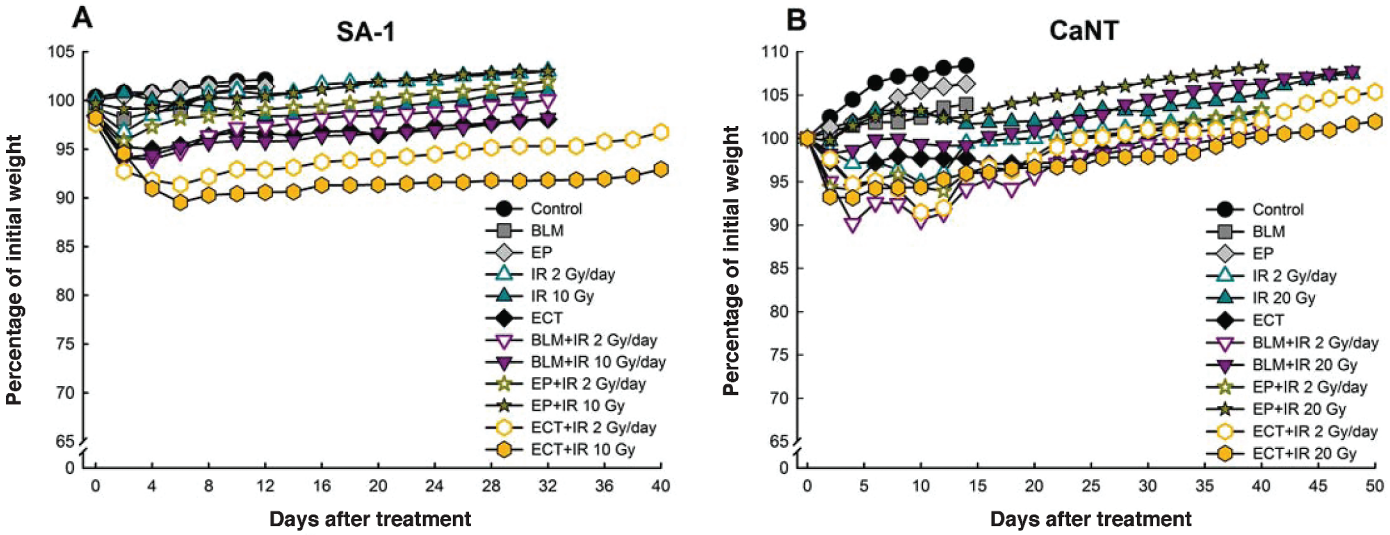

FIG. 5. Changes in animal weight after electrochemotherapy (ECT) with bleomycin (BLM) combined with tumor irradiation (IR) with either the single-dose or fractionated radiation regimen. Panel A: SA-1 sarcoma, panel B: CaNT adenocarcinoma. Points represent mean values. Data are from at least eight animals per treatment group.

serious normal tissue damage than the fractionated irradiation (Fig. 6). The greatest damage was observed 19 days after single-dose irradiation and 30–40 days after the first irradiation in the fractionated regimens. Single-dose irradiation resulted in around four times more skin damage than the fractionated regimens, regardless of the treatment combination. Electrochemotherapy with single-dose radiation induced greater normal tissue damage compared to other irradiated groups, whereas electrochemotherapy with fractionated radiation induced slightly more than or the same amount as in the irradiated groups.

DISCUSSION

This study demonstrated that increased delivery of the radiosensitizing drug bleomycin by electroporation of tumors (electrochemotherapy) enhanced the response of tumors to both a single-dose and fractionated radiation regimen. One electrochemotherapy session produced a 4.6-fold increase in the response of the radiosensitive SA-1 sarcoma tumors, whereas a 3.1-fold increase was observed in the radioresistant CaNT tumors after fractionated irradiation. The combined treatment was safe in that there was no major potentiating effect of electrochemotherapy on radiation-induced skin damage.

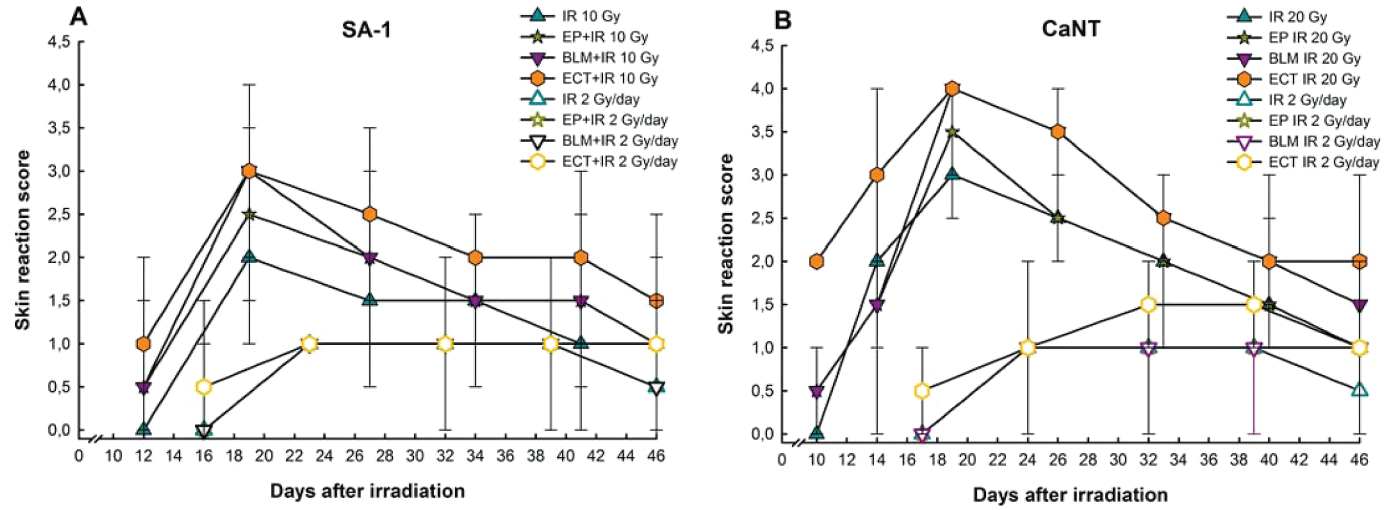

FIG. 6. Reactions of skin surrounding the tumor after irradiation (IR) using the single-dose and fractionated regimen combined with electrochemotherapy (ECT). Panel A: SA-1 tumor, panel B: CaNT tumor. Points are median of at least eight animals per treatment group; error bars represent the 25th and 75th percentiles.

Electrochemotherapy is used in the treatment of recurrent cutaneous and subcutaneous tumor nodules of different histology, predominantly in palliative care (*10, 14–18, 35*). In both preclinical and clinical studies, all tumor types have responded positively to the treatment (*9, 10, 12, 14–18, 35–39*). However, some preclinical studies have demonstrated a variable response to the treatment that was due to differences in intrinsic tumor cell responsiveness to the drug used (cisplatin or bleomycin) in conjunction with electric pulses (*9, 39, 40*). Therefore, the difference in the responsiveness of the tumors to electrochemotherapy obtained in the present study could be ascribed to differences in the intrinsic sensitivity of the tumors to the drug.

The radiosensitizing effect of bleomycin has been demonstrated in preclinical and clinical studies (*20–23, 25, 41–43*). Bleomycin administered 5–20 min prior to a single-dose or fractionated irradiation regimen resulted in sensitized of murine mammary carcinoma C3H and fibrosarcoma FSaIIC tumors; enhancement factors of up to 1.4 were obtained (*41, 43*). When bleomycin was used alone or in combination with other drugs concurrently with fractionated radiation, locoregional control and survival in patients was improved by up to 30% (*19, 20, 22*).

Our previous studies combining electrochemotherapy with either bleomycin or cisplatin demonstrated the radiopotentiating effect of electrochemotherapy (*24–26, 29*). We found that the underlying mechanism was increased delivery of the drug to the tumors by electroporation. Electroporation increased bleomycin uptake by the cells up to fourfold and cisplatin uptake by up to twofold (*8, 25*). Bleomycin affects cells by directly binding to DNA, resulting in reduced synthesis of DNA, RNA and proteins. It induces single- and double-strand DNA breaks, leading to cell death. The effect is dependent on bleomycin dose; thus increased accumulation of bleomycin in the cells by electroporation leads to increased cytotoxicity. The cytotoxic effect of bleomycin is also potentiated by chemicals that produce superoxide as do X rays (*44–46*). Therefore, we speculate that increased DNA damage produced by the increased drug concentration sensitizes the cells to subsequent irradiation. The effect was dramatic; the potentiation factor for the tumor radiation response for single-dose irradiation measured at the TCD_{50} was 1.9 for electrochemotherapy with bleomycin and 1.6 for electrochemotherapy with cisplatin (*25, 26*). In this study, we compared the radiosensitizing effect of electrochemotherapy with bleomycin in two tumor models with different radiosensitivities in conjunction with single-dose or fractionated radiation. The results are in accordance with our previous results; however, the present study included fractionated radiation to bring the combination of electrochemotherapy and radiation closer to clinical use. Radiosensitization after the fractionated tumor irradiation was pronounced in both the radiosensitive sarcoma and radioresistant adenocarcinoma tumor models. In radiosensitive SA-1 tumors, the potentiation was 4.6-fold whereas in radioresistant CaNT tumors, the potentiation was 3.1-fold. The present data indicate that electrochemotherapy can radiosensitize tumors with only one electrochemotherapy treatment before tumor irradiation, which makes its use possible in the clinic.

No major enhancement of toxicity was found with electrochemotherapy. Compared to the single-dose regimen, animals treated with the fractionated regimen lose slightly less weight. Animals that received eletrochemotherapy lost more weight than the other irradiated groups, but the toxicity was still acceptable. Skin damage in the irradiation field around the tumors was affected only slightly in both tumor systems. Overall the skin damage with the fractionated regimen was a fourfold less pronounced than with the single-dose regimen. The maximal observed damages with the fractionated regimen were moderate erythema and dry skin desquamation in less than 20% of the irradiated field, whereas for single-dose irradiation, the damage included edema, severe erythema and moderate moist desquamation with (20–50% of the irradiated field) ulceration. The skin damage had largely resolved in both cases after 46 days of observation.

In conclusion, this is the first preclinical study of the radiosenzitization of tumors by electrochemotherapy with bleomycin in a fractionated radiation regimen. The results indicate that tumors can be radiosensitized with only one electrochemotherapy treatment, which makes it practicable in a clinical setting. Further studies on the radiosensitization of tumors by electrochemotherapy are warranted, and such studies should explore the use of repeated electrochemotherapy sessions integrated in a fractionated regimen. Combined treatment with electrochemotherapy and tumor irradiation could be used to treatment of radioresistant tumors and of larger tumor nodules that are not well controlled with a single modality treatment. Several repeats of electrochemotherapy during a fractionated tumor irradiation would increase the amount of bleomycin in tumor cells. This could selectively increase the effect on tumor cells since electrochemotherapy can be targeted by the application of electric pulses only to the tumor.

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