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Re-Evaluating Gadolinium(III) Texaphyrin as a Radiosensitizing Agent[1]

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ABSTRACT

Gadolinium(III) texaphyrin (Gd-tex) was recently proposed as a radiosensitizing agent that combines preferential tumor uptake with detection of drug localization by magnetic resonance imaging (S. W. Young *et al.*, Proc. Natl. Acad. Sci. USA, *93:* 6610–6615, 1996). In view of the initial report on this compound, four radiobiology laboratories undertook independent efforts to further study radiosensitization by Gd-tex. In addition to repeating the previously reported studies on Gd-tex in HT-29 cells, we tested five other human tumor cell lines (U-87 MG, U251-NCI, SW480, A549, and MCF-7). These studies included a Gd-tex treatment period of 24 h before irradiation (as in the original publication), with concentrations of Gd-tex ranging from 20–500 μM. In neither the HT-29 cells nor any of the other five human cell lines did we see radiation sensitization by Gd-tex. Two cell lines (MCF-7 and U-87 MG) were further tested for radiosensitization by Gd-tex under hypoxic conditions. No radiosensitization was observed in either case. Finally, the radiation response of two tumor lines were assessed *in vivo*. Neither HT-29 xenografts in severe combined immunodeficient (SCID) mice nor RIF-1 tumors growing in C3H mice demonstrated radiosensitization after Gd-tex treatment before single or fractionated doses of radiation. Our results raise questions about the efficacy of Gd-tex as a radiosensitizing agent.

INTRODUCTION

Developing a radiosensitizing agent specific for tumor cells has long been a research goal of radiobiology. Gd-tex[3] was recently proposed as a radiosensitizing agent that combines preferential tumor uptake with detection of drug localization by MRI technique (1). The proposed mechanism of action of Gd-tex as a radiosensitizer was that the molecule would capture hydrated electrons formed by irradiation, thereby increasing the concentration of hydroxyl radicals formed by radiation in areas where the drug was present. This novel mechanism of sensitization, combined with selective concentration in tumors and the ability to detect drug presence with MRI, represented a potentially revolutionary approach to tumor cell radiosensitization. In their report outlining the properties of Gd-tex, Young *et al.* (1) showed a significant radiosensitization of the HT-29 colon carcinoma cell line *in vitro* after incubation with Gd-tex and a prolonged growth delay of EMT-6 tumors given fractionated irradiation combined with i.v. administration of Gd-tex 2 h before irradiation. Improved survival was also reported in DBA/2N mice bearing SMT-F tumors when mice were treated with a single dose of 30 Gy after i.v. administration of 40 μg/kg Gd-tex. On the basis of these initial findings, a Phase I study was conducted, demonstrating that the compound was well tolerated in patients and that it led to selective enhancement of tumor imaging on MRI (2).

These promising results prompted us to study the effects of Gd-tex on radiosensitivity in the cell lines originally reported and to extend the studies to other cell lines *in vitro*. In addition, the radiosensitizing properties of Gd-tex were tested *in vitro* under conditions of hypoxia such as occur in tumors. We also sought to further define the activity of this compound as a radiosensitizer of transplanted tumors *in vivo*. The studies reported here were independently initiated in four separate laboratories. Communication between the authors concerning the data presented here was initiated only after the majority of the studies had been completed, at which time it was decided that these results could best be presented in a single report.

The results we obtained cast doubt on the potential of the Gd-tex as a radiosensitizing agent. No radiosensitization was detected in the HT-29 cell line originally reported by Young *et al.* (1) or in an expanded panel of human and rodent tumor lines *in vitro* or as tumors *in vivo*, irradiated under a variety of conditions.

MATERIALS AND METHODS

Radiosensitizers. Gd-tex (M_r 1148.4) is a green-to-black solid that is soluble in water to a concentration of 200 mg/ml (174 mM). The compound was supplied by Pharmacyclics Inc. (Sunnyvale, CA) in powder form and dissolved in either sterile distilled water at 100 mM or 1× Earle's balanced salt solution at 25 mM. The resulting solution was filter-sterilized by passage through a 0.22 μM filter, stored at 4°C and protected from light, and used within 15 days from the time the stock solution was made. Working solutions of the compound were made from stock solutions immediately before use by dilution into tissue culture medium. The Gd-tex compound used in animal experiments at Stanford was supplied by Pharmacyclics Inc. as a solution of 2 mg/ml and stored at 4°C until needed. This compound corresponds to Gd-tex compound 2 reported by Young *et al.* (1). The structure and properties of SR2508 (tirapazamine) have been reported previously (3, 4).

Cell Culture and Survival Determination. HT-29, SW480, and A549 cells were maintained and assayed in DMEM with high glucose supplemented with 10% fetal bovine serum and penicillin-streptomycin. U251-NCI and U-87 MG were cultured in Eagle's minimal essential medium supplemented with nonessential amino acids, glutamine, and 10% fetal bovine serum. MCF-7 cells and, in one institution, HT-29 cells were cultured in RPMI 1640 with 10% fetal bovine serum. Cultures were kept at 37°C in humidified incubators with 5% CO_2. Clonogenic survival was determined by two methods: (*a*) clonogenic assay at varying radiation doses from 1–10 Gy; and (*b*) limiting dilution cloning to measure the surviving fraction at 2 Gy. For both assays, cells from log-growth cultures were treated for 24 h before irradiation with the indicated concentration of Gd-tex. For growth inhibition studies, Gd-tex was added to cultures established 1 day earlier, and cell counts were determined at the times indicated after drug addition. Gd-tex toxicity was determined by exposing cells to Gd-tex for the time indicated, followed by plating of treated cells for clonogenic survival in the absence of drug.

Clonogenic assays were carried out on exponentially growing cultures. To generate clonogenic survival curves, cells were treated with Gd-tex alone or treated with the drug and irradiated immediately before trypsinization and counting. Known numbers of cells were then seeded in plastic Petri dishes and incubated in a 37°C humidified incubator with 5% CO_2 until colonies were ready for scoring. In experiments on U251-NCI and U-87 MG, cells were plated onto a feeder layer of lethally irradiated SF-126 cells. In experiments measuring the surviving fraction at 2 Gy (Fig. 2, *C* and *D*) and in clonogenic survival determinations for A549 and SW480 (Fig. 3, *C* and *D*), cells that had been pretreated were harvested, and a known number of cells were replated in drug-free medium before irradiation. Cells were either irradiated on ice with a

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[3] The abbreviations used are: Gd-tex, gadolinium(III) texaphyrin; MRI, magnetic resonance imaging; SF_2, surviving fraction after 2 Gy irradiation; PE, plating efficiency.

Table 1 *Effect of 4-day continuous culture in Gd-tex on cell proliferation*

Values shown represent the number of cells recovered after 4 days of culture in the presence of the indicated concentration of Gd-tex relative to the number of cells recovered from cultures that received no drug.

	Growth relative to control cultures	
Dose (μM)	U251-NCI	U-87MG
1	1.02	0.69
10	0.96	0.46
100	0.67	0.34
500	0.39	0.35
1000	ND[a]	0.33

[a] ND, not determined.

Phillips orthovoltage therapeutic X-ray machine at a dose rate of 1.3 Gy/min or irradiated at ambient temperature with a Mark I cesium irradiator (J. L. Shepherd, San Fernando, CA) at a dose rate of 1.6 Gy/min or with a cobalt-60 irradiator at a dose rate of 1.5 Gy/min. Colonies were stained and counted 14–21 days after irradiation. The surviving fraction at a given dose is defined as the ratio of the PE of irradiated cells to that of unirradiated cells exposed to the same concentration of drug. Each point on the survival curves represents the mean surviving fraction from at least three dishes.

The SF_2 was also determined by limiting dilution analysis for HT-29 cells. Cells were pretreated, trypsinized, counted, and plated in 96-well dishes in 200 μl of medium. Dishes were irradiated or sham-irradiated and cultured for 14–21 days, after which individual wells were scored for the presence of colonies. Wells were scored positive for colony formation if they contained one or more colony of 20 or more cells. Linear regression analysis was performed on the natural log of negative wells with the origin as the initial point of the line. The slopes obtained from this regression analysis were compared to determine the surviving fraction in the presence or absence of inhibitor. SF_2 was defined as the slope of the linear regression obtained from irradiated cultures divided by the slope of the linear regression obtained from unirradiated cultures given the same treatment before irradiation.

Hypoxia was induced by flushing cultures in glass Petri dishes or flasks with either 95% air:5% CO_2 or 95% nitrogen:5% CO_2 four times, followed by the addition of Gd-tex through gas-tight valves. After 1 h, cells were irradiated in Gd-tex under hypoxia and subsequently replated for clonogenic survival in drug-free medium.

Determination of Radiosensitivity *in Vivo*. C3H or severe combined immunodeficient (SCID) mice were inoculated in the flank by intradermal injection with RIF-1 or HT-29 tumor, respectively, at approximately 15 weeks of age. The mice were bred and housed under specific pathogen-free conditions in the American Association for Laboratory Animal Science-approved facilities at Stanford Medical Center. Tumors were segregated into experimental groups when they attained a mean diameter of approximately 7 mm. Gd-tex compound 2 was administered as an i.v. injection at a dose of 40 μmol/kg in a volume of 0.02 ml/g before irradiation. SR2508 was dissolved at a concentration of 50 mg/ml and administered i.p. at a dose of 1500 mg/kg. Mice were irradiated at the tumor site with a Phillips 250 kVp orthovoltage therapeutic X-ray machine with a single fraction of 20 Gy to determine the clonogenic survival of HT-29 or with either a single 15-Gy fraction or three fractions of 6 Gy at 24-h intervals to determine the regrowth delay in RIF-1 tumors. In experiments with RIF-1 tumors, animals that were treated with both irradiation and Gd-tex received injections of the drug 4 h before each irradiation. Each treatment group consisted of five animals. Regrowth of RIF-1 tumor was monitored in five mice/treatment group until tumors attained three times their original volume.

Clonogenic survival of HT-29 tumors treated with irradiation and Gd-tex was determined by excision and dissociation of tumors 24 h after irradiation. The relative number of clonogenic cells/tumor was calculated as the product of the clonogenic survival and the number of cells extracted per treated tumor relative to the untreated tumors. This value therefore takes into account any cells that die in the first 24 h after irradiation. HT-29 tumor-bearing animals received Gd-tex at the times indicated before irradiation.

RESULTS

Assessing Gd-tex Cytotoxicity. To determine whether Gd-tex had any toxicity to tumor cells when these were exposed to the drug alone, clonogenicity and cell proliferation were assessed in U251-NCI and U-87 MG cells after treatment with Gd-tex at varying concentrations for up to 96 h. Gd-tex inhibited cell proliferation in a dose-dependent manner over the course of 4 days, as shown in Table 1. The toxicity of Gd-tex was also measured by determining the clonogenic survival of cells treated at 100–500 μM drug concentrations for 24–96 h (Fig. 1). The clonogenic survival of treated cells was decreased in a manner dependent on both the concentration of Gd-tex, and the duration of the exposure. Because the drug itself exhibits some toxicity, the radiation survival determinations reported below are corrected for any decrease in PE observed in unirradiated controls exposed to Gd-tex alone.

Radiation Survival after Gd-tex Treatment of Tumor Cells *in Vitro*. Because clonogenic survival in the presence of Gd-tex was initially tested by Young *et al.* (1) in the HT-29 cell line, three of the laboratories contributing to this report attempted to reproduce the previously reported results using this cell line (Fig. 2). The first notable finding was that although the three laboratories in the current study reported very similar radiosensitivity for HT-29 cells as determined by standard clonogenic assay or by limiting dilution analysis, the survival reported by Young *et al.* (1) was significantly lower at all doses (Fig. 2*A*). This finding was corrected by the authors while the current manuscript was under review (5).

To test the radiosensitizing effect of Gd-tex on HT-29 cells, the clonogenic survival of cultured cells exposed to this drug for 24 h before irradiation was determined. Log growth cell cultures were treated with Gd-tex for 24 h, and then they were either irradiated in drug-containing medium or harvested by trypsinization and washed in medium without drug before irradiation as indicated. Cells pretreated with Gd-tex and rinsed in drug-free medium before irradiation were observed to retain a yellow color after removal of the drug, indicating residual drug presence on the cells or uptake by the cells, although the location of the residual drug (intra- *versus* extracellular) was not

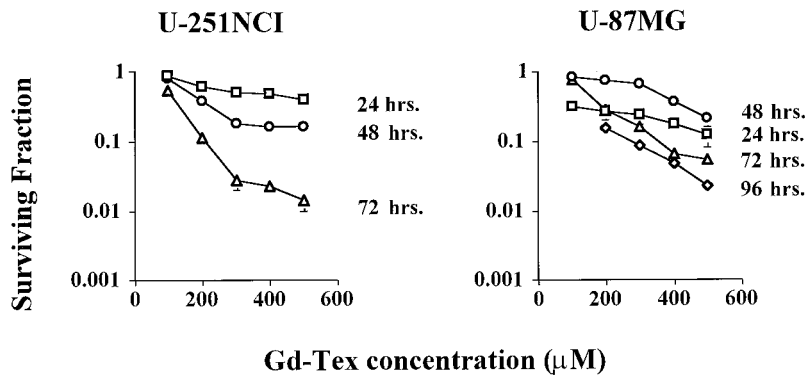

Fig. 1. Assessing the cell toxicity of Gd-tex exposure. Clonogenic survival was assessed in U251-NCI cells (*left*) and U-87 MG cells (*right*) after incubation in 100–500 μM Gd-tex for 24 (□), 48 (○), 72 (△), or 96 h (◇). At the times indicated, cells were rinsed, trypsinized, and replated in drug-free medium for clonogenic survival determination. Values shown are the clonogenic survival relative to untreated control cultures.

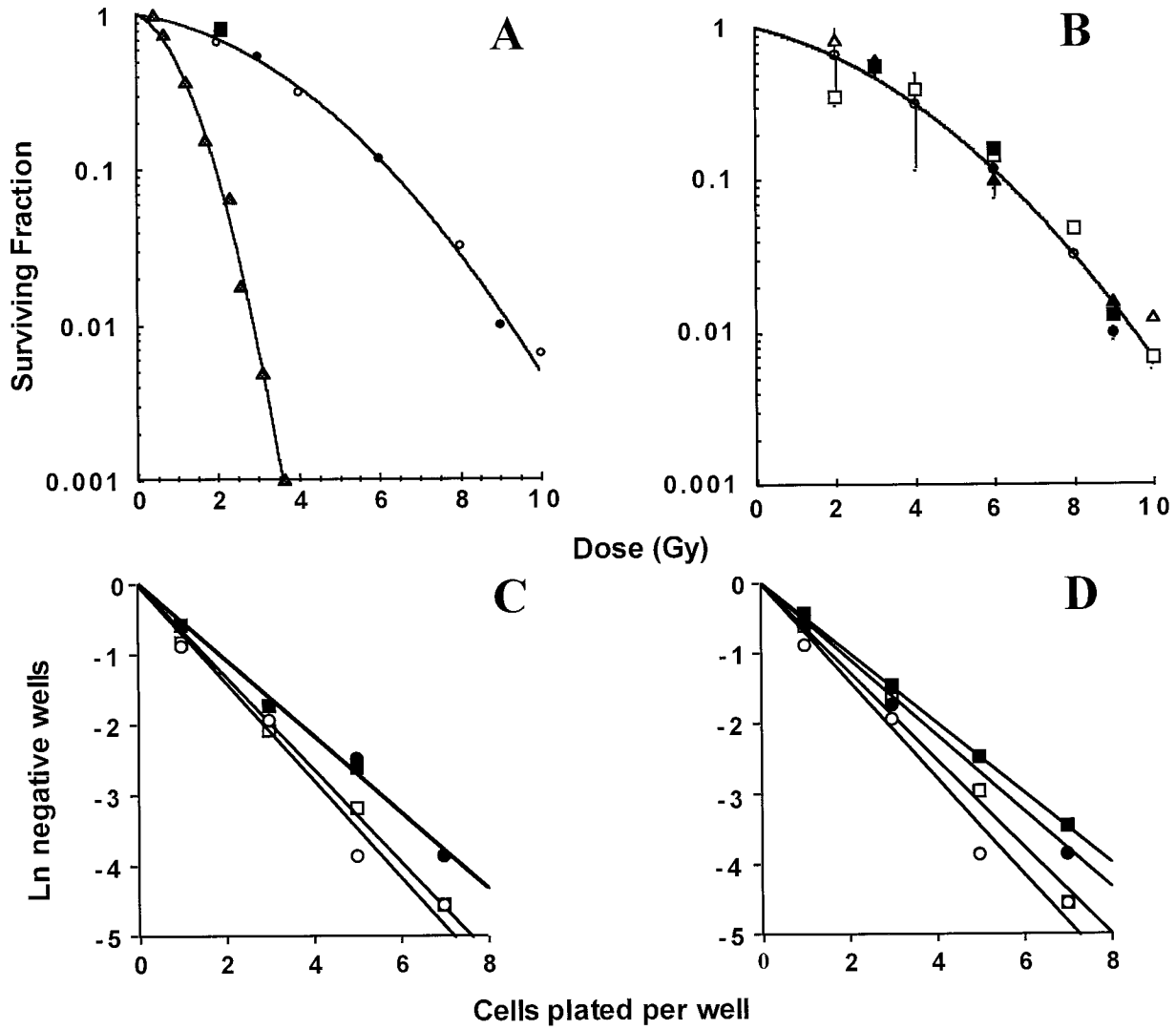

Fig. 2. Effect of Gd-tex pretreatment on HT-29 radiation sensitivity. *A*, HT-29 survival data obtained from three centers in the present study (○, ●, and ■) was compared to the data for untreated HT-29 cells reported by Young *et al.* (▲; Ref. 1). *B*, HT-29 cells were treated with carrier (○ and ●) or 20 (△), 25 (■), 40 (□), or 100 μM (▲) drug before irradiation at the doses indicated. Irradiation was carried out in the presence of Gd-tex before trypsinization and replating in drug-free medium. Clonogenic survival was scored 2 weeks after irradiation. *C*, clonogenic survival was determined by limiting dilution analysis (see "Materials and Methods"). Cells were treated with 20 μM drug (□ and ■) or an equal volume of carrier (○ and ●) for 24 h before irradiation. Cells were trypsinized and plated for clonogenic survival in drug-free medium before irradiation (*filled symbols*). The SF_2 in the presence of drug was 0.82. *D*, HT-29 cells were treated as described in *B*, but with 100 μM drug. The SF_2 in the presence of drug was 0.79. Control cells have a SF_2 of 0.78 (this control result is superimposed on the curves in *A* (■) and the data shown in *C* and *D* for comparison with drug-treated cultures).

determined. As seen in Fig. 2*B*, the radiation survival of HT-29 cells as determined by standard clonogenic assay after a 24-h pretreatment with 20–100 μM Gd-tex was not altered from that seen in control cultures. This finding was reproduced independently in two laboratories. The SF_2 was also measured by limiting dilution analysis in a third laboratory after a 24-h pretreatment with 20 μM (Fig. 2*C*) or 100 μM (Fig. 2*D*) Gd-tex. The SF_2 obtained by this method for control cells was 0.78, in accord with the values obtained by standard clonogenic assay (Fig. 2*A*), and no reduction in this value was seen after pretreatment with either 20 or 100 μM Gd-tex. Thus, in three studies carried out independently in three established radiobiology laboratories, no radiosensitization of HT-29 cells was observed after treatment with Gd-tex.

To test whether Gd-tex could radiosensitize other tumor cell types, clonogenic survival determinations were also carried out with an extended panel of tumor cells of different origins. Two tumor lines derived from central nervous system malignancies, U251-NCI (Fig. 3*A*) and U-87 MG (Fig. 3*B*) were examined for radiation survival after pretreatment with 25–100 μM Gd-tex for 24 h. No radiosensitization was observed in Gd-tex-treated U251-NCI cells. Slightly reduced survival was seen in U-87 MG cells treated with 100 μM Gd-tex in one experiment, but this finding was not reproduced in a replicate experiment, nor was it observed after 48 h pretreatment with Gd-tex (data not shown). Similar negative results were obtained in these two cell lines after a 24-h pretreatment with doses of 250 and 500 μM (data not shown). Gd-tex was also tested as a radiosensitizer in SW480 colon carcinoma cells (Fig. 3*C*), A549 lung cancer cells (Fig. 3*D*), and MCF-7 breast carcinoma cells (Fig. 3*E*) after pretreatment with 20–100 μM Gd-tex for 24 h. None of these cell lines showed significantly reduced clonogenicity as a result of Gd-tex treatment before irradiation at any Gd-tex dose tested. A modest reduction in survival was noted for MCF-7 cells at 100 μM Gd-tex at lower radiation doses.

Effect of Gd-tex on the Radiosensitivity of Hypoxic Cells. Because the redox potential of Gd-tex was reported to be above the threshold for electron affinic hypoxic cell senstizers, experiments were carried out to test whether Gd-tex might act preferentially on hypoxic cells. These experiments were carried out on both MCF-7 cells, in which minor changes in survival had been noted at the highest

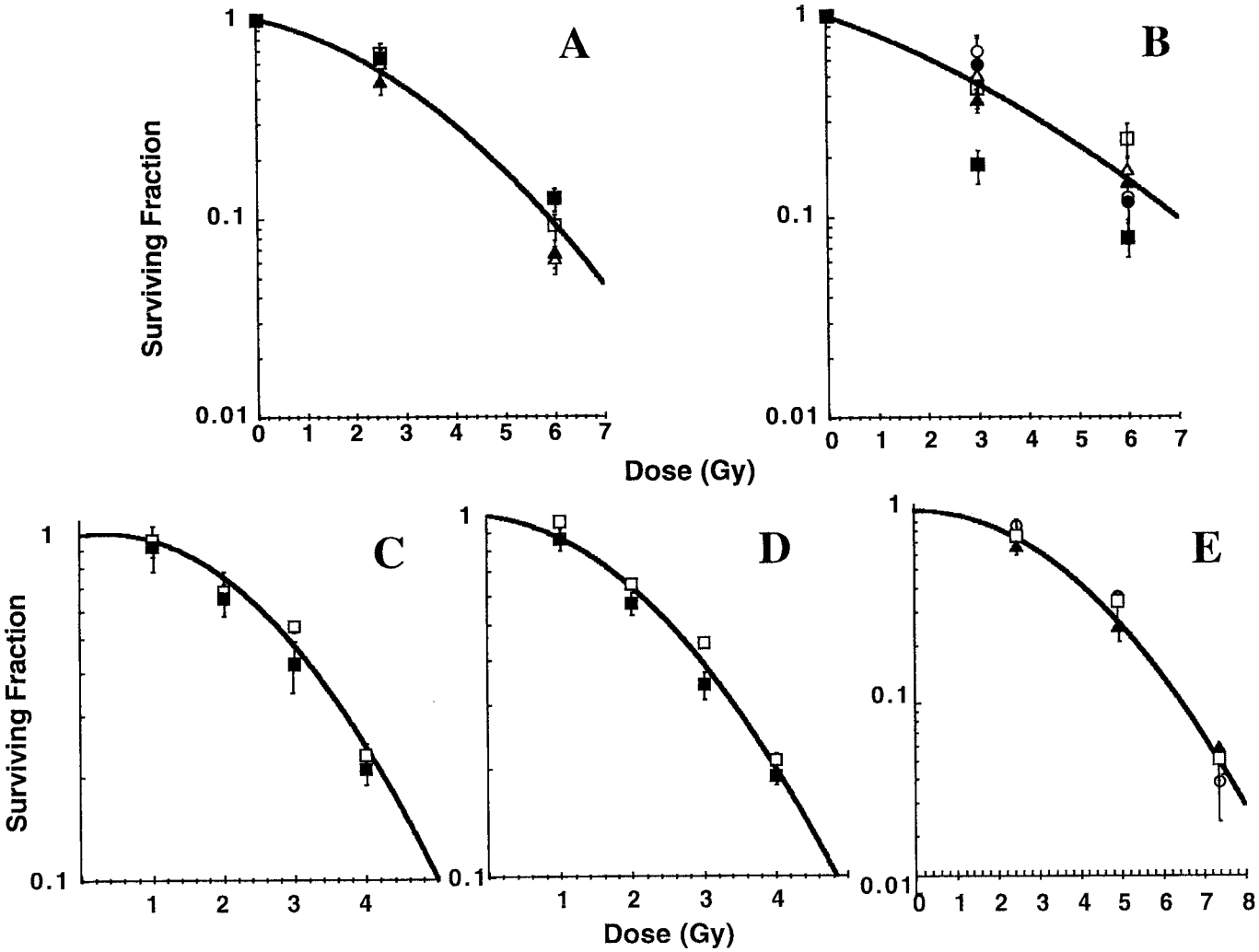

Fig. 3. Radiation survival of tumor cells after 24 h of Gd-tex treatment. U251-NCI cells (*A*) and U-87 MG cells (*B*) were pretreated with 0 (□), 25 (△), 50 (▲), or 100 μM (■) Gd-tex for 24 h. Cells were then irradiated on ice before trypsinization and plating for clonogenic survival in drug-free medium. In *B*, a replicate experiment is shown for treatment with 0 (○) and 100 μM (●) Gd-tex. PEs for U251-NCI cells were as follows: control, 0.47; 25 μM, 0.49; 50 μM, 0.56; and 100 μM, 0.36. The PEs for U-87 MG cells were as follows: control, 0.38 and 0.3; 25 μM, 0.36; 50 μM, 0.48; and 100 μM, 0.34 and 0.12. SW480 cells (*C*) were pretreated with 100 μM Gd-tex for 24 h before trypsinization and plating. Irradiation was carried out after replating in drug-free medium. The PE was 0.51 for control cells and 0.54 for Gd-tex-treated cells. A549 radiation survival (*D*) was determined as described in *C*. The PE was 1.2 for control cells and 0.96 for Gd-tex treated cells. Control cells, □; 100 μM Gd-tex-treated cells, ■. *E*, MCF-7 cells were pretreated with 0 (○), 25 μM (□), or 100 μM (▲) Gd-tex for 24 h before irradiation and plating for clonogenic survival as described in *A*. The PE was 0.72 for control MCF-7 cells and 0.8 and 0.6 for Gd-tex-treated cells at 25 and 100 μM, respectively.

Gd-tex doses tested, and in U-87 MG cells. Cultures were pretreated with Gd-tex for 24 h, subjected to hypoxia, and irradiated under hypoxic conditions. As seen in Fig. 4, hypoxia increased the radioresistance of these cells by a dose-modifying factor of approximately 2.4. However, pretreatment for 24 h with 100 μM Gd-tex failed to alter the radiosensitivity of either the hypoxic or aerobic cells. U-87 MG cell radiosensitivity was also assessed by clonogenic survival after pretreatment with 100–500 μM Gd-tex under oxic and hypoxic conditions as detailed in "Materials and Methods." No difference in radiosensitivity was detected in these cells under either condition after pretreatment with up to 500 μM Gd-tex (data not shown).

Radiation Survival after Gd-tex Treatment of Tumor Cells *in Vivo*. Because significantly increased survival of SMT-F tumor-bearing mice and increased tumor growth delay of EMT-6 tumors were reported after combined radiation plus Gd-tex treatment, we assessed the effect of this drug on tumor cell radiosensitivity *in vivo* by determining the clonogenic survival of tumor cells after irradiation *in vivo* and the tumor regrowth delay. Clonogenicity of HT-29 tumor cells isolated from animals treated with a single dose of Gd-tex administered at various times before irradiation with 20 Gy was determined (Fig. 5). Irradiation alone reduced the survival of isolated tumor cells by 3 logs to 0.0018. Administering Gd-tex at 24, 8, 6, or 2 h before irradiation showed no significant effect on the clonogenic survival of the tumor cells compared to the effect of irradiation alone (0.0017- 0.0044). In contrast, survival was decreased by more than 50-fold (to 3×10^{-5}) after treatment of identical tumors in the same experiment with SR2508 1 h before irradiation. Thus, Gd-tex failed to radiosensitize SMT-F tumors under conditions where radiosensitization was observed using SR2508.

We then tested whether Gd-tex could contribute to radiation-induced tumor growth delay in RIF-1 tumor-bearing animals (Fig. 6). Untreated tumors or those receiving either one or three injections of Gd-tex reached three times the initial tumor volume by 5 to 6 days. Irradiation with either a single fraction of 15 Gy or three fractions of 6 Gy prolonged this interval to 23 and 22 days, respectively. However, treatment with Gd-tex before irradiation had no significant effect on the regrowth delay in either the 15-Gy single dose group or in tumors treated with three 6-Gy fractions.

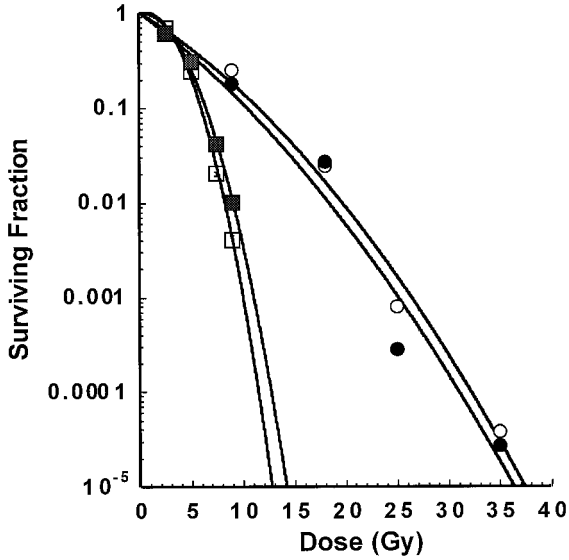

Fig. 4. Clonogenic survival under hypoxic conditions in the presence of Gd-tex. MCF-7 cells were pretreated with 100 μM Gd-tex for 24 h before the induction of hypoxia and irradiation as described in "Materials and Methods." The PE for control MCF-7 cells (□) was 0.66; the PE for Gd-tex-treated oxic cells (■) was 0.48. The PE for hypoxic cells was 0.74 and 0.67 in the presence (●) and absence (○) of Gd-tex, respectively.

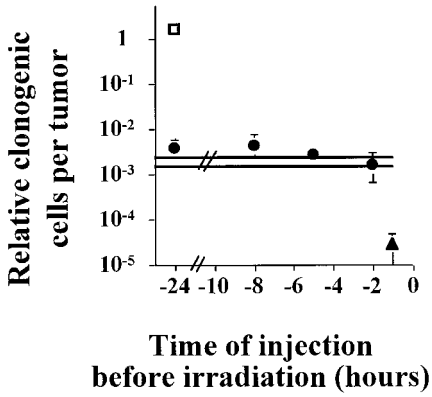

Fig. 5. Clonogenic survival after Gd-tex treatment and irradiation of HT-29 tumors *in vivo*. HT-29 tumor-bearing animals were treated with Gd-tex (○) or SR2508 (▲) at the times indicated before irradiation of the tumor with 20 Gy. Tumors were excised, dissociated, and plated for clonogenic survival determination 24 h after irradiation. Colony formation was scored after 2 weeks in culture. Clonogenic survival of HT-29 tumors treated with Gd-tex alone (□) is shown for comparison. All values are relative to the control number of clonogenic cells/tumor (a value of 1.0). The *upper* and *lower horizontal lines* show the upper and lower SE limits for survival after 20 Gy of irradiation alone. Each point represents the geometric mean ± SE clonogenic survival of tumors from three mice treated independently.

DISCUSSION

This study presents a summary of the work of four different laboratories that independently tested the potential of Gd-tex as a radiosensitizer of tumor cells *in vitro* and *in vivo*. The earlier publication of Young *et al.* (1) and more recent unpublished observations from Pharmacyclics Inc. (6) report that this compound selectively accumulates in transplanted mouse tumors, sensitizes tumor cells to radiation cell kill *in vitro*, and potentiates the response of transplanted tumors to both single and fractionated irradiation. The fact that this compound is selectively localized in tumors and can be imaged through MRI-contrasted enhancement because of the paramagnetic nature of the agent makes this compound particularly appealing for use in clinical trials (7). Indeed, the National Cancer Institute bypass budget document (8) features this compound as a new agent to be used with radiotherapy. A multicenter Phase III clinical trial of Gd-tex as a radiosensitizer combined with radiotherapy for the treatment of patients with brain metastases is being sponsored by Pharmacyclics Inc., the developer of this compound (9). These characteristics and promising preclinical data encouraged each of the four laboratories to undertake studies of the compound as a radiation sensitizer. While the studies were conducted, none of the laboratories was aware of activities in the other laboratories, and only learned of the other data by chance after the studies were largely completed. Because there was general agreement on the results obtained with the cell lines tested in the four laboratories, it was decided to combine the data into the present publication. When the data were combined, the degree of similarity of the results was remarkable. Three of the laboratories, for example, had tested the HT-29 human colon carcinoma cells *in vitro* under conditions similar to those described in the publication of Young *et al.* (1). In the current study, cells were irradiated before trypsinization in medium containing inhibitor as well as after preincubation in inhibitor, followed by trypsinization and replating as in the original report. The data for the control (and Gd-tex-treated) HT-29 cells were essentially indistinguishable among the three laboratories and are typical of those obtained for human tumor cells, whereas the HT-29 cells of the earlier publication (1) were apparently hypersensitive to radiation, even more so than cells with mutations in genes involved in DNA repair such as *ATM* or *DNA-PK* (10, 11). A correction to the original report has since been published (Ref. 5; discussed below).

In addition to attempting to repeat the earlier data showing radiosensitization by Gd-tex in HT-29 cells, we tested five other human tumor cell lines (U-87 MG, U251-NCI, SW480, A549, and MCF-7). These cells were tested with a preirradiation Gd-tex treatment period of 24 h (as in the original publication) with concentrations of Gd-tex from 20–500 μM (*i.e.*, up to concentrations more that 10 times higher than those used *in vivo*). In neither the HT-29 cells nor any of the other five human cell lines did we see any suggestion of radiation sensitization by Gd-tex (Figs. 2–4).

Gd-tex was reported to have a redox potential above the upper threshold proposed for electron affinic hypoxic cell radiosensitizers (1), which led us to test whether the compound would be a sensitizer

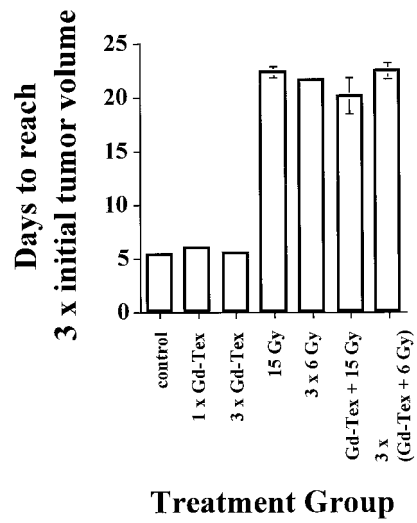

Fig. 6. Regrowth delay after Gd-tex treatment and irradiation of RIF-1 tumors. C3H mice bearing RIF-1 tumors were treated with Gd-tex 4 h before irradiation of the tumor with either 15 Gy or three fractions of 6 Gy administered at 24-h intervals. Tumors were then measured daily, and the volume was determined by the following formula: $(\pi/6) \times a \times b \times c$ (where a, b, and c are mutually perpendicular tumor diameters). The growth of untreated tumors, tumors, and tumors exposed to either drug or radiation alone are shown for comparison. The data represent the geometric mean growth delay ± SE. Five tumor-bearing animals were evaluated for each group.

of hypoxic cells. Although this was not tested specifically in the earlier publication, the positive results obtained *in vivo* could have been the consequence of sensitization of hypoxic cells in the tumors. Also, if the compound were acting as an electron affinic agent, as proposed (1), it would be expected to sensitize hypoxic cells rather than aerobic cells (12). However, the two cell lines tested under hypoxic conditions (U-87 MG and MCF-7) showed no suggestion of any sensitization of hypoxic cells, even at concentrations of up to 500 μM.

Finally, we tested Gd-tex for its ability to potentiate the antitumor efficacy of radiation in the same way as that reported in the previous study using two different tumor models assayed by two different methods. Although it is unlikely that a compound would sensitize tumors *in vivo* without radiosensitization *in vitro*, such a phenomenon is not unprecedented. Nicotinamide, for example, does not sensitize aerobic or hypoxic cells *in vitro*, but because it improves tumor blood flow and oxygenation, it sensitizes tumors to radiation *in vivo* (13). However, this was not the case with the experiments we performed with HT-29 tumors growing in SCID mice and irradiated with a large, single dose of 20 Gy. To assay cell killing in this experiment, we measured the total number of surviving cells in the tumor (by clonogenic assay) 24 h after irradiation. As a positive control, we gave a large, single dose of the 2-nitromidazole hypoxic cell radiosensitizer SR2508, which sensitizes tumors to irradiation by sensitizing hypoxic cells (3). As shown in Fig. 5, we saw no sensitization of the tumors to irradiation with Gd-tex injected i.v. (at 40 μmol/kg) from 2–24 h before irradiation. As a further test of the ability of the compound to sensitize tumors *in vivo*, we performed a growth delay experiment with the RIF-1 tumor-bearing mice injected 4 h before either a single dose of 15 Gy or to three daily doses of 6 Gy to the tumor. Under neither circumstance did we observe any change in the growth delay of the tumors in mice injected with Gd-tex compared to groups treated with irradiation only, despite the fact that larger growth delays can be readily obtained at larger radiation doses (14).

Therefore, the present data show no indication that Gd-tex is a radiation sensitizer of aerobic or hypoxic cells *in vitro* or of tumors *in vivo*. How can these data be reconciled with the earlier study apparently showing highly significant radiosensitization of tumor cells both *in vitro* and *in vivo*? In terms of the *in vitro* data with HT-29 cells, Young *et al.* (1) reported a dose modification factor of 1.9 for HT-29 human colon cancer cells. We have commented above on the unusually sensitive control survival curve published by these authors (Fig. 1 of this report and Fig. 2 of Ref. 1). During review of our manuscript, a correction was published (5) stating that the scale reported in Fig. 2 of the Young *et al.* study (1) was incorrect and that the experiments had been repeated, giving "radiation enhancement comparable to our initial findings at doses between 8 and 20 Gy." Because no other details were given, it is impossible to assess this statement. However, if the radiation sensitivity of the untreated HT29 cells were similar to that found by us (Fig. 2A), the reported sensitization enhancement ratio of 1.9 would have produced a surviving fraction at 10 Gy of less than 10^{-10}. This is well outside the range of survival that can be measured using the clonogenic assay. However, without additional details, it is not possible to reconcile the two data sets.

The explanation that Gd-tex is an electron affinic sensitizer able to "capture" the hydrated electron (e^-_{aq}) could not account for the sensitization because electron affinic radiosensitizers are specific for hypoxic cells and show no radiosensitization of aerobic cells (11). Because we found no radiosensitization of either aerobic or hypoxic cells, this mechanism seems unlikely. However, we feel that there is a likely explanation for the apparent radiosensitization of the EMT-6 tumor transplanted into BALB/C mice and given five consecutive doses of 1, 2, or 4 Gy/fraction with varying doses of Gd-tex. This tumor is antigenic in this strain of mice, a phenomenon that makes growth delay experiments hazardous (15). In particular, any agent that changes the cell-mediated immunity against this tumor could affect growth delay in the absence of any change in the response of the cells to radiation. Thus, a possible explanation for the positive results seen with Gd-tex added to fractionated irradiation is that the concentration of the compound in the tumor potentiated host cell immunity against the tumor, thereby increasing growth delay. Consistent with this interpretation is the large effect reported by Young *et al.* (1). For example, the dose-response curve for 40 μmol/kg Gd-tex added to 5×1 Gy is similar to that of tumors given 5×4 Gy alone, implying a dose modification factor of 4 in these tumors. This is much larger than that reported at even the highest Gd-tex concentrations *in vitro*. Similar considerations may have led to the positive results for mouse survival seen with SMT-F tumors transplanted into DBA/2N mice and treated with 30 Gy.

Thus, our results differ substantially from those originally reported by Young *et al.* (1), although conditions and experimental systems did not exactly reproduce those of the original report, and suggest that further independent study of Gd-tex as a radiosensitizer is indicated.

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