Direct Evidence that Apoptosis Enhances Tumor Responses to Fractionated Radiotherapy

Brent A. Rupnow, Albert D. Murtha, Rodolfo M. Alarcon, Amato J. Giaccia, and Susan J. Knox

Department of Radiation Oncology, Stanford University School of Medicine, Stanford, California 94305-3302

Abstract

Currently, the contribution of cellular apoptotic sensitivity to tumor response after radiation therapy remains controversial. To address this issue, the survival of Rat-1 fibroblasts containing a 4-hydroxytamoxifen-regulated c-Myc allele, c-MycER (T. D. Littlewood et al., Nucleic Acids Res., 23: 1686–1690, 1995), after single and fractionated doses of radiation was investigated. This model system allows pharmacological regulation of apoptosis sensitivity in the same cells in vitro and as xenograft tumors derived from these cells in vivo (G. I. Evan et al., Cell, 69: 119–128, 1992; R. M. Alarcon et al., Cancer Res., 56: 4315–4319, 1996). Activating c-MycER in vitro resulted in marked sensitization of Rat-1 fibroblasts to the effects of both single-dose and fractionated irradiation as measured by the induction of apoptosis and clonogenic survival. Overexpression of the antiapoptosis protein Bcl-2 suppressed the induction of apoptosis and increased clonogenic survival in cells with activated c-Myc after single-dose and fractionated radiation. Systemic time-release implant delivery of 4-hydroxytamoxifen to severe combined immunodeficient mice bearing Rat-1-MycER tumors over the course of either single-dose (10 Gy) or fractionated (five fractions of 2 Gy) radiotherapy resulted in prolonged tumor growth delay relative to identical tumors from mice that received placebo implants. Furthermore, tumors derived from Rat-1-MycER cells that overexpressed Bcl-2 exhibited shorter tumor growth delays relative to similarly treated Rat-1-MycER tumors. The length of tumor growth delay after single-dose or fractionated radiotherapy strongly correlated with the extent of radiation-induced apoptosis in the xenograft tumors as measured by terminal deoxynucleotidyl transferase-mediated nick end labeling. These in vivo results provide direct evidence that increasing the sensitivity of tumor cells to die by apoptosis increases the efficacy of fractionated radiotherapy by reducing tumor cell clonogenic survival.

Introduction

Ionizing radiation is a very effective means of killing tumor cells, because it can be uniformly administered in a very defined and protracted means that is not directly impeded by tumor physiology (1). Decades of research on the biological basis of the antitumor effects of ionizing radiation have identified two major limitations in the successful control of human cancers by radiotherapy: (a) the sensitivity of normal, noncancerous tissue relative to tumor cell sensitivity; and (b) the decreased oxygen levels often present in the tumor microenvironment (2). Nevertheless, radiotherapy is a very efficacious treatment for both lymphoid and solid tumors (1). At present, a great deal of attention is focused on understanding the key molecular pathways that modify the survival of tumor cells to radiotherapy with the long-term goal of developing specific pharmacological inhibitors of this response.

Many genetic regulators of apoptosis (i.e., p53, pRb, and Bax) have been shown to be important in tumor initiation and progression (3–9). Additional studies have shown that these same proteins can affect the apoptotic response of cells to cytotoxic stimuli including ionizing radiation (10–13). Most in vitro assays for tumor cell response to radiation involve measuring either rapid cell killing due to apoptosis or the long-term survival of tumor cells as defined by their ability to form a multicellular colony from a single cell (2). Studies of lymphoid tumors or oncogene-transfected mouse embryo fibroblasts indicate that apoptosis may play an important role in responses to radiotherapy (14, 15), but the effect of apoptosis on clonogenic survival is unknown. Furthermore, human colorectal cancer cells that are homozygous null for the p21Waf1/Cip1 cyclin-dependent kinase inhibitor exhibit substantial increases in radiation-induced apoptosis in vitro but show no associated decrease in their clonogenic survival after ionizing radiation in vitro (16, 17). Although the p21 null tumor cells are more radiosensitive in vivo, it has not been determined whether their sensitivity in vivo is due to a difference in apoptotic potential or clonogenic survival. In light of these reports, recent attention has focused on the potential importance of various cell characteristics (i.e., mitotic potential, drug efflux capacity, functionality of cell cycle checkpoints, apoptotic susceptibility, and clonogenic survival) as predictors of tumor response to conventional cytotoxic cancer therapies (18, 19). To specifically investigate the relationship between apoptosis, clonogenic survival, and tumor control, experiments were performed in vitro and in vivo using clinically relevant doses of ionizing radiation to treat the same cells under conditions in which their apoptotic sensitivity could be pharmacologically modulated.

Materials and Methods

Cell Culture. Rat-1, Rat-1MycER-Neo, and Rat-1MycER-Bcl-2 cell lines have been described previously (9, 20). Rat-1MycER-Neo and Rat-1MycER-Bcl-2 cells express c-MycER that is activated by the addition of 4HT3 (21). All cells were maintained in DMEM containing 10% FBS and no selection antibiotics (Rat-1) or 2.5 μg/ml puromycin and 300 μg/ml G418 (Rat-1MycER-Bcl-2 and Rat-1MycER-Neo). All experiments were performed in DMEM in the absence of selection antibiotics.

Quantitation of Apoptosis. A total of 250,000 cells were plated in 60-mm tissue culture dishes. After cells attached to the dishes, media were changed to fresh DMEM containing 10% FBS and 2 × 10−7 M 4HT or vehicle control solution and incubated for an additional 4 h to allow c-MycER activation. Cultures were then irradiated at the indicated doses using a 137Cs source at a dose rate of 422 cGy/min. At various times after irradiation, cells were permeabilized and stained with PI, and apoptosis was quantitated by flow cytometric DNA content analysis as described previously (22). Cells containing subdiploid DNA content were considered apoptotic. All data points for apoptosis represent the mean ± SD of at least three independent experiments.

1 The abbreviations used are: 4HT, 4-hydroxytamoxifen; c-MycER, c-Myc-estrogen receptor fusion protein; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; FBS, fetal bovine serum; SCID, severe combined immunodeficient; PI, propidium iodide.
Fig. 1. Relationship between apoptotic sensitivity and clonogenic survival in vitro. a–c, dose-response relationship of radiation-induced apoptosis in Rat-1 (a), Rat-1MycER-Neo (b), and Rat-1MycER-Bcl-2 (c) cells 24 h after irradiation with 0–1000 cGy in the absence (squares) or presence (circles) of $2 \times 10^{-7}$ M 4HT. d–f, time course of apoptosis induction in Rat-1 (d), Rat-1MycER-Neo (e), and Rat-1MycER-Bcl-2 (f) cells at 0–5 days after irradiation with 500 cGy in the absence (squares) or presence (circles) of $2 \times 10^{-7}$ M 4HT. The percentage of apoptosis reflects the mean percentage of cells containing subdiploid DNA content as determined by PI staining and flow cytometry. Error bars represent 1 SD from the mean and are shown where larger than the symbols. g–i, clonogenic radiation survival curves of Rat-1 (g), Rat-1MycER-Neo (h), and Rat-1MycER-Bcl-2 (i) cells after irradiation with single fractions ranging from 0–10 Gy in the absence (squares) or presence (triangles) of $2 \times 10^{-7}$ M 4HT. Data points reflect mean clonogenic survival. Error bars representing the SE of the mean are shown when larger than the symbols.
Clonogenic Survival Assay. Colony assays were performed on lethally irradiated feeder layers of parental Rat-1 fibroblasts. Cells (100–50,000) were then plated over the feeder layers and allowed to adhere. The media in each dish were then changed to fresh DMEM containing 10% FBS and 2 × 10⁻⁷ M 4HT or vehicle control solution and incubated for an additional 4 h to allow c-MycER activation. After incubation, cultures were irradiated with single fractions of 0–10 Gy or with five fractions of 2 or 4 Gy each administered every 12 h. Media were changed to provide fresh 4HT 4 h before the third and fifth fraction. After irradiation, plates were incubated for 7–10 days, at which time the colonies were fixed and stained with a solution of 0.2% crystal violet in 70% ethanol. Macropot colonies (>50 cells) were counted in each plate. All data points for clonogenic survival represent the mean surviving fraction (±SE of the mean).

Growth and Analysis of Rat-1 Solid Tumors in SCID Mice. Male 8–10-week-old SCID mice (housed under defined flora conditions) were injected with 1.5 × 10⁶ Rat-1, Rat-1MycER-Neo, or Rat-1MycER-Bcl-2 cells intradermally in the midline of the back of each mouse at a site approximately 1 cm from the base of the tail. After 3–5 weeks, mice were implanted with either time-release pellets containing 10 mg of 4HT that were released over a 10-day period to activate c-Myc expression or matched placebo pellets (Innovative Research of America, Sarasota, FL). We have previously demonstrated the ability of systemic 4HT administration to activate the c-Myc expression and decrease apoptosis in hypoxic regions of Rat-1MycER-derived tumors (20). Twenty-four h after pellet implantation, radiotherapy was initiated. Mice were immobilized in lead jigs that shielded the entire mouse, except for the small portion of the back that harbored the xenograft tumor, from radiation. Tumors averaging 85 mm³ were irradiated with a single dose of 10 Gy or with five fractions of 2 Gy each at 12-h intervals using a 250 kV X-ray machine (Siemens, Munich, Germany) at a dose rate of 140 cGy/min. For immunocytochemistry, tumors were excised 24 h after single-dose radiotherapy or 12 h after the final dose of fractionated radiotherapy. TUNEL staining (fluorescein, bright green) of frozen sections (6-µm thick) was performed using the ApopTag system according to the manufacturer’s instructions (Oncor, Gaithersburg, MD). Sections were counterstained with PI (2.5 µg/ml in mounting medium). Representative photographs of multiple frozen tumors are shown. Tumor growth after in vivo radiotherapy was analyzed by measuring three orthogonal diameters of each tumor every 4 days. Tumor volumes were estimated by the formula \( V = \frac{4}{3} \pi r^3 \), where \( r \) is the mean of the three orthogonal diameters of each tumor. Tumor volume was calculated every other day. The relative tumor volume at various times after irradiation is given by the formula \( V(t)/V(0) = \left(\frac{d_1}{d_0}\right)^3 \), where \( d_1 \) and \( d_0 \) are the diameters of the tumor at time \( t \) and at the start of irradiation, respectively.

**Results and Discussion**

The effect of c-MycER activation on radiation-induced apoptosis at varying doses was determined by irradiating Rat-1, Rat-1MycER-Neo, and Rat-1MycER-Bcl-2 cells in the presence or absence of 4HT. Cells were irradiated with a single dose ranging from 0–10 Gy. Parental Rat-1 cells, which do not express the c-MycER construct, exhibited low levels of apoptosis within 24 h, regardless of the radiation dose. This was true for Rat-1 cells in either the presence or absence of 4HT (Fig. 1a). In the absence of c-MycER activation by 4HT, Rat-1MycER-Neo cells also demonstrated little apoptosis at doses up to 10 Gy (Fig. 1b). However, the percentage of apoptosis observed in these cells was slightly higher than that seen in parental Rat-1 cells, suggesting that the c-MycER chimera may be somewhat active even in the absence of 4HT. In the presence of 4HT, however, Rat-1MycER-Neo cells underwent extensive radiation-induced apoptosis. By 24 h after irradiation at 5 Gy, more than 30% of Rat-1MycER-Neo cells with activated c-MycER had executed apoptosis, and more than 60% of these cells were apoptotic after 10 Gy. Rat-1MycER-Neo cells expressing the antiapoptotic protein Bcl-2 were relatively refractory to radiation-induced apoptosis, regardless of their c-MycER activation status (Fig. 1c). These results demonstrate that activation of c-Myc can profoundly sensitize cells to the rapid induction of apoptosis by ionizing radiation at the same doses that induce little apoptosis in the absence of activated c-MycER. The fact that 4HT increases apoptosis only in cells expressing the c-MycER chimera, and not in parental Rat-1 fibroblasts, establishes the specificity of this compound for c-MycER.

To determine whether the activation of c-MycER increased the overall apoptotic sensitivity of Rat-1MycER-Neo cells or simply resulted in a more rapid execution of apoptosis, we quantitated the fraction of apoptotic cells for 5 days after irradiation with 5 Gy (Fig. 1, d–f). We observed no increase in apoptosis over background levels in parental Rat-1 cells over the entire course of the experiment (Fig. 1d) and in the absence of 4HT. In contrast, the percentage of apoptotic cells in irradiated Rat-1MycER-Neo cultures increased in a time-dependent manner until approximately 3 days postirradiation, at which time the percentage of apoptotic cells declined (Fig. 1e). Whereas the peak percentage of apoptotic cells irradiated with 5 Gy in the absence of 4HT was approximately 20%, identical Rat-1MycER-Neo cells irradiated with 5 Gy in the presence of 4HT demonstrated peak levels of apoptosis exceeding 60% between 2 and 3 days after irradiation. A similar time course for the induction of apoptosis was observed for Rat-1MycER-Bcl-2 cultures, however, the maximum level of apoptosis was less than 10% in the absence of 4HT and less than 20% in its presence (Fig. 1f). Therefore, the effect of c-MycER activation observed at 24 h in the dose-response experiments cannot be explained solely by a more rapid induction of radiation-induced apoptosis. Overexpression of Bcl-2 did not delay the onset of apoptosis but suppressed the magnitude of the apoptotic response after irradiation.

Using a system in which the apoptotic potential of Rat-1 cells expressing the c-MycER construct to ionizing radiation could be altered pharmacologically, we next examined the effects of altering...
sensitivity to radiation-induced apoptosis on overall cell killing. Colony formation assays were performed on Rat-1, Rat-1MycER-Neo, and Rat-1MycER-Bcl-2 cells irradiated with doses ranging from 0–10 Gy in the presence or absence of 4HT. Fig. 1 (g-i) shows the radiation survival curves for these experiments. Insignificant differences were observed between Rat-1 cells irradiated in the presence or absence of 4HT (Fig. 1g). In contrast, the colony-forming ability of Rat-1MycER-Neo cells irradiated in the absence of 4HT differed markedly from the colony-forming ability of the same cells irradiated in the presence of 4HT (Fig. 1h). The presence of 4HT conferred a substantial increase in sensitivity to radiation-induced apoptosis and also resulted in decreased clonogenic survival of Rat-1MycER-Neo cells as compared to cells treated in the absence of c-MycER activation. Interestingly, the Rat-1MycER-Neo cells irradiated without 4HT seemed to be slightly more radiosensitive than the parental Rat-1 fibroblasts. This observation is consistent with the difference in apoptotic potential between these two cell lines even in the absence of c-MycER activation by 4HT. Overexpression of the antiapoptotic oncogene Bcl-2 suppressed the increased radiosensitivity conferred by c-MycER activation, as shown by the similar shapes of the radiation survival curves for Rat-1MycER-Bcl-2 cells, regardless of whether the cells were irradiated with or without c-MycER activation (Fig. 1i). These survival curves also closely resemble the survival curve for Rat-1MycER-Neo cells irradiated in the absence of 4HT. We and others have shown previously that the expression of Bcl-2 in Rat-1 fibroblasts does not directly inhibit the function of the c-Myc protein (20, 23). Therefore, the ability of Bcl-2 to suppress c-Myc-mediated alterations in clonogenic survival suggests that the activation of c-Myc alters clonogenic radiosensitivity via an apoptotic mechanism.

Because clinical radiation therapy is delivered in multiple small fractions to minimize normal tissue toxicity (1), we next examined the effects of c-Myc activity and the resulting apoptotic sensitivity on the clonogenic survival of cells treated with fractionated radiation. Five fractions of 2 or 4 Gy were administered to cells at 12-h intervals. The surviving fractions calculated from these experiments as well as the differences in clonogenic survival between cells plated in the presence...
and absence of 4HT are shown in Table 1. There was no difference in clonogenic survival between parental Rat-1 fibroblasts treated in the presence or absence of 4HT after fractionated irradiation. Conversely, Rat-1MycER-Neo cells were considerably more sensitive when irradiated after c-MycER activation than they were when irradiated in the absence of 4HT. In fact, the relative increase in cell killing observed when Rat-1MycER-Neo cells were treated with five fractions of 2 or 4 Gy closely resembled the expected difference in killing calculated by expanding the surviving fraction at 2 or 4 Gy to the fifth power. Furthermore, Bcl-2 overexpression, which diminished apoptosis in the presence of 4HT, also eliminated c-MycER-mediated differences in clonogenic survival after fractionated radiotherapy. Therefore, alteration of the apoptotic sensitivity of these cells to radiation-induced apoptosis resulted in increased radiosensitivity as measured by clonogenic survival after fractionated radiation. Furthermore, because the observed differences in cell survival were very similar to the differences predicted if one assumes that there would be equal killing in each of five fractions of 2 or 4 Gy, it seems that apoptotic sensitivity remains relatively constant during fractionated irradiation, and that the relative contribution of apoptosis to overall cell killing is similar for each fraction of radiation.

The contribution of radiation-induced apoptosis to the efficacy of radiation therapy in vivo was studied using xenograft tumors in mice. SCID mice were injected intradermally with $1.5 \times 10^7$ Rat-1, Rat-1MycER-Neo, or Rat-1MycER-Bcl-2 cells. Tumors averaging 85 mm$^3$ in volume were treated with 10 Gy of radiotherapy delivered either as a single fraction or in five fractions of 2 Gy each at 12-h intervals. Tumor cryosections from Rat-1 tumors treated with either single-dose or fractionated radiotherapy demonstrated few apoptotic nuclei, regardless of whether animals received 4HT or placebo implants (Fig. 2, a and d and Fig. 3, a and d). In contrast, TUNEL stained sections from Rat-1MycER-Neo tumors demonstrated a marked increase in the number of apoptotic cells relative to Rat-1 tumors (Fig. 2, b and e and Fig. 3, b and e). Furthermore, cryosections from Rat-1MycER-Neo tumors excised from animals that received 4HT implants (Figs. 2e and 3e) demonstrated more TUNEL-positive cells.
than genetically similar tumors from animals that received placebo pellets before single-dose or fractionated radiotherapy (Figs. 2b and 3b). In contrast to our previous report that activation of c-MycER in unirradiated tumors resulted in apoptosis that clustered in regions of tumor hypoxia (20), apoptosis observed after radiotherapy appeared to be evenly distributed throughout, especially in regions surrounding blood vessels. Bcl-2-overexpressing Rat-1MycER-Bcl-2 tumors were refractory to radiotherapy-induced apoptosis in vivo (Fig. 2, c and f, and Fig. 3, c and f). The relative levels of apoptosis observed in vivo after radiotherapy in the presence or absence of 4HT closely paralleled the relative levels of apoptosis observed after irradiation in vitro.

Next, the growth kinetics of these tumors were assessed after in vivo radiotherapy with or without 4HT administration (Fig. 2, g-i, and Fig. 3, g-i). Rat-1MycER-Neo-derived tumors demonstrated prolonged tumor regrowth delay relative to Rat-1 or Rat-1MycER-Bcl-2 tumors after irradiation with a single dose of 10 Gy (Fig. 2, g-i). Additionally, Rat-1MycER-Neo tumors from mice that received 4HT pellet implants were relatively more sensitive to 10 Gy of radiation than were genetically identical tumors from mice that received placebo pellets. Administration of 4HT had little effect on the tumor growth kinetics of Rat-1 and Rat-1MycER-Bcl-2 tumors after single-dose radiotherapy (Fig. 2, g and i). In general, the length of tumor regrowth delay correlated strongly with the extent of apoptosis observed in TUNEL-stained cryosections after single-dose radiotherapy in vivo (Fig. 2). Furthermore, after fractionated radiotherapy administered in five fractions of 2 Gy each, the growth of Rat-1MycER-Neo tumors from mice that received 4HT was substantially delayed relative to any other tumor type or treatment group. 4HT administration had no measurable effect on Rat-1-derived tumors (Fig. 3g) and caused only a slight delay in Bcl-2-overexpressing Rat-1MycER tumors (Fig. 3i). Therefore, tumor regrowth kinetics can be significantly influenced by the level of apoptosis induced during fractionated radiation therapy (Fig. 3).

The results presented here demonstrate that pharmacological regulation of the susceptibility of cells to apoptotic death can increase the overall in vitro sensitivity of otherwise identical cells to single-dose and fractionated irradiation. Also, apoptotic sensitivity contributes similarly to overall cell killing from each of at least five fractions of radiation. Therefore, even small increases in tumor cell killing from individual fractions of radiation could have profound effects on overall cell killing throughout an extended, clinically relevant course (i.e., 30 fractions) of radiation therapy. Most importantly, this study provides direct genetic evidence that increasing tumor cell apoptotic sensitivity, in vivo potentiates the response of tumors to treatment with doses of ionizing radiation that are typically given during fractionated radiotherapy for the treatment of human cancers. Because approximately 50% of cancer patients are treated with radiation therapy, therapeutic interventions that allow for pharmacological alteration of radiation-induced apoptosis (either by enhancing tumor cell apoptosis or by suppressing normal tissue apoptosis) may have the potential to enhance the therapeutic index of radiation therapy and thereby have profound effects on the ability to control and cure human cancers.

References

Direct Evidence That Apoptosis Enhances Tumor Responses to Fractionated Radiotherapy


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/9/1779

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.