

Radiation Abscopal Antitumor Effect Is Mediated through p53¹

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ABSTRACT

The observation that radiation treatment to a local area of the body results in an antitumor effect for tumors distant to the radiation site has been termed the "abscopal effect." To understand the mechanism of this unusual phenomenon, we examined whether the effect was mediated through p53, a protein complex up-regulated in irradiated cells. Non-tumor-bearing legs of C57BL/6 (wild-type p53) and p53 null B6.129S2-*Trp53^{tm1Tyj}* mice were irradiated to determine whether an abscopal effect could be observed against Lewis lung carcinoma (LLC) and T241 (fibrosarcoma) implanted at a distant site. In mice with wild-type p53, both LLC and T241 tumors implanted into the midline dorsum grew at a significantly slower rate when the leg of the animal was exposed to five 10-Gy fractions of radiation compared with sham-irradiated animals, suggesting that the abscopal effect is not tumor specific. When the radiation dose to the leg was reduced (twelve fractions of 2 Gy each), the inhibition of LLC tumor growth was decreased indicating a radiation-dose dependency for the abscopal effect. In contrast, when the legs of p53 null animals or wild-type p53 mice treated with pifithrin- α (a p53 blocker) were irradiated (five 10-Gy fractions), tumor growth was not delayed. These data implicate p53 as a key mediator of the radiation-induced abscopal effect and suggest that pathways downstream of p53 are important in eliciting this response.

INTRODUCTION

The term "abscopal effect" caused by radiotherapy was first coined by Mole in 1953 (1). The word abscopal was derived from the Latin *ab* meaning "position away from" and *scopos*, which means "a target for shooting at." Mole used this term to describe systemic effects that were observed at nonirradiated sites in an animal after treatment with localized radiotherapy. Multiple case reports describing an abscopal effect observed after radiotherapy have been published with a variety of malignancies including lymphoma, papillary adenocarcinoma, melanoma, adenocarcinoma of the esophagus, chronic lymphocytic leukemia, and hepatocellular carcinoma (2–9).

There have been two main theories proposed to explain the abscopal antitumor effect. The first applies to leukemias and lymphomas and hypothesizes that diseased lymphocytes circulate through the irradiated volume during local therapy, giving the false impression of a systemic antitumor effect from local treatment (3, 4). The second applies to solid tumors and postulates that local radiation induces a release of cytokines into the circulation that mediate a systemic antitumor effect. Support for this hypothesis has been provided by Ohba *et al.* (9), who demonstrated an elevation of circulating tumor necrosis factor- α after radiotherapy that coincided with the regression of a hepatocellular carcinoma situated away from the radiation field.

Others have also proposed that the abscopal effect is mediated by the immune system. For example, local radiation of a lesion could induce the release of circulating tumor antigen or of inflammatory factors that could then mediate an augmented immune response against other, unirradiated, malignant lesions expressing similar tumor antigens. It has been shown that local radiotherapy increases the activity of natural killer cells (10). However, to date, none of these explanations for the abscopal antitumor effect have been verified in either the laboratory or the clinic.

In this study, we developed a reproducible and reliable model to study the radiation abscopal effect by irradiating the normal leg of a mouse and observing the growth of a tumor implanted at a site distant from the radiation portal. We here demonstrate that the abscopal antitumor effect in C57BL/6 mice is dependent on the function of p53 and that it is not observed in the absence of functional p53 or in mice in which p53 has been pharmacologically inhibited.

MATERIALS AND METHODS

Cell Lines and Cell Culture. LLC-LM and T241 tumor cell lines were grown at 37°C in 10% CO₂ in DMEM with 10% heat-inactivated fetal bovine serum plus 1% glutamine-penicillin-streptomycin, as has been described previously (11).

Animals and Tumor Model. Male 4–6-week-old C57BL/6 mice or p53 null B6.129S2-*Trp53^{tm1Tyj}* mice (Jackson Labs, Bar Harbor, ME) were used. Mice were caged in groups of five or less, and their backs and hind limbs shaved. All of the animals were fed a diet of animal chow and water *ad libitum*. Animals were anesthetized in an isofluorane chamber before all procedures and were observed until fully recovered. Animals were sacrificed by lethal inhalation of carbon monoxide. Animals with tumors \sim 1000 mm³ were sacrificed, and the skin overlying the tumor was cleaned with Betadine and ethanol. A suspension of tumor cells in 0.9% normal saline was made by passing viable tumor through a sieve, followed by a series of sequentially smaller hypodermic needles (22–30-gauge) as reported previously (12). Tumor cells (1×10^6 cells) were injected s.c. into the midline dorsum. Tumor diameters were measured with calipers and a volume was calculated using the formula (length * width * width)/2.

Tumor Irradiation. Mice were implanted with LLC-LM or T241 cells and when a palpable tumor formed, the mice were randomized and radiation was started. During the radiation therapy, mice were immobilized in a customized harness that allowed the right hind leg to be exposed whereas the remainder of the body, including the midline dorsal tumor site, was shielded by 3.5 cm of lead. The right hind legs were irradiated in a Gammacell Cesium 137 (Atomic Energy of Canada) source operating at a rate of 100 cGy/min. Whole body irradiation was performed in a Plexiglas jig in the same irradiator. Tumors were measured in two dimensions every 3rd day, unless stated otherwise, and tumor volume was determined as described above.

Drug Delivery. Pifithrin- α (Tocris, Bristol, United Kingdom) was diluted in DMSO and was delivered as a daily dose of 2.5 mg/kg of body weight as an i.p. injection. DMSO carrier was given as a daily i.p. injection in the control group.

Irradiation Dose Calculation. A TLD³ was placed into the irradiation jig within the lead block. The apparatus was irradiated with a dose of 10 Gy. The TLD was then queried for dose delivered.

³ The abbreviations used are: TLD, thermoluminescence dosimeter; LLC, Lewis lung carcinoma; TBI, total body irradiation.

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Statistics. Tumor volumes were compared using a paired, 2-tailed Student *t* test with a significance value of $P < 0.05$.

RESULTS

Observation of the Abscopal Effect in Mice. In one experiment, LLC was implanted in the midline dorsum of C57BL/6 mice. At day 10, postimplantation animals with tumors were randomized to treatment and control groups [radiation group, $360 \pm 88 \text{ mm}^3$ ($n = 5$), versus unirradiated group, $391 \pm 97 \text{ mm}^3$ ($n = 5$)]. Irradiation was started on day 10 and 10 Gy were delivered for 5 consecutive days. The control group received sham treatment consisting of placement in the jig but no radiation. The tumors in the mice that received radiation treatment to the leg grew at a significantly slower rate than those in the nonirradiated group. On day 15 postimplantation, the day after completion of radiation, there was already a growth difference between the sham-treated controls and the irradiated animals ($2244 \pm 815 \text{ mm}^3$ versus $1506 \pm 745 \text{ mm}^3$, respectively). By day 18 postimplantation, tumor volume in the sham-irradiated mice was almost 3-fold higher than that of the irradiated mice. In the sham-irradiated group, a mean tumor volume of $6058 \pm 1447 \text{ mm}^3$ was observed as compared with a mean tumor volume of only $2201 \pm 816 \text{ mm}^3$ in the irradiated group ($P = 0.003$).

This experiment was repeated with the addition of a third group of mice treated with a low-dose fractionation schedule of 2 Gy/fraction, two fractions per day, for 6 consecutive days. Five C57BL/6 mice, implanted with LLC, were assigned to each of the three groups in a randomized fashion on the day of tumor implantation. Randomization was performed at the day of implantation to remove any potential observer bias caused by randomization at a later time point. Radiation was initiated on day 5 postimplantation for each of the randomized animals. The control mice received sham treatment (*i.e.*, no irradiation) concurrently with the 10 Gy/fraction group. Tumors were measured and mouse body weight was obtained every 5th day. An inhibition of tumor growth was observed in both of the radiation treatment groups compared with the control group. The abscopal effect of the radiation therapy was dose dependent (Fig. 1). An early effect of the radiotherapy could be seen on day 10 postimplantation, the last day of irradiation. The mean tumor volumes on that day for the 2-Gy- \times -12, 10-Gy- \times -5, and control groups were $571 \pm 278 \text{ mm}^3$, $148 \pm 58 \text{ mm}^3$, and $1262 \pm 547 \text{ mm}^3$, respectively. The effect was more pronounced

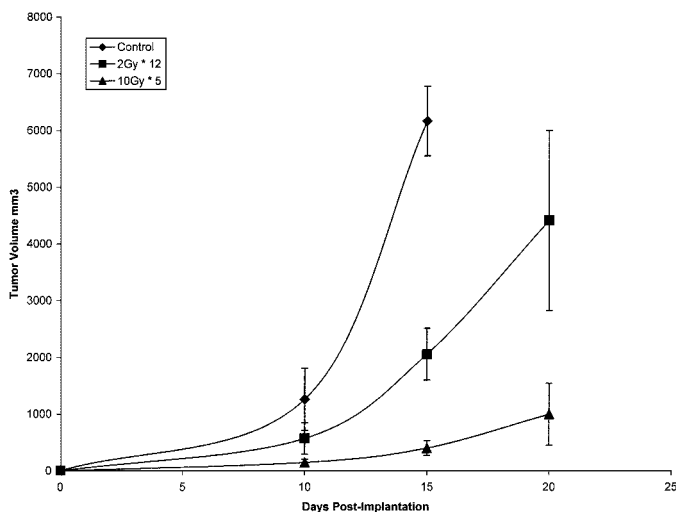


Fig. 1. The radiation abscopal effect is dose dependent. Five mice in each treatment group, 10 Gy \times 5, and 2 Gy \times 12, were irradiated, and the tumor was measured in each group, as well as the sham-irradiated group, and plotted versus days postimplantation. At 15 days, the abscopal effect is dose dependent.

at day 15 postimplantation, when the mean tumor volume measured between the 2 Gy \times 12 groups and the 10 Gy \times 5 groups was $2060 \text{ mm}^3 \pm 130 \text{ mm}^3$ versus $400 \text{ mm}^3 \pm 130 \text{ mm}^3$, respectively ($P = 0.004$). The mean tumor volume on that day for control animals was $6167 \text{ mm}^3 \pm 613 \text{ mm}^3$.

After accounting for tumor weight, the body weights of the animals in each of the three groups were not significantly different (Fig. 2), indicating that the antitumor effect observed with hind leg irradiation was not caused by weight loss.

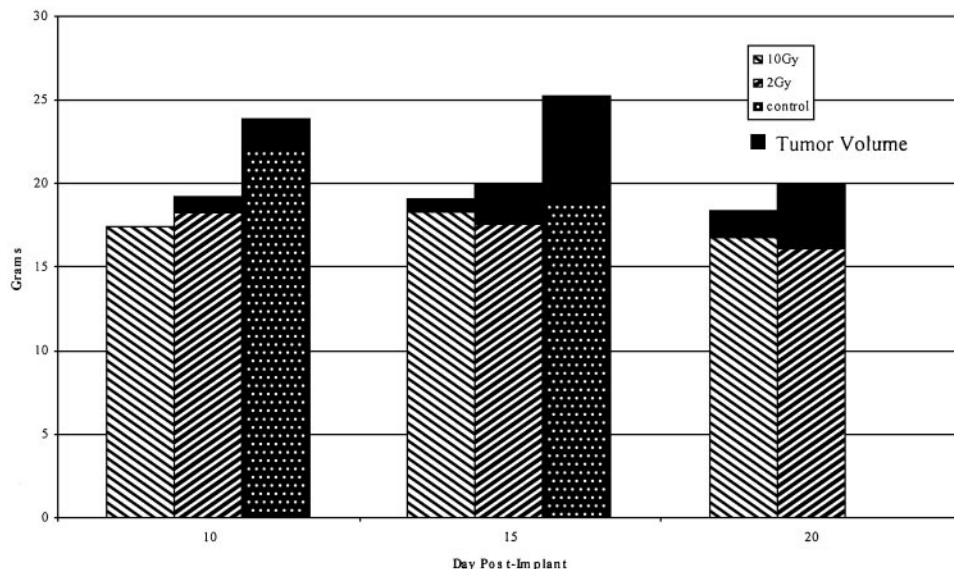
The Abscopal Effect Can Be Observed for Multiple Tumor Types. To determine whether the abscopal antitumor effect observed was tumor-type dependent, the experiments described above were repeated using a murine fibrosarcoma cell line, T241. Again, two groups with five animals in each group were implanted with T241 tumor cells according to the methods described previously. T241 tumors grew to $5000 \pm 822 \text{ mm}^3$ in the unirradiated mice within 15 days of s.c. implantation compared with only $1750 \pm 568 \text{ mm}^3$ in the irradiated mice ($P < 0.001$). These data show that the abscopal antitumor effect is not specific to LLC and suggest that it may be independent of tumor histology.

The Abscopal Effect Is Not Caused By Scatter Dose. In the previous experiments, a TLD placed in the irradiation jig at the level of the dorsal tumor consistently measured less than 1% (10 cGy) of the total daily dose delivered to the right hind leg. To determine whether this scattered radiation dose contributed to the inhibition of growth from the shielded midline dorsal tumor, five mice were treated with TBI to a dose of 50 cGy per fraction for five fractions. The midline tumor, therefore, received five times the scatter dose measured from the previous experiment. The mean tumor volume was virtually identical at 18 days postimplantation between the unirradiated control group and the low-dose TBI group ($4300 \pm 437 \text{ mm}^3$ versus $4354 \pm 1571 \text{ mm}^3$, respectively). Therefore, low doses of radiation, in the range of that deposited by scatter, do not inhibit tumor growth and cannot explain the distant antitumor effect observed with hind leg irradiation.

The Abscopal Effect Is Dependent On Functional p53. We hypothesized that the observed abscopal antitumor effect may be mediated by the induction of p53 by the radiation treatments, given that p53 is a radiation-responsive element. To test this hypothesis, we implanted 10 mice that were null for p53 (B6.129S2-*Trp53tm1Tyj*; Ref. 13) with LLC tumor cells in the midline dorsum and randomized them into two groups. One group was irradiated to the right hind leg, and the other received sham treatment only. We noted that the LLC cell line had been previously shown to be p53 mutant (14). Unlike the studies in mice with wild-type p53, there was no evidence of the abscopal antitumor effect in the p53 null mice. The tumors growing in unirradiated p53 null mice and those growing in the irradiated p53 null mice grew to similar volumes at day 20 (5800 mm^3 versus 6500 mm^3 , respectively).

To further test our hypothesis that the abscopal effect is dependent on p53, we used a second method of suppressing p53 as described previously by Komarov *et al.* (15). The chemical pifithrin- α can pharmacologically inhibit p53-driven effects after radiotherapy, as demonstrated by the reversal of lethal whole-body-radiation treatments in C57BL/6 mice. Twenty mice were given injections of LLC tumor cells in the dorsum and were randomized into one of four groups: no treatment, vehicle (DMSO) alone, pifithrin- α alone, or the combination of pifithrin- α and radiotherapy. Radiation treatments and pifithrin- α injections, given 1 h before radiotherapy, were initiated on day 8 postimplantation. On that day, the mean tumor volumes for the untreated, vehicle-alone, pifithrin- α -alone, and combination groups were $486 \pm 111 \text{ mm}^3$, $355 \pm 90 \text{ mm}^3$, $431 \pm 131 \text{ mm}^3$, and $516 \pm 174 \text{ mm}^3$, respectively. The final tumor volumes, at day 16

Fig. 2. The radiation abscopal effect is not caused by weight loss. Two of three groups, 10 Gy * 5, and 2 Gy * 12, were irradiated, and the mean weights for these, plus the sham irradiated group, were plotted *versus* days postimplantation. ■, the mean tumor volume assuming a density of 1 mm³ equaling 1 mg. There is no significant difference in overall weight when corrected for tumor weights.



postimplantation, were 3500 ± 926 mm³ in the untreated group, 4100 ± 847 mm³ for vehicle alone, 3250 ± 923 mm³ for pifithrin- α alone, and 4700 ± 1787 mm³ for pifithrin- α and radiotherapy (Fig. 3). There was no statistically significant difference between any of the groups, ($P = 0.19$ for untreated control *versus* combination therapy, $P = 0.61$ for carrier *versus* combination therapy, and $P = 0.27$ for pifithrin- α -alone *versus* combination therapy); and no abscopal antitumor effect was observed. The pifithrin- α study was repeated, and, again, no abscopal effect was seen when pifithrin- α was combined with radiotherapy. When taken together with the results of the p53-null-mice experiments, these data show that the radiation abscopal effect is mediated through p53 and can be negated by blocking this protein complex.

DISCUSSION

Cells receiving radiation are characterized by DNA damage, the induction of apoptosis, and an up-regulation of a multitude of transcription factors including, but not limited to, p53. Radiotherapy can

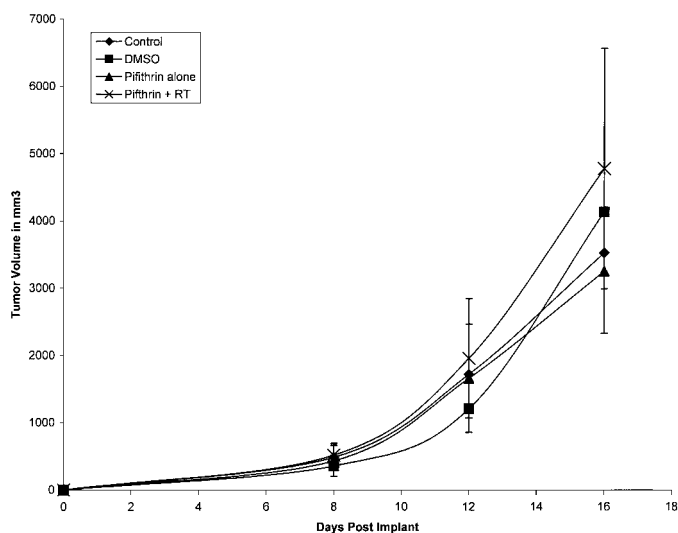


Fig. 3. The radiation abscopal effect is reversed by treatment with pifithrin- α . Five mice per group were randomized to no treatment, carrier alone, pifithrin- α alone, or radiation plus pifithrin- α . Tumor volumes were plotted *versus* days postimplantation. Pifithrin- α can reverse the radiation abscopal effect.

also have a significant impact on the local microenvironment of tissues within the radiation portal. Such effects from radiotherapy include increased vascular permeability, altered cytokine levels, and local inflammation (16, 17). More recently, the concept of a bystander effect, in which radiotherapy to one cell has direct impact on an adjacent cell, has been described (18). These cellular and supracellular effects of radiotherapy are distinct from the abscopal effect coined by Mole, which is an "... effect observed at a site distant to that irradiated within the same organism" (1).

Several hypotheses have been proposed for the mechanism of the abscopal effect. These have included a systemic release of specific cytokines, a systemic immune reaction generated against local tumor antigens, or local inflammation leading to a distant effect. However, none of these postulated mechanisms have stood the test of time nor have they been validated in the laboratory or the clinic. In this study, we choose to use solid murine tumors in immunocompetent mice. We choose solid tumors so that tumor cells circulating through the radiation portal could not account for our observed abscopal effect as hypothesized by Antoniadis *et al.* (3) and Rees (4). C57BL/6 mice were chosen as the host murine strain because we desired an intact immune system, a mouse with a known p53 status, and a commercial supply of p53 knockout mice.

By using the LLC and T241 tumors in C57BL/6 mice, we were able to demonstrate that local irradiation to normal tissue has a systemic antitumor effect against both LLC and T241 tumors. The antitumor effect was not attributable to weight loss because the irradiated and nonirradiated mice had comparable adjusted body weights. Nor was the effect attributable to scatter dose of radiation therapy, because we did not observe any effect on tumor growth after low-dose TBI. However, in this model system, the abscopal effect was dependent on p53, because the effect was not observed in p53 null mice nor with concurrent administration of pifithrin- α , a drug shown to block p53 after radiation treatments. Although the exact mechanism for the abscopal effect is still unknown, we have demonstrated that the effect is reproducible and is mediated through p53.

To test the hypothesis that the abscopal effect is, in part, caused by the release of tumor antigens into the circulation after radiotherapy, we chose to irradiate normal tissue *versus* a second tumor site. Because we saw the abscopal effect after radiation to normal tissue and not after tumor antigen release, our data strongly suggest that the mobilization of tumor antigens does not contribute to the abscopal

antitumor effect in our model. However, our data are consistent with the hypothesis that, after radiation therapy, p53 may act as a transcription factor leading to the expression of cytokines or other factors, possibly because of local inflammation. These factors may be produced locally within the irradiated tissues and then released systemically leading to a systemic antitumor effect. These factors could exert a direct or indirect effect on the tumors at a distant site. It is tempting to speculate that angiogenesis inhibition could contribute to the effects. A recent study demonstrated that circulating levels of the angiogenesis inhibitor endostatin significantly increased after radiotherapy in mice (19). Alternatively, p53 is also known to increase the expression of thrombospondin, another inhibitor of angiogenesis, and to decrease the expression of vascular endothelial growth factor after radiotherapy (20, 21). When taken together with our studies, these findings lend credence to the hypothesis that radiotherapy may lead to a systemic antiangiogenic effect mediated through p53, and that this may be one of the mechanisms for the abscopal effect.

In conclusion, we have developed a model for the radiation abscopal effect in C57BL/6 mice by using the LLC and T241 tumors, a model that is, in part, mediated through p53, as demonstrated both in p53 knockout mice and through pharmacological inhibition of p53 by pifithrin- α . We have demonstrated that in our model the effect is not caused by weight loss, scatter dose, irradiated circulating tumor cells, or a release of tumor antigens. We have not defined the entire mechanism for the abscopal effect and future studies will use additional tumor lines and mouse strains, as well as additional p53 inhibitors, to further elucidate this interesting effect.

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