Influence of Tumor Cell and Stroma Sensitivity on Tumor Response to Radiation

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Abstract

In this study, we evaluated the role of tumor cell and tumor stroma sensitivity as determinants of radiation-induced tumor growth delay. A DNA double-strand break repair–defective DNA-PKcs$^{-/-}$ tumor cell line and its radioresistant DNA-PKcs$^{+/+}$–transfected counterpart were used to initiate tumors in nude and hypersensitive severe combined immunodeficient (SCID) mice. Insertion of the human DNA-PKcs$^{+/+}$ gene substantially increased the intrinsic radioresistance of the DNA-PKcs$^{-/-}$ tumor cells and substantially decreased tumor response to radiation in both nude and hypersensitive SCID mice. Tumor cell radiosensitivity was the major determinant of tumor response in nude mice. In SCID mice, both tumor cell sensitivity and radiation-induced stromal damage contributed to response. The relative contribution of host and tumor cell sensitivity on tumor response was unchanged for single doses of 1 × 15 and 6 × 3 Gy–fractionated dose irradiation. [Cancer Res 2007;67(9):4016–21]

Introduction

Identification of the determinants of tumor response to radiation has long been a goal of radiation oncologists and biologists. Tumors of different histology exhibit markedly different responses to radiation, and the variability in response of tumors of the same histology, stage, and grade is especially puzzling (1, 2). Local failure may not only directly cause or contribute to life shortening, but importantly, the rate of distant metastasis clearly increases in patients with uncontrolled primaries (3, 4). In principle, tumor response to radiation is determined by radiation damage of tumor cells or the tumor stroma, or a combination of the two. Substantial, although largely indirect, evidence suggests that tumor cell radiosensitivity is the principal determinant of tumor response (5–7). However, some studies, especially recent studies, challenge this hypothesis (8, 9). These studies, employing tumor growth delay as the end point, show that tumor response in severe combined immunodeficient (SCID) mice, the cells and tissues of which are hypersensitive to radiation, is greater than the response of the same tumors grown in wild-type mice (9). Similarly, responses were greater when tumors were grown and irradiated in acid sphingomyelinase$^{+/+}$ host mice than in their endothelial cell apoptosis–resistant acid sphingomyelinase$^{-/-}$ littermates (8). In these studies, it was also reported that the contribution of stromal damage versus tumor cell killing to tumor response was dose-dependent: at lower single fraction dose levels, damage to tumor stroma dominated tumor responses.

We recently addressed this issue by assessing the response of tumors initiated from a DNA-PKcs$^{-/-}$ tumor line and tumors from the same line transfected with DNA-PKcs$^{+/+}$ (10). The increase in tumor cell resistance conferred by the introduction of DNA-PKcs$^{+/+}$ caused a proportionate decrease in tumor response to radiation. If stromal damage were the sole or predominant determinant of response, then for isogenic tumors implanted in the same strain of mice and exposed to the same dose of radiation, the tumors may be expected to exhibit similar responses to radiation. This was not observed. The 1.5-fold difference in the radiosensitivity of the isogenic tumor lines caused a 1.5-fold change in tumor response. In the present study, we evaluate the influence of hypersensitive tumor stroma on tumor response. The effect of a 2.5-fold difference in tumor cell radiosensitivity is evaluated on tumors in nude and hypersensitive SCID mice. The normal tissues and cells including vascular endothelial cells of DNA double-strand break repair–deficient SCID mice are 2.5- to 3-fold more sensitive to radiation than the tissues and cells of normal repair–proficient nude mice and humans (5, 11, 12).

Materials and Methods

Tumor induction, transfection, and selection. Isogenic tumor lines were prepared as previously described (10). Briefly, a tumor was induced in SCID mice with an i.m. injection of 0.1 mg of methylcholanthrene. The tumor was excised, a single cell suspension was prepared and transfected by the calcium phosphate procedure (Invitrogen). A full-length human DNA-PKcs cDNA clone (pKDPJ1) was created in a pBluescript II KS vector (Stratagene). The DNA-PKcs expression vector was cotransfected with the pSV2neo plasmid for selection purposes. Seventy-five neoresistant clones were screened for sensitivity to 4 Gy of irradiation. Four clones exhibited increased radioresistance and were analyzed for the integration and expression of human DNA-PKcs transgene by genomic PCR and reverse transcription-PCR (RT-PCR) reactions. PCR primer pairs specifically amplifying only the human DNA-PKcs cDNA or mouse glyceraldehyde-3-phosphate dehydrogenase were employed for genomic PCR and RT-PCR. The genomic DNA and total RNA from the parental FSC1-3 cells and FSaII mouse tumor cells were used as negative controls in both the PCR and RT-PCR reactions.

Tumor cell radiosensitivity. Intrinsic tumor cell radiosensitivity was evaluated with the clonogenic assay as previously described (6). Following irradiation, the cells were incubated 7 to 11 days for colony formation depending on the dose administered. The surviving fraction data were corrected for initial and final multiplicities determined 4 to 6 h after plating and at the time of irradiation. The radiosensitivity of the parental tumor line FSC1-3, and its transfected counterpart T43, was periodically evaluated and unchanged for >20 passages. The *in vivo* stability of the tumor cells was evaluated. Source FSC1-3 and T43 tumors were excised, a single cell suspension was prepared and plated, and the cells were irradiated 20 h later.

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Functional vascular density. Functional tumor vessels were labeled via tail vein injection of 10 μg of biotinylated lectin (Vector Laboratories) per gram of body weight (13, 14). Five minutes following lectin injection, the thorax of anesthetized mice was opened and the tissue fixed by systemic perfusion with 4% paraformaldehyde in PBS via the left ventricle, at a pressure of 80 mm Hg for 5 min (13, 14). Following paraffin embedding, 5-μm sections were stained with peroxidase-conjugated streptavidin (KPL), and vascular endothelial cells of functional vessels were visualized with true blue chromagen (KPL; ref. 8). The number of true blue–labeled profiles per high-power field were determined in 15 randomly selected fields/tumor without discrimination between necrotic and non-necrotic areas.

Tumor growth delay. F1 generation tumors were initiated from *in vitro* cultures. For experimental studies, ≈2 mm source tumor fragments were transplanted to the s.c. tissue of the right rear gastrocnemius of male NCr(*nu/nu*) mice. A single FSC1-3 or T43 source tumor was used to initiate all tumors for each experiment. Upon reaching a diameter of ~140 to 180 mm^3, the tumors were irradiated with ^{137}Cs in a specially designed jig as previously described (6). Tumor volumes were measured daily. For calculation of tumor growth, tumor volume was normalized to each tumor's volume at the time treatment was initiated. Individual growth curves for each of six control and seven to eight irradiated tumors were plotted and the time for the tumors to grow to a specified factor greater than their initial volumes was calculated as the mean time for all tumors within the group to achieve that volume. Tumor growth delay was calculated as the days for irradiated tumors to achieve four times their initial volume, minus the time for control tumors to achieve four times their initial volume over the same volume range. The unpaired *t* test was used to analyze for differences in growth delay between the isogenic tumor pair. The data shown are for one experiment done for each radiation dose schedule. Pilot studies were done at 15 Gy in T43 tumors in nude mice, as well as FSC1-3 tumors in nude and SCID mice. The results did not differ between the preliminary experiments and those reported here.

Results

Following bulk transfection of the parental DNA-PKcs$^{-/-}$ tumor line FSC1-3, 75 neoresistant clones were isolated and screened for assessment of their intrinsic radiosensitivity. Four clones exhibited increased resistance to radiation relative to the parental line FSC1-3, and all resistant clones contained human DNA-PKcs. Genomic and RT-PCR analysis of two of the more resistant clones, T43 and T53, confirmed the integration and expression of human DNA-PKcs as shown in Fig. 1*A*. *In vitro* survival curve analyses showed that clone T43 was ~2.5-fold more resistant to radiation than FSC1-3 as seen in Fig. 1*B*, and was used in the present study. The *in vivo* stability of the parental FSC1-3 and the transfected T43 tumor clone was confirmed by evaluation of the cells' radiosensitivity following *in vivo* growth. A single cell tumor suspension was prepared, plated *in vitro* and irradiated. Following a dose of 4 Gy, the plating efficiency adjusted surviving fractions were 0.4 for cells from T43 tumors and 0.02 for cells from FSC1-3 tumors.

The growth of control and irradiated T43 and FSC1-3 tumors in nude mice following single and fractionated dose irradiations was evaluated as shown in Fig. 2*A* and *B*. Consistent with their similar *in vitro* proliferative rates, tumors initiated from the isogenic tumors grew at a similar rate *in vivo*. Following 15 Gy of irradiation, tumors arising from the radiosensitive FSC1-3 DNA-PKcs$^{-/-}$ cells exhibit a substantially longer growth delay than their DNA-PKcs$^{+/+}$ counterpart (*A*). To determine if the influence of tumor cell

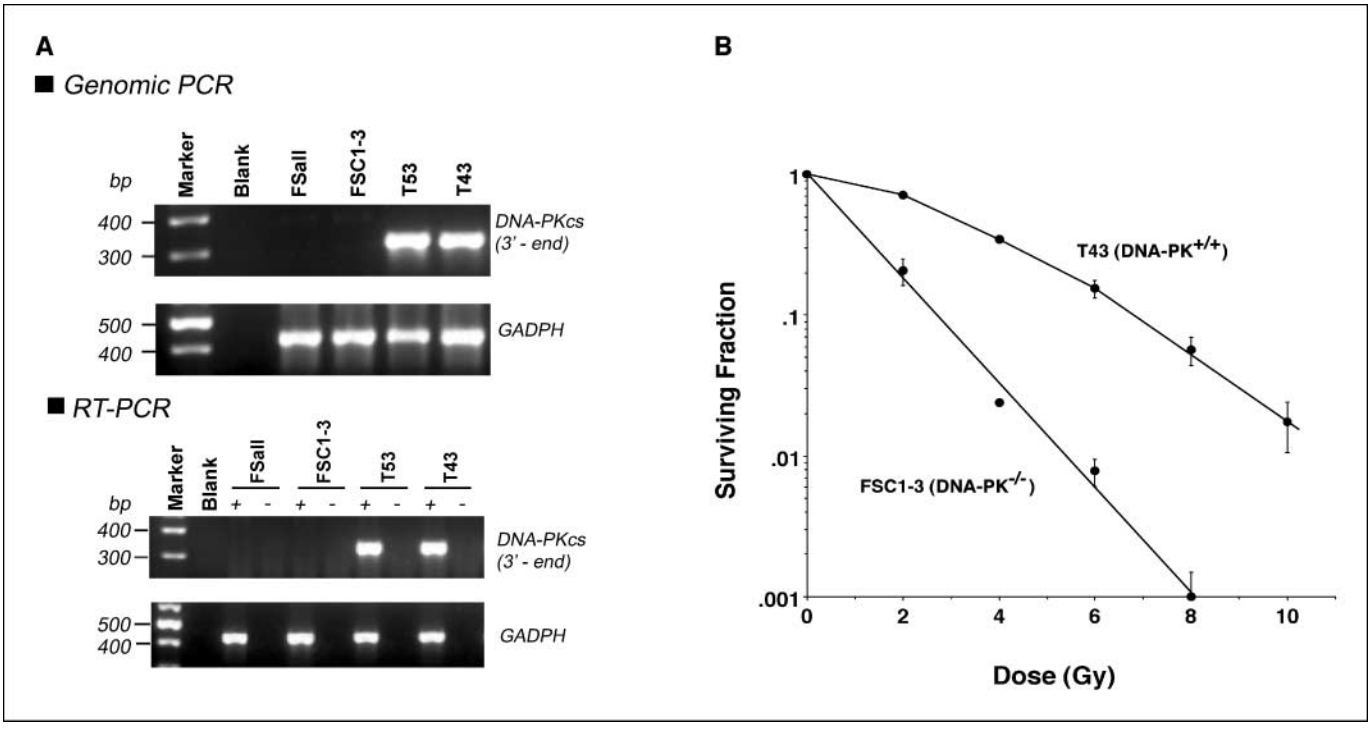

Figure 1. *A,* the absence of human DNA-PKcs in FSC1-3 and the integration of DNA-PKcs into the genome of transfected FSC1-3 (T53 and T43) cells. The primer sequences employed do not recognize murine DNA-PKcs of mutated FSC1-3 or nonmutated murine FSaII cells. To determine if the integrated DNA-PKcs is expressed (e.g., intact cDNA promoter sequences), RT-PCR analysis of mRNA employing the human primers DP-28 and DP-29 was done. Samples in the (−) lanes were prepared without the addition of reverse transcriptase prior to PCR. The absence of bands in these lanes shows the absence of contaminating genomic DNA in the mRNA extracts. *B,* the clonogenic survival of DNA-PKcs$^{-/-}$ FSC1-3 cells, and its more resistant DNA-PKcs$^{+/+}$ transfectant T43, following ^{137}Cs irradiation *in vitro*. Confidence intervals are 1 SD.

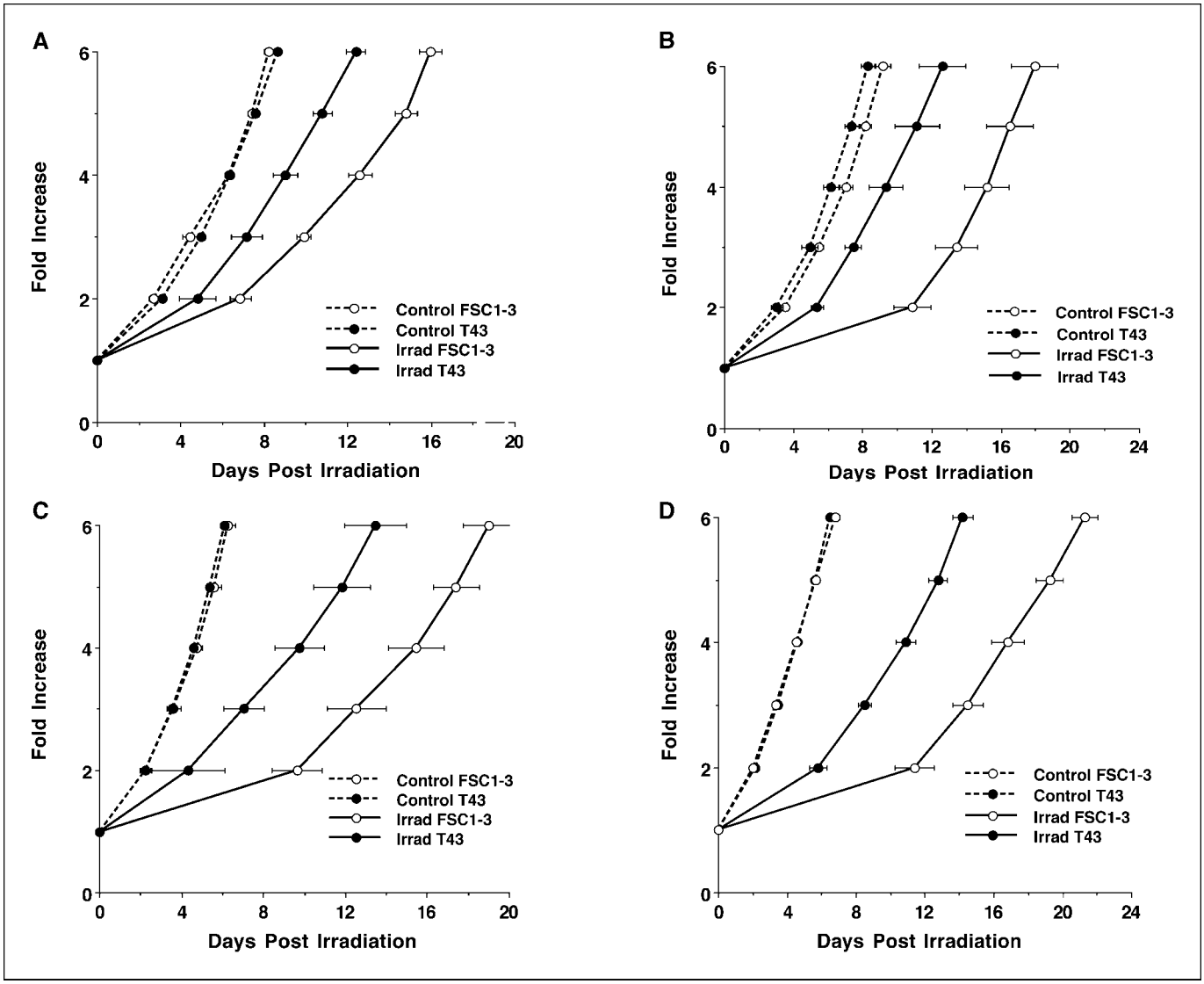

Figure 2. *A* and *B,* the growth of control (nonirradiated) and irradiated FSC1-3 and T43 tumors in nude mice. *C* and *D,* the growth of control and irradiated FSC1-3 and T43 tumors in SCID mice. Irradiated tumors in (*A*) and (*C*) received 15 Gy as a single dose; tumors in (*B*) and (*D*) received 6 × 3 Gy doses in 3 d with >8 h between fractions. *Points,* mean number of days from six control and seven to eight irradiated tumors to achieve the specified fold increase in volumes, relative to their volumes at the initiation of treatment; *bars,* 1 SE.

radiosensitivity was dose-dependent, we also irradiated FSC1-3 and T43 tumors with 6 × 3 Gy fractions administered over 3 days (>8 h between fractions). As observed following a single fraction dose of 15 Gy, tumors arising from FSC1-3 cells were substantially more sensitive to radiation when exposed to fractionated dose irradiation (*B*). With one exception (FSC1-3, 15 Gy, $P < 0.05$), the doubling times of unirradiated control and regrowing irradiated tumors in nude mice did not significantly differ over the volume range of 2 to 4 Vo ($P = 0.3$, T43, 15 Gy; $P > 0.5$, T43, 6 × 3 Gy; $P > 0.5$ FSC1-3, 6 × 3 Gy).

We next examined the influence of tumor cell sensitivity on the response of tumors growing in hyper-radiosensitive DNA-PKcs$^{-/-}$ SCID mice. To confirm the greater radiation sensitivity of SCID versus nude mice, including greater endothelial cell sensitivity, as previously reported (9, 11, 12), we evaluated the number of functional tumor vessels in 15 randomly selected fields from each of three control and three irradiated T43 and FSC1-3 tumors in nude and SCID mice, at 5 days following 15 Gy irradiation. At the time of fixation, control tumor volumes ranged between ~180 and 260 mm^3; the volume of irradiated regrowing T43 and FSC1-3 tumors in nude and SCID mice at 5 days following irradiation was 170 to 360 mm^3.

Representative images showing the influence of irradiation on the functional vasculature of tumors in nude and SCID mice are shown in Fig. 3. As suggested in Fig. 3, irradiation resulted in a pronounced decrease in the functional vessels of both T43 and FSC1-3 tumors in SCID mice compared with unirradiated tumors, as well as compared with irradiated tumors grown in nude mice. In nude mice, the mean number of vessels per field was 6.5 ± 0.6 (SE) in control FSC1-3 tumors, and 5.8 ± 0.9 in irradiated tumors ($P > 0.2$). For T43 tumors in nude mice, the number of functional vessels was 8.8 ± 1.4 in control and 6.7 ± 1.1 in irradiated tumors ($P > 0.2$). For the same tumors in SCID mice, irradiation markedly reduced the number of functional vessels from 9.1 ± 0.6 to

3.5 ± 0.8 in FSC1-3 tumors ($P < 0.01$), and from 11.3 ± 1.3 to 2.2 ± 0.6 in T43 tumors ($P < 0.01$).

The intrinsic radiosensitivity of the tumor cells substantially affects tumor response in SCID as well as nude mice (Fig. 2). However, in contrast with the results observed in nude mice, the doubling times of irradiated regrowing tumors in SCID mice substantially exceeded the doubling times of control unirradiated tumors. Damage to the hypersensitive stroma of SCID mice, and the resultant substantial slowing of the growth rate of irradiated tumors, increases growth delay by an average of 3.5 ± 0.8 days. The additional growth delay for both isogenic tumors in SCID mice reduces the average FSC1-3 to T43 growth delay ratio from ~2.5 in nude mice, to 2.1 in SCID mice, indicating that in SCID mice, factors other than the tumor cells' 2.5-fold difference in intrinsic radiosensitivity, i.e., damage to tumor stroma, influences tumor response. Radiation-induced growth delays for the various host tumor models and dosing schemes are summarized in Fig. 4. Tumor cell radiosensitivity is

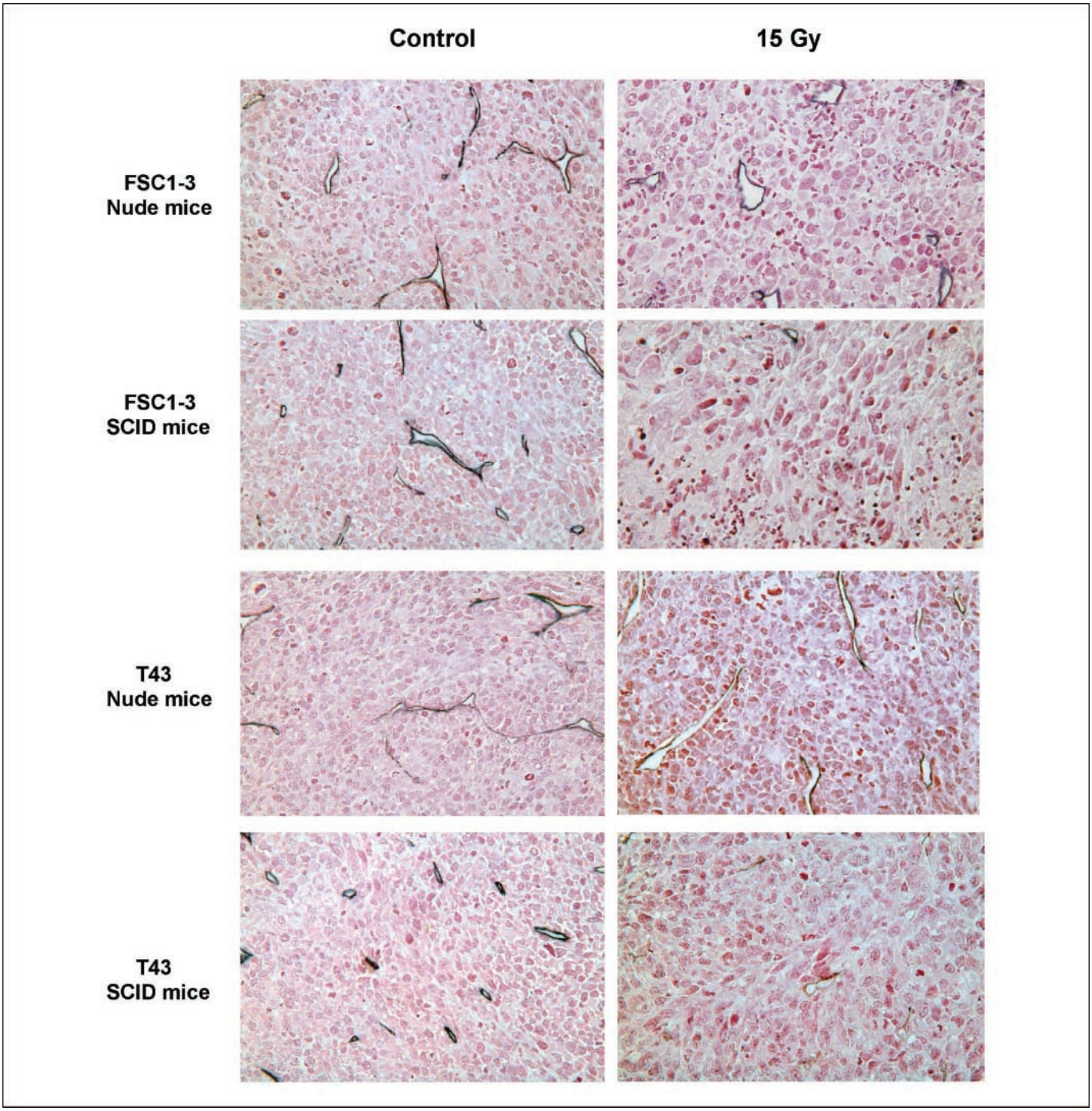

Figure 3. Pronounced effect of irradiation on the functional vascular density of tumors in SCID versus nude mice. Functional tumor vessels were identified by the i.v. injection of biotinylated lectin 5 min prior to perfusion fixation. The decrease in the functional vascular density of tumors in SCID, but not nude mice, was similar in both FSC1-3 and T43 tumors.

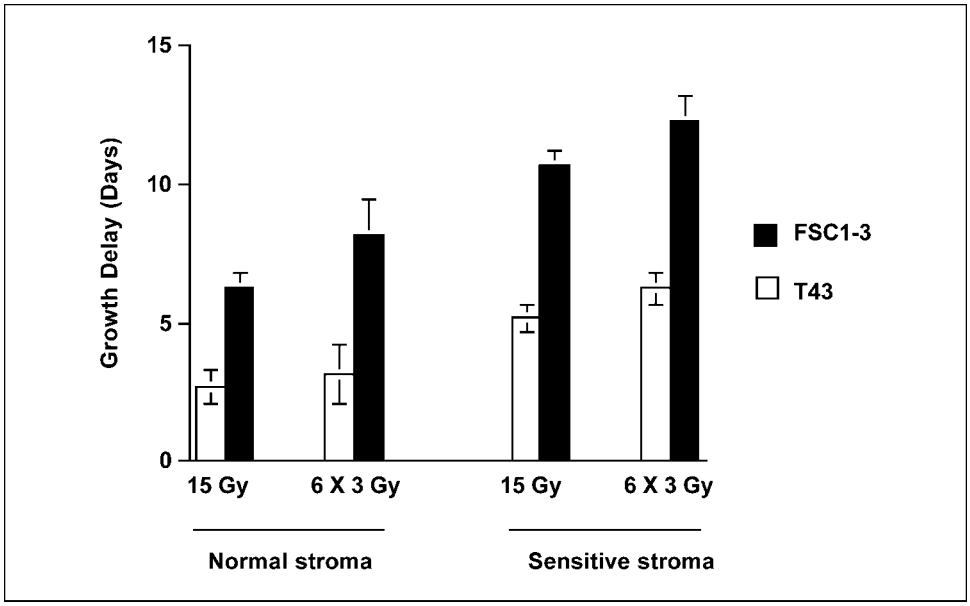

Figure 4. Growth delay (in days) of isogenic tumors receiving a single dose of 15 Gy or 6 × 3 Gy irradiation in mice exhibiting normal (nude) or hypersensitive (SCID) stromal response to radiation.

a major determinant of tumor response regardless of the host stromal sensitivity.

Discussion

The results of this study show a major influence of tumor cell radiosensitivity on tumor response to radiation in nude and radiosensitive SCID mice. The influence of tumor cell sensitivity is not dose- or dose per fraction–dependent over the dose range of 1 × 15 to 6 × 3 Gy. Tumor response measured in terms of growth delay was greater in SCID than in nude mice, likely due to the substantial vascular damage observed in SCID mice following irradiation. Additionally, the results of the present study indicate that stroma and tumor cell sensitivity are not interdependent, as previously suggested (8), at least as reflected by the tumors' functional vascular response at 5 days following irradiation. In the same strain of mice, the response of the vasculature was similar in tumors that exhibited markedly different responses to radiation. Similarly, the vascular response to radiation markedly differs for the same tumors implanted in different host mice.

Variations in tumor and normal tissue response to radiation are clinically well documented, with tumor response variability substantially exceeding that observed for several dose-limiting normal tissue reactions (15). As the stromal elements of normal and tumor tissue are derived from the host, the present results suggest that tumor response variability primarily arise from the substantial and well-documented variability in intrinsic tumor cell sensitivity (5). Although factors such as variable tumor hypoxia and differences in the tumor clonogenic fraction may occur between tumors and may influence response, the dose required to sterilize tumor cells is directly dependent on the cells' intrinsic radiosensitivity, be they oxic or hypoxic. However, it has been noted that the stromal element of human tumors, such as tumor-associated macrophages, may be substantially higher than is commonly observed in rodent tumor models (16). One cannot exclude the possibility that stromal cells may play a more prominent role in human cancers than is observed in rodent tumors.

Irradiation alters the activity of a wide array of transcription factors, chemokines, growth factors and cytokines that influence tissue responses to radiation, including tissue edema, fibrosis, inflammation, and apoptosis (17). Consistent with the studies of others (8, 9, 18, 19), the results obtained in the present study suggest that changes in the activity of factors that influence the sensitivity of tumor stroma or its constituent elements would likely influence radiation-induced tumor growth delay.

Although the severe stromal damage of tumors in SCID mice increases tumor growth delay, it cannot be concluded that such damage will reduce the dose required for permanent local tumor control. Budach et al. found that for large subcurative single doses of radiation, tumor growth delay was greater for the same tumors in SCID versus nude mice, but the doses required for permanent local control did not differ (20). Kozin et al. observed that anti–vascular endothelial growth factor receptor monoclonal antibodies significantly decreased the dose required for local tumor control (21), whereas Zips et al. found that adjuvant inhibition of vascular endothelial growth factor receptor kinases increased tumor growth delay, but not local tumor control (22). In the present study, the relatively slower regrowth of irradiated tumors in SCID mice, rather than increased radiation killing of tumor clonogens, accounts for their enhanced growth delay. Additional studies will be required to resolve the roles of tumor cell and tumor stroma damage as determinants of response for the end point of permanent local tumor control, achieved by clinically relevant fractionated dose irradiation.

In summary, tumor cell radiosensitivity is the major determinant of tumor response to radiation in repair-proficient nude mice for the end point of tumor growth delay. The effect of tumor cell sensitivity is also prominent for tumors in SCID mice, but the greater stromal sensitivity of SCID mice, and the resulting reduction in functional tumor vasculature, substantially increases tumor response to radiation.

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