Tumor Cell Radiosensitivity Is a Major Determinant of Tumor Response to Radiation

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Abstract
Substantial evidence suggests that the radiosensitivity of the tumor cells is the primary determinant of tumor response to radiation. More recent studies suggest that tumor stroma radiosensitivity is the principle determinant of response. To assess the relationship between intrinsic tumor cell radiosensitivity and tumor response, we altered the intrinsic radiosensitivity of a cloned tumor cell line and analyzed the effect of this alteration on tumor response. A cloned tumor cell line derived from DNA double-strand break repair–deficient severe combined immunodeficient mice was transfected with the double-strand break repair gene DNA-PKcs. The intrinsic radiosensitivity of the transfected tumor line was decreased by a factor of ~1.5. The isogenic lines were used to initiate tumors in NCr-nu/nu mice. When transplanted in the same strain of mice and exposed to the same dose of radiation, the isogenic tumors may be expected to exhibit a similar response to radiation if radiation damage to host stroma is the principle determinant of response. This was not observed. Over the dose range of 20 Gy in four 5-Gy fractions to a single dose of 30 Gy, the 1.5-fold increase in intrinsic tumor cell radioresistance conferred by the introduction of DNA-PKcs caused a 1.5-fold decrease in tumor growth delay. The results show that the intrinsic radiosensitivity of tumor cells is a major determinant of tumor response to radiation. (Cancer Res 2006; 66(17): 8352-5)

Introduction
Substantial although largely correlative evidence suggests that the intrinsic radiosensitivity of tumor cells is the primary determinant of tumor response to radiation. Tumor cells derived from radioresistant tumor types exhibit substantial radiosensitivity when examined in vitro, whereas cells derived from largely incurable tumor types exhibit radioresistance in vitro (1). In rodent and xenografted human tumor models, the experimentally estimated number of clonogens per tumor combined with their in vitro measured radiosensitivity predicts the single fraction radiation dose required for permanent local tumor control (2), and changes in the number of clonogens per tumor result in a proportionate change in the tumor control dose (3).

The relationship between tumor cell radiation sensitivity and tumor response has been challenged by recent experimental studies indicating that at doses or doses per fraction that approximate those employed clinically, tumor response is governed by the radiation response of the tumor microvasculature, and not by tumor cell radiosensitivity. These conclusions are based on the longer radiation-induced growth delay of tumors grown in mice in which normal tissues and tumor vascular endothelium are hypersensitive to radiation [i.e., severe combined immunodeficient (SCID) mice; ref. 4]. In addition, it has been shown that radiation response is reduced when tumors are grown in acid sphingomyelinase–deficient (asmase−/−) mice. The vascular endothelium of asmase−/− mice exhibits a substantially reduced apoptotic response to radiation compared with wild-type mice (5).

To evaluate the role of tumor cell radiosensitivity in tumor response, we prepared isogenic tumor cell lines that differ by the presence of a functional DNA double-strand break repair gene, DNA-PKcs. The tumor cell lines were implanted and studied in wild-type mice that are neither genetically nor phenotypically hypersensitive nor resistant to radiation (i.e., NCr-nu/nu mice). If host stroma is the sole determinant of response, the response of the isogenic tumors implanted in the same strain of mice and exposed to the same dose of radiation will not differ. If tumor cell radiosensitivity is the primary determinant of tumor response, the response of the isogenic tumors may be expected to differ in direct proportion to the difference in the tumor cell radiosensitivity. If both factors contribute to tumor response, then the difference in response of the isogenic tumors may be expected to lie between these extremes.

Materials and Methods
SCID tumor induction, transfection, and selection. Tumors were induced in SCID mice with an i.m. injection of 0.1 mg methylcholanthrene. The tumors were excised and a single-cell suspension was prepared and plated. A clone was expanded and transfected by the Ca-phosphate procedure (Invitrogen, Carlsbad, CA). A full-length human DNA-PKcs cDNA clone (pKDP11) was created in a pblessurect II KS vector (Stratagene, La Jolla, CA). A phosphoglycerate kinase promoter sequence was introduced in the unique NotI site located ~90 bp upstream of the DNA-PKcs translation initiation codon. The DNA-PKcs expression vector was cotransfected with the pSV2neo plasmid for selection purposes. Seventy-five neo-resistant clones were screened for sensitivity to 4-Gy 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 amplifying specifically the human DNA-PKcs cDNA (forward primer, 5′-CCTCCGGGCTTCCGCTCAGAC-3′; reverse primer, 5′-GTTCTTGGCCAC-GAATGTTTGCG-3′) or mouse GAPDH (forward primer, 5′-AGAAGACTCTGG-GATGGCCCC-3′; reverse primer, 5′-AGGTACCCACCCCTGTGC-3′) were employed for both genomic PCR and RT-PCR. In RT-PCR analysis, isolated total RNA was preincubated with DNaseI before the reverse transcription reaction to eliminate possible contamination of genomic DNA. 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 radiosensitivity was evaluated with the clonogenic assay as previously described (2). A single-cell suspension of test cells was plated and 137Cs irradiated at a nominal dose rate of 0.5 Gy/min 18 to 20 hours later. To assess for possible dose rate effects, the results were compared with those obtained with 250-kVp X-rays at 1.7 Gy/min. The ratio of doses required to produce the same surviving fraction did not differ at the two dose rates. The cells were incubated for 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 hours after plating and at the time of irradiation (2). The radiosensitivity of the parental and transfected cells was periodically evaluated and unchanged for >20 passages. The radiosensitivity of cells derived from FSC1-3 and T53 tumors was also evaluated by the clonogenic assay. Source tumors in NCr-nu/nu mice were excised, rendered into a single-cell suspension, plated, and irradiated 20 hours later.

**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 T53 source tumor was used to initiate all tumors for each experiment. On reaching a diameter of 135 to 185 mm3, the tumors were irradiated with 137Cs in a specially designed jig as previously described (2). For calculation of tumor growth, tumor volume versus time was normalized to initial volume of each tumor at the time of treatment (Vo), and the time to achieve 3Vo was calculated from a best fit exponential for five to six control tumors and seven to eight irradiated tumors over a volume range that flanked 3Vo (i.e., ∼250-550 mm3). Tumor growth delay was calculated as the days for irradiated tumors to achieve 3Vo minus the time for control tumors to achieve 3Vo. The unpaired *t* test was used to analyze for the significance of differences in growth delay between the isogenic tumor pair. The data shown are for one experiment done for each radiation dose schedule. An initial pilot study was done at 15 Gy in T53 tumors, as well as repeat experiments at 15 Gy in FSC1-3 tumors. The results did not differ among the repeat experiments.

**Results**

Following bulk transfection of the parental DNA-PKcs−/− tumor line FSC1-3, 75 clones were isolated and screened for assessment of their intrinsic radiosensitivity. Four clones exhibited a 1.1- to 2.5-fold increased resistance compared with the parental DNA-PKcs−/− line. All radioresistant clones contained human DNA-PKcs. Transfected clone T53 exhibited moderately increased radioresistance and was used in the present study. The *in vitro* radiosensitivity of the parental FSC1-3 cell line and clone T53 is shown in Fig. 1A. T53

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**Figure 1.** A, clonogenic survival of DNA-PKcs−/− FSC1-3 cells, and the same cells transfected with DNA-PKcs+/* (T53) following 137Cs irradiation in vitro. Confidence intervals are 1 SD. B, absence of human DNA-PKcs in FSC1-3 and the integration of DNA-PKcs into the genome of transfected FSC1-3 (T53) 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 also employing the human primers DP-28 and DP-29 was done. Samples in the (−) lanes were prepared without the addition of reverse transcriptase before PCR. The absence of bands in these lanes show the absence of contaminating genomic DNA in the mRNA extracts.

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**Figure 2.** A and B, growth of control (nonirradiated) and irradiated tumors. Irradiated tumors in (A) received 30 Gy as a single dose; those in (B) received 4 × 5 Gy fractions in 2 days with >8 hours between fractions. Points, mean volume of five to six control tumors and seven to eight irradiated tumors at the specified days; bars, SE. Horizontal bars (1 SE) and data points are the days to achieve the specified volumes in the control and regrowing tumors.

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was ~1.5-fold more resistant to radiation than FSC1-3 at a survival level of 0.01. The integration of human DNA-PKcs+/− and the production of DNA-PKcs mRNA in T53 cells were confirmed by genomic and RT-PCR as shown in Fig. 1B. The DNA-PKcs primer sequence employed in these studies recognizes human, but not murine, DNA-PKcs. The in vivo stability of the parental FSC1-3 and T53 transfectant lines was confirmed by evaluation of the cell radiosensitivity following in vivo growth. Tumors were initiated from stock cultures and, on reaching a diameter of 9 mm, 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 0.16 for cells from T53 tumors and 0.017 for cells from FSC1-3 tumors.

The growth of control and irradiated tumors following a single dose of 30 Gy is shown Fig. 2A. Consistent with their similar in vitro proliferative rates, tumors initiated from FSC1-3 and T53 tumors also grow at a similar rate. Following a single 30-Gy dose of radiation, DNA-PKcs−/− FSC1-3 tumors exhibited a substantially longer growth delay than their DNA-PKcs+/+ T53 counterpart. To determine if the difference in tumor response was dose or dose per fraction dependent, we next irradiated FSC1-3 and T53 tumors with 4 × 5 Gy fractions administered over 2 days (>8 hours between fractions). As observed with 30-Gy single fractions, tumors arising from FSC1-3 cells were substantially more sensitive to radiation than tumors arising from radiosensitive T53 tumor cells when exposed to fractionated dose irradiation (Fig. 2B). In addition to assessing tumor growth following 30-Gy single dose and 4 × 5 Gy fractionated dose irradiation, we also assessed tumor growth following 15 Gy under normal tumor blood flow and hypoxic conditions elicited by vascular clamping. Although variations in tumor oxygenation between these isogenic tumors were judged to be unlikely, this procedure ensured that potential differences in tumor response were not due to differences in the oxygen status between the models.

For all irradiation protocols, tumor response was evaluated as tumor growth delay. As seen in Table 1, all dose and fractionation schemes resulted in significantly longer growth delays in DNA-PKcs−/− than in DNA-PKcs+/+ tumors. Growth delay observed under conditions of normal tumor blood flow was not markedly greater than observed under conditions of 100% hypoxia, indicating that both isogenic tumors possess a radiobiological hypoxic fraction.

As shown in Fig. 1, the in vitro intrinsic radiosensitivity of FSC1-3 and T53 tumor cells differed by a factor of ~1.5. The 1.5-fold decrease in the intrinsic radiosensitivity of FSC1-3 cells resulted in a similar decrease in tumor growth delay as seen in Fig. 3. The effect and its magnitude were comparable for radiation regimens of 4 × 5 Gy fractions and single-dose fractions of 15 and 30 Gy, and 15 Gy under uniform hypoxic conditions.

### Discussion

Insertion of the DNA-PKcs double-strand break repair gene into a tumor cell line lacking a functional repair gene decreased the cell radiosensitivity and caused a proportionate decrease in tumor response to radiation. The results provide direct evidence that tumor response to radiation is substantially mediated by the radiosensitivity of the constituent tumor cells. If vascular endothelial or other stromal damage was the determinant of response, growth delay would be identical in isogenic tumors exposed to the same dose of radiation in the same strain of mice. If host stroma and clonogen radiosensitivity both contributed to growth delay, the ratio of growth delays in the isogenic tumors would be <1.5. The ratio of growth delays was ~1.5 for all dosing schemes.

The results of the present study differ from recent reports suggesting that for single fraction doses of 10 and 15 Gy, damage to host stroma, and in particular vascular endothelial apoptosis, dictates tumor response (5). In this previous study, tumors were transplanted into apoptosis-proficient and apoptosis-resistant asmasmic mice. In apoptosis-proficient mice, tumor irradiation resulted in a markedly greater endothelial apoptotic response and longer growth delay than was observed in apoptosis-deficient mice. However, as unirradiated control tumors grew at a substantially slower rate in the apoptosis-proficient mice, a substantially longer radiation-induced tumor growth delay for tumors in apoptosis-proficient mice would be expected. Nevertheless, it is clear that severe damage to tumor vasculature, whether induced chemically or by radiation, may delay tumor growth. SCID mice and various

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### Table 1. Tumor growth delay in DNA-PKcs+/+ and DNA-PKcs−/− isogenic tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor growth delay (d)</th>
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<tbody>
<tr>
<td></td>
<td>T53</td>
</tr>
<tr>
<td>4 × 5 Gy</td>
<td>7.51 (6.51-8.67)</td>
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<tr>
<td>15 Gy</td>
<td>3.98 (3.77-4.20)</td>
</tr>
<tr>
<td>15 Gy (H)</td>
<td>2.70 (2.25-3.24)</td>
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<tr>
<td>30 Gy</td>
<td>12.8 (11.0-14.9)</td>
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**NOTE:** Values in parentheses are 95% confidence intervals.
*P < 0.001, FSC1-3 versus T53.
†P = 0.02.
‡15 Gy (H), 15 Gy administered under conditions of acute tumor hypoxia.
§P < 0.01.
tissues and cell types derived from SCID mice, including vascular endothelial cells, exhibit a 2.5- to 3-fold hypersensitivity to radiation (4, 6, 7). Previous studies have shown that tumor growth delay is increased in SCID versus wild-type mice treated by large subcurative as well as smaller 3-Gy fractionated doses of radiation (4, 7). It is noteworthy, however, that in contrast to tumor growth delay, the single fraction dose of radiation required for permanent local control of 50% of treated tumors is unchanged for tumors treated in SCID compared with wild-type mice (7). These studies in hypersensitive SCID mice show that severe stromal damage delays the onset of tumor regrowth but does not result in substantial tumor cell death. Following the onset of regrowth, the doubling time of recurrent tumors is increased versus unirradiated tumors in a dose-dependent manner, but is similar for the same tumors in sensitive SCID or nude mice (7).

To summarize, the intrinsic radiosensitivity of tumor cells is a major determinant of tumor response to radiation over the dose range of 15 to 30 Gy administered as a single fraction to 20 Gy administered in $4 \times 5$ Gy fractions.

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