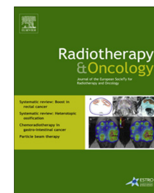
Radiotherapy and Oncology 113 (2014) 146–149

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com



Experimental radiobiology

Determinates of tumor response to radiation: Tumor cells, tumor stroma and permanent local control

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ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form 11 September 2014
Accepted 14 September 2014
Available online 2 October 2014

Keywords:
Radiation
Tumor control dose
Intrinsic radiosensitivity
Tumor vasculature
Predictive assays

ABSTRACT

Background and purpose: The causes of tumor response variation to radiation remain obscure, thus hampering the development of predictive assays and strategies to decrease resistance. The present study evaluates the impact of host tumor stromal elements and the *in vivo* environment on tumor cell kill, and relationship between tumor cell radiosensitivity and the tumor control dose.

Material and methods: Five endpoints were evaluated and compared in a radiosensitive DNA double-strand break repair-defective (DNA-PKcs$^{-/-}$) tumor line, and its DNA-PKcs repair competent transfected counterpart. *In vitro* colony formation assays were performed on *in vitro* cultured cells, on cells obtained directly from tumors, and on cells irradiated *in situ*. Permanent local control was assessed by the TCD_{50} assay. Vascular effects were evaluated by functional vascular density assays.

Results: The fraction of repair competent and repair deficient tumor cells surviving radiation did not substantially differ whether irradiated *in vitro*, *i.e.*, in the absence of host stromal elements and factors, from the fraction of cells killed following *in vivo* irradiation. Additionally, the altered tumor cell sensitivity resulted in a proportional change in the dose required to achieve permanent local control. The estimated number of tumor cells per tumor, their cloning efficiency and radiosensitivity, all assessed by *in vitro* assays, were used to predict successfully, the measured tumor control doses.

Conclusion: The number of clonogens per tumor and their radiosensitivity govern the permanent local control dose.

Published by Elsevier Ireland Ltd. Radiotherapy and Oncology 113 (2014) 146–149

Although employed as a principal method for the treatment of cancer for over 60 years, the biological causes of local treatment failure following radiation therapy remain obscure. The absence of such knowledge hampers the development of assays for identifying likely resistant *vs.* sensitive tumors as well as strategies to target and reduce resistance. Especially puzzling is the range of responses obtained in tumors matched for histology, and grade [1,2]. Local failure not only directly causes or contributes to life shortening, but importantly, increases the probability of distant metastases [3–6].

Relatively recent studies have reported that the dose to achieve permanent local tumor control is determined by radiation damage of the tumor stroma, and more specifically, the tumor vasculature [7,8]. This contrasts with studies indicating that the radiation sensitivity of the tumor cells is the primary determinant of response [9–12] .

Garcia-Barros et al. [7,8] reported that permanent local control was achieved in tumors exposed to 15 Gy, when transplanted and treated in acid sphingomyelinase (asmase$^{+/+}$) proficient mice, but was without effect on the growth of the same tumors in their asmase$^{-/-}$ littermates. The difference in tumor response was attributed to a more pronounced vascular endothelial apoptotic response for tumors in asmase$^{+/+}$ mice, than was observed in tumors grown and treated in their Niemann–Picks analog asmase$^{-/-}$ littermates.

In apparent conflict with these results, Budach et al. measured the dose of radiation required to achieve permanent local control of 50% of treated spontaneous FSaII tumors (TCD_{50}) isotransplanted into C3H mice, as well as into Nude and SCID mice [9]. SCID mice are hypersensitive to radiation due to the absence of a competent DNA double-strand break repair gene, DNA-PKcs [13–15]. These investigators found that the TCD_{50} values did not differ for tumors in C3H, Nude and SCID mice. The role of tumor cell sensitivity *vs.* vascular endothelial sensitivity was further augmented by studies showing that as pertained to other cells and tissues derived from SCID mice, SCID vascular endothelium was hypersensitive to radiation compared to the endothelium and vasculature of DNA-PKcs proficient mice [10,16].

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http://dx.doi.org/10.1016/j.radonc.2014.09.006
0167-8140/Published by Elsevier Ireland Ltd.

The present study determines if and the extent to which the radiation killing of tumor cells *in vitro* is altered by the tumor host stroma and *in vivo* environment. The study utilizes an isogenic tumor pair, *i.e.,* a sensitive parental DNA-PKcs defective tumor line from SCID mice, and the same cell clone transfected with a competent DNA-PKcs$^{+/+}$ repair gene. The isogenic tumors were grown and treated in wild type, asmase$^{+/+}$ Nude mice. The radiosensitivity of the two tumor lines following irradiation *in vitro*, *i.e.,* in the absence of stromal elements, was compared to the sensitivity of the tumor cells when irradiated *in vivo*. Concurrently, the clonogenic surviving fraction and the functional vascular density of the irradiated tumors was assessed at various times following *in vivo* irradiation. To further assess the role of the clonogens per tumor and their intrinsic radiosensitivity as determinants of the tumor control dose, we calculated and compared the predicted tumor control doses based on the *in vitro* estimated number of clonogens per tumor and their radiosensitivity, to the measured 50% control doses.

Materials and methods

Mice, isogenic tumor cells and tumors

The DNA double-strand break repair deficient and proficient tumor lines used in this study, have been previously described [10,11] (Supplementary methods). For selection purposes the DNA-PKcs expression vector was co-transfected with the pSV2 neo plasmid. Source tumors were initiated from *in vitro* cultured cells. Experimental tumors were initiated by subcutaneous chunk transfer of source tumors to the hind leg of 8–9 week old male NCr/Sed *nu/nu* mice provided by the Cox-7 defined flora animal facility in MGH. The tumor volume doubling-times of both isogenic FSC and T53 tumors were two days over the caliper measured size range of 5–8 mm average diameter. All animal care and procedures were carried out and performed following the guidelines of *Public Health Service Policy on Humane Care of Laboratory Animals* and approved by the Institution Animal Care and Use Committee at the MGH.

Irradiation

Radiation was administered with 320 kVp X-rays (half value layer = 1.5 mm copper), at 1.66 Gray (Gy) per minute for cells and 3.76 Gy per minute for tumors. For irradiation of tumors under conditions of uniform hypoxia, a spring loaded brass clamp was applied to the root of the thigh for 3 min prior to and during radiation [17]. All cells and tumors were processed identically, including control tumors which were clamped and sham irradiated [18].

Colony formation assay

Regardless of the source of the cells, *i.e.,* from *in vitro* cell cultures or from tumors, single cell suspensions were prepared and irradiated 18–24 h later. Lethally irradiated feeder cells (20 Gy) were used to maintain a constant number of cells per flask [19]. All surviving fraction data were multiplicity corrected. Tumors were excised, weighted, cut into approximately 1 mm pieces and single cell suspensions were prepared enzymatically (Supplementary methods). Five to 6 mm average diameter FSC and T53 tumors (approximately 25 mg tumor tissue) yielded approximately 5×10^6 trypan excluding cells. Cells obtained from FSC initiated tumors failed to form colonies in G418 containing selection medium, whereas the *in vivo* to *in vitro* cloning efficiency of cells from T53 initiated tumors, did not significantly differ ±G418.

Tumor control dose (TCD_{50}) assay

Following a pilot assay, 56 mice bearing 5–6 mm diameter FSC tumors and 56 bearing T53 tumors were entered into the TCD_{50} assay. Prior studies with the FSC and T53 tumor have shown that they contain a similar hypoxic fraction [11]. However, to reduce any tumor-to-tumor variability in tumor oxygenation, a spring-loaded clamp was placed around the upper thigh for 3 min prior to and during irradiation. Following irradiation, the mice were examined twice weekly for the presence of tumor and progressive tumor growth. Tumor bearing mice were sacrificed when tumors reached an average diameter of 7–8 mm. In the absence of palpable tumor and progressive growth, mice were followed for 120 days. Eight mice were irradiated at each of seven dose levels. The TCD_{50} values and their 95% confidence intervals were calculated as previously described [20].

Functional vascular density

Functional tumor vessels were labeled via tail vein injection of 10 μg of biotinylated lectin (Vector Laboratories, Burlingame, CA) as previously described [10,21,22]. Vessel density was assessed in control and irradiated mice at 24, 96 and 288 h following irradiation. Additional details are provided in Supplementary methods.

Statistics

Survival curve terminal slopes were calculated by least square fitting of log transformed surviving fraction data. Tests of significance were calculated with unpaired, two tailed *T* tests. Confidence intervals are 95% standard error of the mean, unless otherwise indicated.

Results

Stability and radiation sensitivity of cells obtained from tumors

The clonogenic survival of the irradiated DNA-PKcs$^{-/-}$ cell line, FSC, and its DNA-PKcs$^{+/+}$counterpart, T53, is shown in Fig. 1. Panel A shows the response of cells that were cultured and irradiated in the absence of stromal elements and factors. The clonogenic response of cells obtained directly from tumors is shown in panel B. For both sets of data, FSC cells exhibit minimal resistance to low doses of radiation, whereas DNA-PKcs$^{+/+}$ T53 cells are characterized by a relatively resistant shoulder region between the dose range of 0–4 Gy.

To further characterize and assess the stability of the cells when grown *in vivo*, the data in Fig. 1 were fit to the equation: $\ln \mathrm{Sf} = -\mathrm{D}/\mathrm{Do} + \ln(n)$, over the dose range of 2–8 Gy for FSC cells and 4–10 Gy for T53 cells, where Sf = surviving fraction, Do = the dose of radiation that reduces the surviving fraction on the exponential portion of the survival curve by a factor of "e", D = the administered dose, and "n" equals the constant (survival curve extrapolation number). The coefficients of determination of the survival curve slopes, R^2, ranged from 0.992 to 0.998 for the 4 curves shown in Fig. 1. For both cell lines, neither the survival curve slopes nor constants significantly differed, regardless of whether the cells were from *in vitro* cultures or directly from tumors (p value range = 0.15–0.80), whereas the slopes and constants significantly differed between the FSC and T53 lines ($p < 0.05$). The values of the parameter "n" and 95% confidence intervals of the mean are: 1.56 (1.49–1.64) and 6.36 (2.91–13.87) for FSC and T53 respectively, and "Do" = 1.00 (0.92–1.08) and 1.21 (1.01–1.38) Gy for FSC and T53 cells respectively.

Tumor cell survival following in vivo irradiation

To determine if the *in vivo* environment altered the fraction of tumor cells sterilized by radiation, tumors were treated with

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