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Effects of Brain Irradiation in Immune-Competent and Immune-Compromised Mouse Models

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Abstract

Patient-derived orthotopic xenografts (PDOXs) closely recapitulate primary human glioblastoma (GBM) tumors in terms of histology and genotype. Compared to other mouse strains, NOD-*scid* IL2Rgamma^{null} (NSG) mice show excellent tumor take rates, which makes them an ideal host for PDOXs. However, NSG mice harbor a mutation in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which renders them relatively radiosensitive. This has been a frequently voiced concern in studies involving ionizing radiation. In this study, we assessed brain toxicity in NSG mice compared to three other different mouse strains frequently used in radiation studies at radiation doses commonly used in experimental combination therapy studies. C3H/Sed/Kam, C57Bl/6, nude and NOD-*scid* IL2Rgamma^{null} mice received a single dose of 4 Gy to the right brain hemispheres using an image-guided small animal irradiator. Brains were stained using H&E, luxol fast blue, and antibodies against IBA1 and GFAP one, two, four or six months postirradiation. Additional animals of all four strains were exposed to five daily fractions of 2 Gy (5 × 2 Gy), and tissue sections were stained 72 h later against gH2AX, NeuN, GFAP and IBA1.

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None of the mouse strains displayed radiation-induced toxicity at any of the time points tested. Radiation doses relevant for testing combination therapies can be safely applied to the brains of NSG mice without the occurrence of radiation-induced normal tissue toxicity.

Editor's note

The online version of this article (DOI: <https://doi.org/10.1667/RR15373.1.S1>) contains supplementary information that is available to all authorized users.

INTRODUCTION

Mouse models of brain cancer are essential in the development of novel combination therapies against glioblastoma (GBM). While some spontaneous GBM mouse models (1) and syngeneic glioma cell lines exist (2) and allow for testing of novel drugs in the presence of an intact immune system, patient-derived orthotopic xenografts (PDOXs) have become the mainstay in current preclinical testing of experimental therapeutics because they maintain the histological and genetic features of their originating tumor (3).

The current standard of care for GBM patients relies on postoperative radiotherapy, which improves progression-free survival by 6 months over surgery alone (4, 5). Every novel therapy against GBM must be benchmarked against this standard of care and will most likely be combined with radiation. Patient-derived GBM samples engraft easily in severely immune-compromised NOD-*scid* IL2Rgamma^{null} (NSG) mice (6). However, this mouse strain carries a mutation in murine homolog of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (7) that renders it unable to repair DNA damage via the nonhomologous end-joining (NHEJ) pathway (8). Consequently, these mice are relatively radiosensitive with an LD_{50/30} between 6.0 (100% survival) and 7.5 Gy (LD_{100/21}) total-body irradiation (TBI) (9). Results from these TBI experiments have led to concerns over whether or not NSG mice can be used in xenograft experiments employing local irradiation of the tumor and if they need to be replaced by athymic nude mice instead.

In this study, we compared the brain tissue response to a single-fraction 4 Gy dose or five daily fractions of 2 Gy (5×2 Gy) between the immune-competent C3H and C57Bl/6 mice to those of the immune-compromised nude/nude and NSG strains.

MATERIALS AND METHODS

Animals

Male, 6-to-8-week-old, immune-competent C57BL/6 and C3Hf/Sed/Kam mice, originally obtained from MD Anderson Cancer Center, and immune-compromised NOD-*scid* IL2Rgamma^{null} (NSG) and nude mice, originally obtained from Jackson Laboratory (Bar Harbor, ME), were re-derived, bred and maintained in a pathogen-free environment in the American Association of Laboratory Animal Care-accredited Animal Facilities of Department of Radiation Oncology, University of California (Los Angeles, CA) in accordance to all local and national guidelines for the care of animals.

Half-Brain Irradiation

All treatments were performed using an image-guided small animal irradiator (X-RAD SmART, Precision X-ray Inc., North Branford, CT) with an integrated cone beam computed tomography (CT; 60 kVp, 1 mA) and a bioluminescence-imaging unit. The X-ray beam was operated at 225 KV and calibrated with a micro-ionization chamber using NIST-traceable dosimetry. Cone beam CT images were acquired with a 2-mm aluminum filter. For radiation delivery the beam was hardened using a 0.3-mm copper filter.

During the entire procedure the interior of the irradiator cabinet was maintained at 35°C to prevent hypothermia of the animals. Anesthesia of the animals was initiated in an induction chamber. Once deeply anesthetized, the animals were then immobilized using a custom 3D-printed mouse holder (MakerBot Replicator+, PLA filament) with ear pins and teeth bar, which slides onto the irradiator couch. Cone beam CT images were acquired for each individual animal. Individual treatment plans were calculated for each animal using the SmART-Plan treatment planning software (Precision X-ray) with the right brain hemisphere of the animals as target volume. Irradiation was performed using a square 1×1 cm collimator and opposing anterior and posterior fields, angulated at the brain's midline to obtain a planar radiation field border and to spare the left hemisphere from radiation, which served as internal nonirradiated control tissue. Animals were treated in groups of 3 mice per strain and time point, and received either a single-fraction 4 Gy dose or five fractions of 2 Gy (5×2 Gy). Animals were euthanized and the brains were harvested at 1, 2, 4 or 6 months after single-fraction 4 Gy irradiation or 72 h after five fractions of 2 Gy (5×Gy).

Hematoxylin and Eosin Staining

Sections of 4 μ M thickness were cut and deparaffinization was performed by immersing the slides in three changes of xylenes for 2 min each. Slides were next rehydrated by immersion in 100%, 95%, then 70% ethanol for 1 min each for 3 times. The slides were washed under running tap water and stained with hematoxylin for 8 min. After rinsing the slides under running tap water, the slides were dipped in acid alcohol 3 times, rinsed again, dipped in ammonia water 3 times, rinsed and dipped 30 times in 70% ethanol. They were then incubated with eosin for 3 min, and dehydrated by immersing in 95%, 100% ethanol and xylene for 1 min each for 3 times.

Immunohistochemistry

Immunohistochemistry was performed using standard protocols. Briefly, brains were explanted, fixed in formalin for 24 h and embedded in paraffin. Sections (4 μ m) were cut, baked for 1 h at 65°C, de-waxed in 2 successive xylene baths of 5 min and hydrated for 5 min using an alcohol gradient (ethanol 100%, 90%, 70%, 50%, 25%). The slides were incubated in 3% hydrogen peroxide/methanol solution for 10 min. Antigen retrieval was performed using citrate buffer (10 mM sodium citrate, 0.05% Tween™ 20, pH 6) with heating to 95°C in a steamer for 25 min. After cooling down to room temperature, the slides were blocked and incubated overnight at 4°C with a primary antibody against GFAP (1:50; Cell Signaling Technology®, Danvers, MA), IBA1 (1:8,000; Abcam®, Cambridge, MA), NeuN (1:3,500; Abcam) or γ -H2AX (1:1,000; Cell Signaling Technology). The slides were then incubated with ready-to-use IHC detection reagent (100 μ l; Cell Signaling Technology)

at room temperature for 1 h, rinsed, and incubated with DAB (Cell Signaling Technology) for 3–5 min. Tissues were counterstained or not with Harris modified Hematoxylin (Fisher Scientific™, Waltham, MA) for 30 s, dehydrated via an alcohol gradient (ethanol 25%, 50%, 70%, 90%, 100%) and soaked twice in xylene. A drop of Permount™ aqueous mounting media (Fisher Scientific) was added on the top of the section and covered.

Quantification of Gamma-H2AX-Positive Nuclei

The number of residual DNA double-strand breaks (DSBs) was evaluated using γ -H2AX foci as a surrogate marker. The total number of nuclei within each tissue cross section was recorded, the number of γ -H2AX foci per individual nucleus was counted manually and results were expressed as average number of foci per nucleus.

Myelin Staining

Formalin-fixed, paraffin-embedded brain tissue sections were deparaffinized and hydrated to 95% ethyl alcohol and left in luxol fast blue (Sigma-Aldrich® LLC, MO) solution at 56°C overnight. Slides were then rinsed in the lithium carbonate solution and 70% ethanol (10 s each) followed by counterstaining in Cresyl Violet (Sigma-Aldrich). Tissues were dehydrated in 100% alcohol, soaked in xylene (2 × 5 min) and mounted with Permount aqueous mounting media (Fisher Scientific).

All microscopic images were acquired using a Keyence BZ9000 digital microscope.

RESULTS

The single-fraction 4 Gy dose prescribed to the right brain hemisphere was well tolerated in all animals and in all strains. A representative treatment plan is shown in Fig. 1. No hair loss or hair discoloration was observed within the radiation fields. During the observation time no animal in any of the strains displayed signs of central nervous system (CNS) toxicity or obvious behavioral changes. No treatment-related death occurred in any of the strains and no animal reached an end point for premature euthanasia.

To compare the radiation toxicity between the different mouse strains more closely we first compared H&E-stained sections of the brains of all four strains to examine if radiation treatment caused structural changes to the brain tissue. For consistency, we imaged a cortical region, the hippocampus (dentate gyrus and CA1sp) and the corpus callosum, as shown in Fig. 2. We were unable to detect such changes in any of the four strains when comparing the irradiated right brain hemisphere with its nonirradiated left counterpart (Fig. 3A and B).

Next we assessed the number of activated microglia based on IBA1 staining. Analogous to H&E-stained sections, we were unable to detect differences between the nonirradiated and irradiated hemispheres of the animals (Fig. 4A and B). Likewise, staining for GFAP (Fig. 5A and B) and myelin (Fig. 6A and B) did not reveal differences between the nonirradiated and irradiated brain hemispheres nor between the mouse strains. Figures 3–6 are representative images. Images for the remaining 2 animals per time point are presented in Supplementary Fig. S1 (<https://doi.org/10.1667/RR15373.1.S1>).

To test the acute effects of fractionated radiation on the CNS, we irradiated animals of all four strains with five daily fractions of 2 Gy (5×2 Gy) to the right hemisphere. At 72 h after the final fraction, animals were euthanized, brains harvested and coronal sections cut and stained for IBA1, GFA and NeuN. Again, no differences in staining were found between the nonirradiated and irradiated hemispheres of the animals or between mouse strains (Fig. 7). Finally, we stained these sections with an antibody against the replacement histone γ -H2AX. The number of γ -H2AX foci per nucleus was counted and interpreted as a surrogate marker for residual DNA DSBs (10). Fractionated doses (5×2 Gy) led to a trend of increased numbers of residual γ -H2AX foci in the irradiated hemispheres of all animals. On average, NSG mice showed slightly higher γ -H2AX foci background levels than the three NHEJ-proficient mouse strains. However, none of these differences reached statistical significance using two-way analysis of variance (ANOVA).

DISCUSSION

Patient-derived orthotopic xenografts (PDOXs) in murine immune-deficient hosts are an required foundation of the *in vivo* testing workflow for the discovery of novel anticancer therapies (6, 11). Currently available immune-deficient mouse strains include nude mice, *Scid* or Rag-deficient mice and higher-order immune-deficient mice such as the NSG strain. While nude mice show excellent take rates for established, fast-growing cancer cell lines (12), the successful establishment of PDOXs is more reliably achieved in NSG mice (6). This is highly relevant because patient-derived samples are often small and not repeatedly available. NSG mice have become the standard mouse model for many PDOX studies, with outstanding tumor take rates that often allow for limiting dilution assays down to single-digit cell numbers. However, NSG mice, like all *Scid* mice, are homozygous for the *Prkdc*^{*scid*} mutation. The *Prkdc* genes encode for the catalytic subunit of DNA-PK, a kinase involved in DNA DSB repair through NHEJ. As a consequence, all mouse strains carrying the *Prkdc*^{*scid*} mutation are more radiosensitive than wild-type or nude mice (9). Therefore, the utility of NSG mice in radiation studies has frequently been called into question. The main concerns have been the potentially increased normal tissue toxicity and possible influence of the radiation-sensitive tumor microenvironment on the tumor treatment responses to radiation. The latter turned out not to be the case as radiation tumor control doses (TCD₅₀) in *Scid* (13) or ATMKO (14) mice did not differ from those in wild-type animals.

It is well established that moderate radiation doses cause neuroinflammation in the CNS and lead to cognitive impairment in rodents (15, 16). Furthermore, high-dose irradiation can cause demyelination, necrosis, seizures and ultimately premature death of the animals (17).

In preclinical studies the number of fractions and total radiation doses given to PDOXs when testing novel combination therapies depend on the research questions asked. Classical radiosensitizer studies are best served by fractionation schemes close to the clinical setting when the applied drugs enhance the efficacy of each fraction of radiation (18). On the other hand, combination treatments, in which a drug works independently of DNA damage or its repair but still synergistically with radiation, must ensure that radiation doses are not too high to allow detection of the drug effects (19). The latter studies are often performed using smaller, single radiation doses, which cause only moderate growth delay. Here we have

chosen a single dose of 4 Gy for our long-term studies and 5×2 Gy for our acute studies. These irradiation schemes are frequently used in mouse models of glioblastoma and while they by far do not cover the spectrum of radiation dosing in all preclinical studies, our results are a good indicator of the lack of differences between NSG mice and NHEJ-competent mice after low-dose irradiation. Our findings help to address whether radiation-induced normal tissue toxicity in the CNS of NSG mice, after exposure to these radiation doses, affects the animals' survival and is a confounding factor when studying the effect of combination therapies on survival in tumor-bearing animals.

Normal tissue toxicity is of particular concern in the event that novel anti-cancer treatments are highly effective and animals are cured or tumors are controlled over a long period of time. In TBI experiments, *Scid* mice show decreased LD₅₀ values, which reflect the increased radio-sensitivity of their hematopoietic cells. However, when irradiating mostly post-mitotic tissues such as those in the CNS, this could be of less concern. Here we present a comparison of microglia activation, astroglial GFAP expression and myelination in C3H/Sed/Kam, C57Bl/6, nude and NSG mice at 1, 2, 4 and 6 months after a single dose of 4 Gy. Our study demonstrates that a single dose of 4 Gy or five daily doses of 2 Gy are well tolerated and do not induce long-term or acute normal tissue toxicity, in brains of NSG mice, that would greatly exceed toxicity observed in *Prkdc^{scid}* wild-type mouse strains.

Our study differs from many older studies in that we used an image-guided small animal irradiator, which allowed us to irradiate the tumor region while sparing the contralateral hemisphere but also the fast overturning oral mucosa and the esophagus. In the latter two tissues, deficiencies in NHEJ could have more severe effects than in the CNS and could lead to reduced water and food intake and potentially premature unrelated to the tumor or the irradiation of the CNS. In studies in which NSG mice are irradiated without image-guidance using whole- or partial-brain irradiation, the defect in NHEJ in this strain could make it prohibitive for using this strain in radiation studies, if oral and esophageal mucosa cannot be excluded from the radiation fields.

We conclude that a single-fraction 4 Gy dose or five fractions of 2 Gy from an image-guided small animal irradiator can be safely administered to the brains of NSG mice, which makes this mouse strain an ideal model system for PDOXs in brain cancer research studies involving radiotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Axial, sagittal and coronal CT scan views with corresponding radiation field covering right hemisphere of the brain. The bottom right-side panel represents the percentage of dose to volume in both irradiated (blue curve) and nonirradiated (white curve) hemispheres. CT images for each individual mouse were acquired and transferred to SmART Plan software for review and segmentation.

FIG. 2. Schematic representation of the coronal section of a mouse brain with the imaged regions for the current study indicated. Cortex, corpus callosum, dentate gyrus and CA1sp were imaged at 20× and 40× magnifications.

FIG. 3.

Coronal section images of (panel A) C57BL/6 and C3H, and (panel B) NSG and nude mice showing four different brain regions: dentate gyrus, CA1sp, cortex and corpus callosum. Right brain hemispheres of 6-to-8-week-old male mice received a single dose of 4 Gy and the left hemispheres served as nonirradiated control. Animals were sacrificed at 6 months postirradiation, and brain tissues were collected and stained using H&E. Overview images were taken at 4× magnification (scale bar = 100 μ M). Images of the individual brain regions were taken at 40× magnification (scale bar = 40 μ M). All images are representative of 3 different animals per time point.

FIG. 4.

Coronal sections of brains of (panel A) C57BL/6 and C3H, and (panel B) NSG and nude mice showing four different brain regions stained for IBA1. The right hemisphere received a single dose of 4 Gy and the left hemisphere was not irradiated. All images are representative of 3 different animals per time point. Scale bars = 100 μ M and 40 μ M.

FIG. 5.

Coronal sections of brains of (panel A) C57BL/6 and C3H, and (panel B) NSG and nude mice showing four different brain regions stained for GFAP. The right hemisphere received a single dose of 4 Gy and the left hemisphere was not irradiated. All images are representative of 3 different animals per time point. Scale bars = 100 μ M and 40 μ M.

FIG. 6.

Micrographs of coronal sections (4× magnification, scale bar=100 μM ; 40× magnification, scale bar = 40 μM) from right (4 Gy dose) and left hemispheres of (panel A) NSG and nude, and (panel B) C57Bl/6 and C3H mice stained with luxol fast blue. All images are representative of 3 different animals per time point.

FIG. 7.

Quantification of residual DNA DSBs (γ -H2AX foci) in coronal sections of the right (5×2 Gy irradiated) and left hemispheres of C57BL/6, C3H, NSG and nude mice. Panel A: Average number of γ -H2AX foci per nucleus in the four different brain regions, the dentate gyrus, CA1sp, cortex and corpus callosum in nonirradiated and irradiated mice (upper and lower panels, respectively). Data points represent the mean foci number per nucleus $\pm$ SD in three mouse brains. The differences between the groups were analyzed using a two-way ANOVA. Panel B: Images of the corresponding brain regions in coronal sections of brains of NSG mice stained for γ -H2AX (60 $\times$ magnification). Scale bar = 20 μ M. All images are representative of 3 different animals per time point.