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Maximizing Tumor Immunity With Fractionated Radiation

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Abstract

PURPOSE—Technological advances have led to increased clinical use of higher sized fractions of radiation dose and higher total doses. How these modify the pathways involved in tumor cell death, normal tissue response, and signaling to the immune system has been inadequately explored. Here we ask how radiation dose and fraction size affect anti-tumor immunity, the suppression thereof and how this might relate to tumor control.

MATERIALS and METHODS—Mice bearing B16-OVA murine melanoma were treated with up to 15Gy radiation given in various sized fractions and tumor growth followed. The tumor-specific immune response in the spleen was assessed by IFNγ-Enzyme-Linked Immuno-Spot (ELISPOT) assay with ovalbumin (OVA) as the surrogate tumor antigen and the contribution of regulatory T cells (Tregs) determined by the proportion of $CD4^{+}CD25^{hi}Foxp3^{+}$ T cells.

RESULTS—After single doses, tumor control increased with the size of radiation dose, as did the number of tumor-reactive T cells. This was offset at the highest dose by an increase in Treg representation. Fractionated treatment with medium-size radiation doses of 7.5Gy/fraction gave the best tumor control and tumor immunity while maintaining low Treg numbers.

CONCLUSIONS—Radiation can be an immune adjuvant but the response varies with the size of dose per fraction. The ultimate challenge is to optimally integrate cancer immunotherapy into radiation therapy.

Keywords

radiation therapy; immune response; B16-OVA melanoma; fractionation; regulatory T cells

INTRODUCTION

Recent technological advances, such as image-guided, high-precision Intensity-Modulated Radiation Therapy (IMRT), Stereotactic Radiation Therapy (SBRT), and protons allow radiation dose to be delivered more precisely to the tumor and decreasing the amount to critical normal tissues. These improvements, along with a reevaluation of the dose fractionation response of certain common cancers, has led a move towards the delivery of dose fraction sizes that are higher than conventional, and in some cases tissue ablative. However, RT has systemic consequences [1] that are mediated through “danger” signaling [2] and systemic endothelial cells, monocytes, lymphocytes and other immune cells can be activated to infiltrate and affect the growth of both the primary tumor and metastatic deposits.

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It is unclear how the host-tumor relationship is affected by radiation, in particular when moving away from the 2Gy/fraction, 5-fractions-a-week conventional schedule. The potential exists for higher peak integrated “danger” signals as a result of more rapid cell kill, more vascular damage, and inflammatory cytokine induction at higher radiation doses. However, abscopal effects have been reported to be greater at moderate fractionated doses [3] and the systemic consequences at different radiation fractionation schemes clearly need further evaluation. These considerations are highly relevant to decisions as to how best to deliver RT as well as how to integrate it with other more systemic therapies, including chemo- (CT) and immunotherapy (IT). Here we examine one read-out of “danger” signaling, the development of tumor-specific immune responses, in an attempt to better understand the systemic consequences of different fraction sizes of locally delivered RT.

METHODS and MATERIALS

Mice, cell lines and tumor model

Female 6- to 8- week old C57BL/6 mice were bred and maintained in the defined-flora American Association of Laboratory Animal Care-accredited facility of the Department of Radiation Oncology at the University of California, Los Angeles. All experimental protocols adhered to local and national animal care guidelines.

The B16-OVA tumor model that uses ovalbumin (OVA) as a surrogate tumor antigen has been used extensively to accurately determine immune responses without affecting tumor growth if left untreated [4]. B16-OVA was a gift from Dr. Economou, University of California, Los Angeles and E.G7-OVA cells were purchased from the American Type Culture Collection (Manassas, VA). Both were cultured in RPMI-1640 media (Mediatech, Hernden, VA) with 10% fetal bovine serum (Sigma-Aldrich, St.Louis, MO), 10,000 IU penicillin, 10,000μg/ml streptomycin, 25μg/ml amphotericin (Mediatech), 0.05mM 2-mercaptoethanol (Sigma), and kept under 0.4mg/ml G418 selection (Research Products International Corp., Mt. Prospect, IL). B16-OVA cells were maintained at or below 75% confluency before injection of 0.8×10^6 cells in 100μl PBS s.c. on the right thigh. Tumors were used for experimentation in groups of 4 mice when they became palpable at 8-10days. Growth was assessed by measurements in two dimensions every other day using Vernier calipers. Experiments were repeated up to 4 times and student’s t-test was used for statistical analyses with $P<0.05$ as indicating a significantly difference from 0Gy.

Radiation Treatment

Radiation was administered 10 days after implantation when tumors reached approximately 4mm in diameter. Mice were anaesthetized with an i.p. injection of 2.4mg ketamine/0.12mg xylazine (Ketaject, Xyla-Ject, Pheonix Pharmaceutical, Inc., St. Joseph, MO) and positioned on a platform with cerrobend jig shielding the body, except for the right leg, which was irradiated at 300kV and 10mA with a dose rate of 1.84Gy/min using a Gulmay RS320 X-ray unit filtered with 1.5mmCu and 3mm Al (Gulmay Medical LtD., Camberley, Surrey, UK). The X-rays were administered vertically with a focus to surface distance of 24cm. Dosimetry was performed with Harshaw TLD-100H (LiF:Mg, Cu, P) and film (GAFCHROMIC EBT2, International Specialty Products, Wayne, NJ) and calibrated against a clinical cobalt-60 irradiator (Theratron-1000, MDS Nordion, Ontario). Fractionated exposures were given with 6h intervals to limit the impact of the more rapid tumor cell proliferation associated with mouse tumor grafts in the interpretation of results.

Spleen harvest

Spleens were harvested 7 days after irradiation and splenocytes were depleted of red blood cells by ammonium chloride treatment (ACK Lonza Walkersville Inc., Walkersville, MD) in serum-free RPMI-1640. Single cell suspensions were used for ELISPOT and Treg assay.

ELISPOT assay

OVA-specific T cell responses were assessed by an indirect Enzyme-Linked Immuno-Spot (ELISPOT) assay for interferon-γ (IFNγ) production. Splenocytes (2.5×10^6/ml) were stimulated with heavily irradiated (50Gy) EG.7-OVA at a 25:1 ratio in complete RPMI-1640 with 10U/ml recombinant human interleukin-2 (IL-2, Invitrogen, Carlsbad, CA) for 48h 37°C. Re-stimulated splenocytes were then harvested and plated in X-VIVO 10 (Lonza) at 2×10^5/well in anti-IFNγ-coated (BD Pharmingen, Franklin Lakes, NJ) MultiScreen-HA plates (Millipore Corp, Billerica, MA) for another 24h incubation at 37°C. Cells secreting IFNγ were detected using biotinylated anti-mouse IFNγ (BD Pharmingen), horseradish peroxidase avidin D (1:200 dilution, Vector Laboratories, Burlingame, CA), and 0.4mg/ml 3-amino-9-ethyla-carbazole substrate (AEC, Sigma) in 0.04% (v/v) formamide (Boehringer Mannheim, Indianapolis, IN), 0.05M sodium acetate buffer (pH 5.0), and 0.012% H_2O_2 (Fisher Scientific, Pittsburgh, PA). Spots were counted using the ImmunoSpot Image Analyzer (Cellular Technology Ltd, Cleveland, OH).

Treg Cell Enumeration

Two million splenocytes in normal goat serum were stained with PE-anti CD25 (clone PC61, BD Pharmingen) and PE-Cy5-anti CD4 (clone L3T4, BD Pharmingen) for 30mins on ice and then fixed in Fixation/Permeabilization buffer (eBioscience, San Diego, CA) for 1h on ice prior to staining with FITC-anti Foxp3 (clone FJK-16s, eBioscience) in normal rat serum for 30mins on ice. $CD4^+CD25^{hi}Foxp3^+$ cells were enumerated per 100,000 events using a flow cytometer (FACSCalibur; BD Biosciences, Mountain View, CA).

RESULTS

B16-OVA grows fast in vivo. By day 7 after treatment B16-OVA control tumors had grown from 4 to 8mm diameter but this was slowed by local single-dose radiation between 7.5 and 15Gy, while 5Gy had little effect (Figure 1A). At this time-point, anti-OVA T cell responses in the spleens of control mice were barely present (Figure 1B) while splenic Treg representation increased above the expected baseline of 5% of total white cells (Figure 1C) [5]. Radiation-induced tumor growth delay after 7.5 and 10Gy was associated with an increase in anti-OVA T cell responses and a decrease in the proportion of Tregs in the spleen. Surprisingly, a single dose of 15Gy increased both effector and regulatory T cells. The latter was not statistically significant but is in line with our previous experience where radiation-induced changes in Treg populations were studied in more depth [6]. In other experiments with even higher single doses (not shown), we found inferior anti-tumor immune responses and no improvement in tumor control. Clearly, in this model radiation can act as an immune adjuvant provided the dose is right.

Doses of 15Gy were fractionated into 5, 3, or 2 doses separated by 6 hours to allow for sub-lethal damage repair while minimizing repopulation. No attempt was made to alter fraction size to account for variation in the extent of repair with size of dose per fraction, because such an effect is likely to be small. In vitro clonogenic assays showed this cell line to have a relative high α/β ratio of 36Gy (not shown) and this is consistent with the modest differences between tumor responses of the different groups in figure 2A. Importantly, tumor diameter (Figure 2A) was inversely proportional to IFNγ-producing T cells (Figure 2B). In fact, tumor-specific T cell responses were at their highest when overall tumor control

was maximal and this was following 2 fractions of 7.5Gy. Of note, Tregs were at their lowest at this very same dose/fractionation although this was beyond statistical power (Figure 2C).

DISCUSSION

Tumor cell kill correlates strongly with radiation dose in the absence of an immune response, but if systemic immunity is generated in response to radiation-induced tumor cell kill, it greatly enhances the chances of controlling both local and distant disease [7-9]. In our study, the presence of B16-OVA tumor by itself generated little tumor immunity, while single doses of 7.5Gy and above, but not 5Gy, were immunostimulatory. This is consistent with the findings of Lee at al. [10] who suggested that higher than conventional 2Gy doses may be required. Protective cell-mediated anti-tumor responses are generally ascribed to T cells able to secrete IFNγ and the finding of a threshold dose for radiation to act as an adjuvant is consistent with a radiation-induced switch to a more pro-inflammatory response and with its ability to generate IFNγ and cytotoxic immune responses rather than a IL-4-based anti-inflammatory immune profile [11, 12].

The creation of an immunologically permissive environment by radiation is a complex process with a number of negative regulatory barriers to be overcome and may not be possible to be achieved in all tumor-host environments. Tregs are clearly one of these barriers. They are pivotal in the maintenance of immune tolerance to self [13]. They are also known to suppress tumor-specific immunity and serve as a target for therapeutic intervention [14]. Hence, it came as no surprise that a B16-OVA tumor growing in the leg led to increased Treg representation in the spleen. Median doses of 7.5 or 10Gy, but not 5Gy, given to the tumor were able to prevent this increase. These findings are in keeping with the concept that Treg cells and tumor-specific immunity are two opposite sides of the same coin, Treg representation being a negative image of the IFNγ-producing T cells, as has been suggested by others [15].

We also know from our own experience and from others that Tregs dose-dependently increase following whole-body and leg-only irradiation, in part because Tregs are less radiation-sensitive than other T cells [6, 16, 17]. However, when the rest of the body is heavily shielded, as was done here, local tumor irradiation still raises the fraction of Tregs in the spleen if the dose is high enough, namely 15Gy or greater, and this is presumably unrelated to radiation sensitivity. We hypothesize that a high level of tissue damage over a short time period triggers a tissue protective response while still enabling the development of immunity. To explore this relationship further, we fractionated the 15Gy dose, predicting that several moderate doses would be superior to high single doses at favoring immunity over tolerance and assuming that sub-lethal damage repair would not be a major factor. Indeed, 2x 7.5Gy and 3x 5Gy were generally superior to a single dose of 15Gy in generating immunity and Treg representation was generally again a negative image of the IFNγ-producing T cells. The fact that 3x 5Gy and even 5x 3Gy triggered responses is also important with respect to the single-dose experiments as it suggests to us that immune tolerance was not induced by a single dose of 5Gy (or less) and that the immune system simply did not “see” the damage caused by lower doses when given once. The immunological benefit to be derived from radiation dose fractionation and its contribution to tumor control may not always be obvious [10] and will depend upon many factors but it is clearly worth exploring in a clinical model.

In conclusion, radiation dose and fraction size matter in terms of the tumor-host interactions and this influence extends well beyond the tumor site. Radiation can clearly be an immunological adjuvant if the dose is right but successful integration of IT into RT

protocols will also require detailed consideration of the role of other factors such as regulatory cells and tumor antigenicity. Our experimental system was established using a defined antigen to investigate the concept. Extensive immunoediting in human tumors may make any specific immune-targeting effort in the clinic challenging, but we have shown that patients can make T cell tumor-specific immune responses following standard radiation therapy [18] and are studying whether dose per fraction can be a factor. However, this is only one variable and attempts to combine RT with broader immune intervention may be the way forward such as the phase III trial combining the anti-CTLA-4 antibody Ipilimumab (NCT00861614, Bristol-Myers Squibb) with radiotherapy that is currently under way in patients with advanced prostate cancer.

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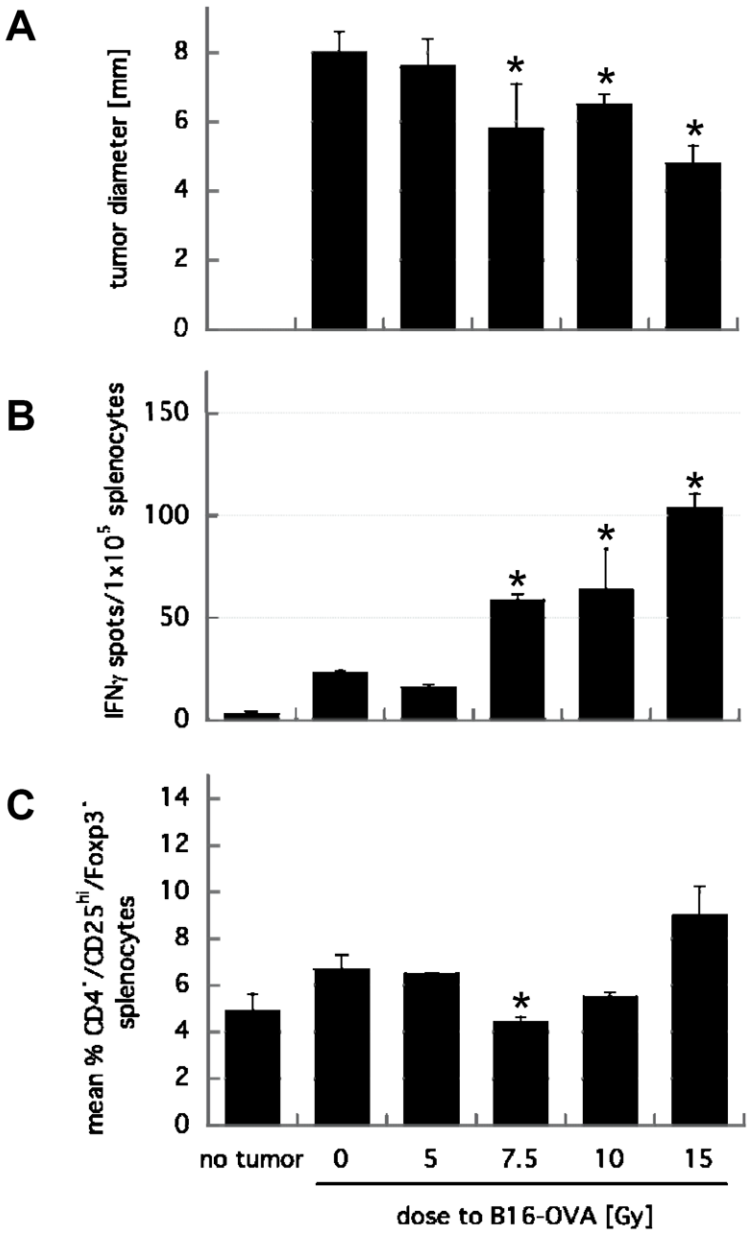

Figure 1.

Radiation dose-dependently increases tumor control, tumor-specific IFNγ^+ splenocytes and alters splenic Tregs. Mice were implanted with 0.8×10^6 B16-OVA cells s.c. in the leg and treated 10 days later with various doses of radiation. 7 days after treatment tumor size and splenic responses were measured. **A)** Tumor size as mean mm diameter (2 dimension) of n=4-16 ± s.e.m. **B)** Splenocytes were mixed ex vivo with EG7.OVA cells and the number of IFNγ-producing cells determined by ELISPOT. Data are mean number of spots per 10^5 splenocytes of n=3-16 ± s.e.m. **C)** Splenocytes were stained for CD4, CD25 and Foxp3 and enumerated by flow cytometry. Data are mean CD4+, CD25+ and Foxp3+ Tregs as fraction of CD4+ Splenocytes of n=1-11 ± s.e.m. * $p<0.05$ compared to 0Gy.

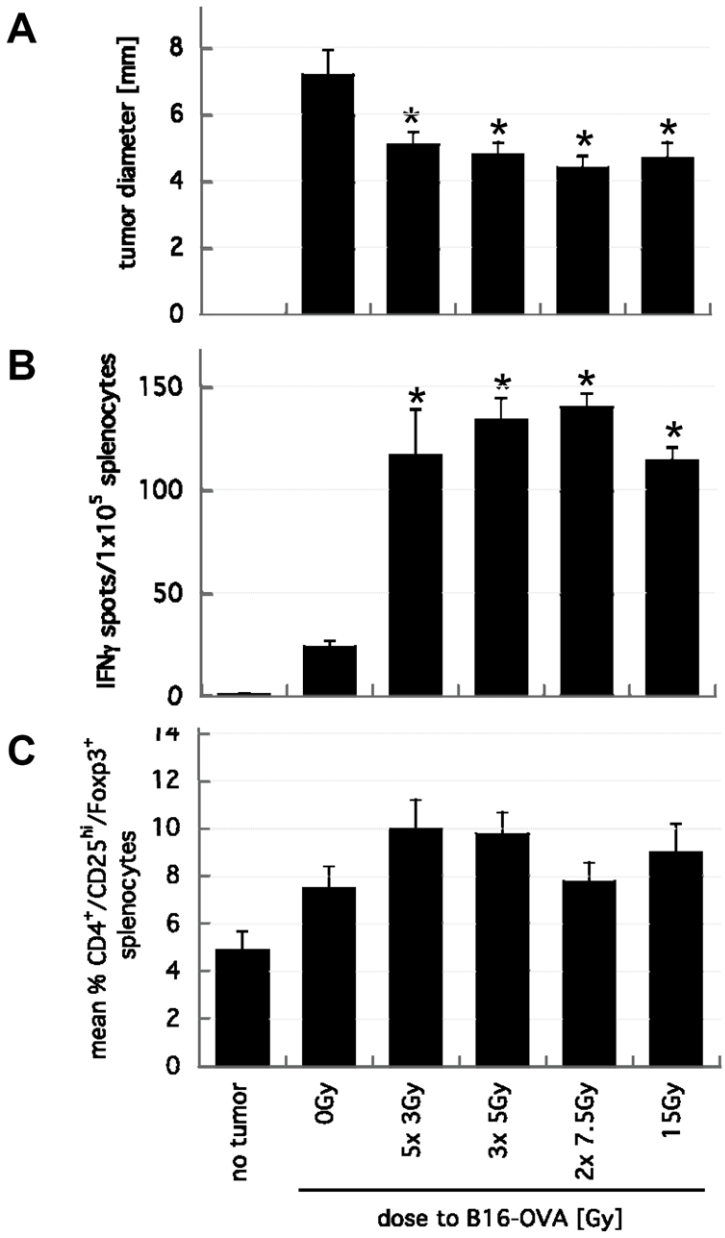

Figure 2.

Fractionated radiation affects tumor control, tumor-specific IFNγ^+ splenocytes and alters splenic Tregs. Mice bearing B16-OVA tumors of 4mm in size were given fractionated radiation in 6h intervals of a total dose of 15Gy and left for 7 days to recover. **A)** Tumor size as mean diameter of n=12 ± s.e.m. **B)** Mean number of IFNγ- ELISpots per 10^5 splenocytes of n=3-6 ± s.e.m. in response to EG7.OVA ex vivo re-stimulation. **C)** Mean percent of splenic $CD4^+CD25^+Foxp3^+$ Tregs as analyzed by FACS of n=8-12 ± s.e.m. * $p<0.05$ compared to 0Gy.