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Adrenergic Receptor Signaling Regulates the Response of Tumors to Ionizing Radiation

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Abstract

While ionizing radiation is a major form of cancer therapy, radioresistance remains a therapeutic obstacle. We have previously shown that the mandated housing temperature for laboratory mice (~22°C) induces mild, but chronic, cold stress resulting in increased circulating norepinephrine, which binds to, and triggers activation of, beta-adrenergic receptors (β -AR) on tumor and immune cells. This adrenergic signaling increases tumor cell intrinsic resistance to chemotherapy and suppression of the anti-tumor immune response. These findings led us to hypothesize that adrenergic stress signaling increases radioresistance in tumor cells in addition to suppressing T-cell-mediated anti-tumor immunity, thus suppressing the overall sensitivity of tumors to radiation. We used three strategies to test the effect of adrenergic signaling on responsiveness to radiation. For one strategy, mice implanted with CT26 murine colon adenocarcinoma were housed at either 22°C or at thermoneutrality (30°C), which reduces physiological adrenergic stress. For a second strategy, we used a β -AR antagonist (“beta blocker”) to block adrenergic signaling in mice housed at 22°C. In either case, tumors were then irradiated with a single 6 Gy dose and the response was compared to mice whose adrenergic stress signaling was not reduced. For the third strategy, we used an *in vitro* approach in which several different tumor cell lines were treated with a β -AR agonist and irradiated, and cell survival was then assessed by clonogenic assay. Overall, we found that adrenergic stress significantly impaired the anti-tumor efficacy of radiation by inducing tumor cell resistance to radiation-induced cell killing and by suppression of anti-tumor immunity. Treatment using beta blockers is a promising strategy for increasing the anti-tumor efficacy of radiotherapy.

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INTRODUCTION

Radioresistance is a major challenge in the field of radiation oncology in that some patients' tumors are inherently resistant, and others, after an initial response, develop resistance (1, 2). Elucidating the mechanisms of radioresistance and developing methods to overcome this problem is an active area of research.

Recent evidence has implicated stress-induced sympathetic nervous system responses in promoting tumor growth through the release of norepinephrine and epinephrine. These catecholamines signal through β -adrenergic receptors (β -ARs) on a variety of cell types to promote tumor cell proliferation, angiogenesis, epithelial-mesenchymal transition, metastasis and resistance to apoptosis (3–5). Our laboratory has shown that the mandated ambient housing temperature of laboratory mice ($\sim 22^{\circ}\text{C}$) induces increased sympathetic nerve activity because of a mild, but chronic, cold stress that increases circulating norepinephrine. This cold stress can be alleviated by housing mice at a thermoneutral temperature ($\sim 30^{\circ}\text{C}$). Our previously published work has shown that elevated norepinephrine in mice housed at 22°C promotes resistance to cytotoxic chemotherapies by altering the ratio of pro- and anti-apoptotic molecules (6). However, the effects of adrenergic signaling on responses to radiation have not been investigated. Additionally, it has been reported that adrenergic signaling in immune cells suppresses anti-tumor immune responses (7, 8), while blockade of β -AR signaling with beta blockers enhances development of anti-tumor immune responses and improves the anti-tumor efficacy of immunotherapy (7, 9, 10). A recently developed anti-tumor immune response has been recognized as a critical contributor to overall outcomes of radiation treatment (11–15). These findings led us to hypothesize that β -AR signaling promotes intrinsic radioresistance in tumor cells while also suppressing CD8^{+} T-cell-mediated anti-tumor immunity, thus suppressing the overall sensitivity of tumors to radiation. We investigated the role of adrenergic signaling in radioresistance. Using both physiological (housing temperature) and pharmacological (adrenergic receptor antagonists, “beta blockers”) approaches, here we show the novel findings that adrenergic receptor signaling significantly impairs the efficacy of radiation, both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cells

The cell lines CT26.CL25, 4T1, B16.F10 and MIA-PaCa-2 were obtained from the American Type Culture Collection (ATCC®, Rockville, MD) and Pan02 from the National Cancer Institutes (NCI) Tissue Repository. Cells were cultured at 37°C in a humidified incubator with 5% CO_2 as described elsewhere (6, 7, 16). CT26.CL25 murine colon carcinoma (ATCC) was cultured in RPMI 1640 medium (Gibco®, Grand Island, NY) adjusted to contain 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino-acids, 2.5 units/ml penicillin, 2.5 mg/ml streptomycin, 0.4 mg/ml G418 and 10% fetal bovine serum (FBS); 4T1 murine mammary carcinoma (ATCC) was cultured in RPMI 1640 medium adjusted to contain 2 mM L-glutamine, 2.5 units/ml penicillin, 2.5 mg/ml streptomycin and 10% FBS. B16.F10 murine melanoma (ATCC) was cultured in DMEM (Corning® Inc., Corning, NY) adjusted to contain 2.5 units/ml penicillin, 2.5 mg/ml streptomycin and 10% FBS; MIA-

PaCa human pancreatic carcinoma (ATCC) was cultured in DMEM (Corning Inc.) adjusted to contain 2.5 units/ml penicillin, 2.5 mg/ml streptomycin, 2.5% horse serum and 10% FBS; Pan02 murine pancreatic ductal adenocarcinoma was cultured in RPMI 1640 adjusted to contain 2 mM L-glutamine, 2.5 units/ml penicillin, 2.5 mg/ml streptomycin and 10% FBS.

Mouse Tumor Models

Female BALB/cAnNcr (BALB/c) mice (6–8 weeks old) were purchased from Charles River Laboratories (Ashland, OH). For all *in vivo* experiments, 5×10^5 CT26.CL25 cells were injected subcutaneously in the left-hind leg. For ambient housing temperature experiments, mice were housed five/cage in Precision Refrigerated Plant-Growth Incubators (Thermo Fisher Scientific™ Inc., Rockford, IL) maintained at either 22°C or 30°C as described elsewhere (6, 7, 16). Mice were acclimated to this housing temperature for at least two weeks prior to tumor implantation. On day 7 after tumor cell implantation, tumor volumes were approximately 100 mm³, and mice received 6 Gy local irradiation using an orthovoltage X-ray machine (Philips RT250; Philips Healthcare, Bothell, WA) at 200 kV using a 1 × 2-cm cone. A lead shield was used to protect normal tissue while irradiating the tumor exposed on the hind leg. For beta-blocker experiments, all mice were housed at 22°C. Mice were randomized to receive daily intraperitoneal injections of 10 mg/kg propranolol (Sigma-Aldrich® LLC, St. Louis, MO) or PBS when tumors became palpable on day 4 after implantation. After 3 days of treatment injections, mice received 6 Gy of local irradiation, as above. Perpendicular-linear dimensions of tumors “S” and “L” were obtained by caliper measurement throughout the experiments. Tumor volume was calculated using the formula, $\text{Tumor volume (mm}^3\text{)} = \frac{S * S * L}{2}$, where “L” is the large dimension and “S” is the small.

Clonogenic Assays

Cells were cultured in media for 48 h. Isoproterenol (Sigma-Aldrich) was added at a dose of 1 μM at 0 and 24 h. Cells were harvested by trypsinization, washed in PBS and counted using a cell counter via trypan blue exclusion. Single cell suspensions were serially diluted in PBS, and 100 cells were added in triplicate to six-well plates containing 4 ml of standard media. Plating efficiency wells were also seeded with 100, 50 and 25 cells upon further serial dilution. Adherent cells received 2, 4, 6 and 8 Gy of radiation (Faxitron 43855D RX-650 X-Ray Generator; Tucson, AZ). Plates were incubated for several days until surviving cells in control wells had proliferated to form colonies of approximately 50 cells. Cells were then fixed with methanol and stained with crystal violet for counting via light microscopy. All plates were coded prior to staining to de-identify samples before to counting. Data from each radiation dose were normalized to plating efficiency wells, which received no radiation (0 Gy) and graphed on a log10 scale. The surviving fraction (SF) value for each group was calculated as the average of the three triplicate wells. In cases where this average was <0.01, the value was recorded as 0. Groups with a surviving fraction less than 0.01 are shown as 0 in Fig. 2B, C and E.

Flow Cytometry

Single cell suspensions were prepared. The following antibodies were used: anti-CD16/CD32 (Fc blocker), anti-CD45 FITC (30-F11), anti-CD3 APC-Cy7 (145-2C11), anti-CD8

BUV395 (53-6.7) and anti-CD4 BV786 (GK1.5) (all from BD Biosciences, Franklin Lakes, NJ); anti-Granzyme B Ax647 (BioLegend® Inc., San Diego, CA); anti-IFN- γ APC (eBioscience™ Inc., San Diego, CA); and live-dead violet was used to identify live cells (Invitrogen™, Carlsbad, CA). Flow data were collected on the Fortessa™ B flow cytometer (BD Biosciences) and analyzed using FlowJo version 10 software (Tree Star Inc., Ashland, OR).

Statistical Analysis

Data between two groups were compared using Student's *t* test and tumor growth statistics were calculated using two-way analysis of variance (ANOVA). All data are graphed as mean $\pm$ SEM.

RESULTS

To investigate the effect of adrenergic stress and adrenergic receptor signaling on tumor responses to radiation *in vivo*, we used a physiological approach to induce adrenergic stress in which we compared radiation responses of CT26 tumors in mice housed at 22°C [in which mice experience chronic mild cold stress and elevated norepinephrine levels (17)] with those in mice housed at 30°C (which is the thermoneutral temperature, alleviating cold stress). As shown in Fig. 1A, thermoneutral housing significantly improved tumor response to radiation, resulting in slower tumor growth compared to housing at 22°C. Next, we sought to determine whether this benefit could be duplicated by blocking adrenergic signaling with the pan β -AR antagonist propranolol in mice housed at 22°C. We found that propranolol treatment also significantly improved the efficacy of radiation in mice undergoing chronic adrenergic stress (Fig. 1B). Furthermore, when CD4⁺ and CD8⁺ T cells from the tumors of these mice were analyzed by flow cytometry, we observed an increase in the percentage of cells expressing IFN- γ and granzyme B (markers of effector function) in tumors from mice that had received propranolol in combination with irradiation (Fig. 1C, D, F and G). An increase in IFN- γ expression is correlated with a more robust anti-tumor immune phenotype (18, 19), while an increase in granzyme B suggests that those T cells within the tumor microenvironment are more effective at inducing apoptosis in tumor cells (19). In addition, of the CD4⁺ and CD8⁺ T cells that were granzyme B⁺, those in the radiation and propranolol treatment group expressed significantly higher levels of granzyme B on a per cell basis than those from the radiation and PBS group (Fig. 1E and H).

Since previously published work has demonstrated that adrenergic signaling increases tumor cell resistance to cytotoxic chemotherapies *in vivo* (6), we sought to determine whether the improved efficacy of radiation seen *in vivo* here was solely the result of an improved anti-tumor immune response, or if adrenergic signaling in tumor cells was also contributing directly to increased tumor cell survival. To address this question, we treated several tumor cell lines *in vitro* with the pan β -AR agonist isoproterenol (ISO) and then exposed to increasing doses. The surviving fractions of CT26, B16, Pan02 and MIA-PaCa-2 were significantly increased at cell line-specific radiation doses by ISO treatment (Fig. 2A–E). However, in 4T1, a cell line that lacks functional β -ARs (20), ISO had no effect (Fig. 2F). To validate these results, experiments with Pan02 were conducted at two separate institutions,

Roswell Park Comprehensive Cancer Center (Buffalo, NY) and University of Rochester Medical Center (Rochester, NY); sensitivity to lower doses of radiation was enhanced by ISO treatment in both cases, although the results at higher doses differed (Fig. 2C and D).

DISCUSSION

The significant improvement in radiation efficacy achieved by the reduction (Fig. 1A) and/or pharmacological blockade (Fig. 1B) of adrenergic signaling *in vivo* likely involves multiple mechanisms. Our previously published data showed that adrenergic signaling increases the ratio of pro- and anti-apoptotic molecules, which leads to tumor cell resistance to chemotherapy (6). Since chemotherapies that result in DNA damage synergize with radiation through the common mechanism of DNA damage, we speculate that the same pro-survival alteration of apoptotic molecules by adrenergic signaling after chemotherapy treatments could be present in tumor cells after irradiation. Thus, this could be one survival mechanism that impairs the ability of radiation to kill several cell lines *in vitro* (Fig. 2A–E). Studies are currently underway to address this possibility.

In the future, it will be interesting to determine whether the radiation response of tumors in mice housed at thermoneutrality can be further improved by combination with propranolol (as shown in Fig. 1B in mice housed at 22°C). As shown in Fig. 1A, reducing adrenergic stress by housing at thermoneutrality resulted in almost complete control of tumor growth in the time frame of this experiment, but it is possible that in a regrowth/recurrence experiment, propranolol could extend progression-free survival.

Since immune cells and tumor cells both express β -ARs, systemic norepinephrine can bind to these receptors and affect functions of both cell types. Thus, a second mechanism triggering the enhanced tumor control seen after reduced or blocked adrenergic stress signaling could involve the anti-tumor immune response. For example, the change in immune contexture that we observed, in addition to reversal of suppressive mechanisms, could encompass an increase in the release of tumor antigen as a result of increased tumor cell radiosensitivity. This increase in tumor antigens could increase the *in situ* vaccine effect and enhance the anti-tumor immune response. Here we also found that blocking adrenergic stress signaling with the beta blocker propranolol resulted in an increase in IFN- γ and granzyme B expression by T cells reflecting an increased effector phenotype. IFN- γ is a cytokine that is important in generating adaptive immune responses, and granzyme B is a molecule secreted by CD8⁺ T cells to induce apoptosis in target cells (18, 19). Thus, an increase in these two markers within CD8⁺ and CD4⁺ T-cell populations suggests a more robust and effective anti-tumor immune response. Here, we attribute the improvement in the immune contexture of these tumors to the combination of propranolol with radiation because we have previously observed and reported that in the context of short-term therapeutic experiments in which mice are treated with propranolol only after tumors reached approximately 100 mm³, propranolol alone does not increase the numbers of T cells with an effector phenotype in tumors (7).

Future efforts will be directed toward exploring and identifying these mechanisms. Importantly, however, since retrospective epidemiological analyses have shown that patients

receiving beta blockers for hypertension or other indications while undergoing chemotherapy (21) and radiotherapy (22) have better outcomes, our studies suggest that radiation treatment would be improved by a prospective combination with beta blockers. This strategy can be tested in the clinic by repurposing widely prescribed and safe beta blockers for cancer patients undergoing radiation therapy.

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FIG. 1.

Alleviating adrenergic stress improves the efficacy of radiation. Panel A: Growth of CT26 in mice housed at 22°C or 30°C, with/without 6 Gy irradiation. Panel B: CT26 tumor-bearing mice housed at 22°C were treated with PBS with/without radiation or beta blocker with or without radiation. Panels C and D: Percentage of CD8⁺ T cells expressing IFN-γ and granzyme B, respectively. Panel E: MFI of CD8⁺ T cells expressing granzyme B. Panels F and G: Percentage of CD4⁺ T cells expressing IFN-γ and granzyme B, respectively. Panel H: MFI of CD4⁺ T cells expressing granzyme B. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

FIG. 2.

Beta-adrenergic receptor (β -AR) signaling increases radioresistance *in vitro* (clonogenic assay). Cells were treated with the β -AR agonist isoproterenol (1 μ M) or PBS and exposed to increasing doses (0–8 Gy). Surviving fraction was then calculated. Logarithmic scaling was used to graphically represent surviving fractions of colonies. Panel A: CT26.CL25. Panel B: B16.F10. Panel C: Pan02 (RPCCC). Panel D: Pan02 (Rochester). Panel E: MIA-

PaCa-2. Panel F: No effect was seen in 4T1, which lacks functional β -ARs. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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