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Original article

Transcriptional-mediated effects of radiation on the expression of immune susceptibility markers in melanoma

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ABSTRACT

Background and purpose: We recently reported a time-sensitive, cooperative, anti-tumor effect elicited by radiation (RT) and intra-tumoral-immunocytokine injection *in vivo*. We hypothesized that RT triggers transcriptional-mediated changes in tumor expression of immune susceptibility markers at delayed time points, which may explain these previously observed time-dependent effects.

Materials and methods: We examined the time course of changes in expression of immune susceptibility markers following *in vitro* or *in vivo* RT in B78 murine melanoma and A375 human melanoma using flow cytometry, immunoblotting, and qPCR.

Results: Flow cytometry and immunoblot revealed time-dependent increases in expression of death receptors and T cell co-stimulatory/co-inhibitory ligands following RT in murine and human melanoma. Using high-throughput qPCR, we observed comparable time courses of RT-induced transcriptional upregulation for multiple immune susceptibility markers. We confirmed analogous changes in B78 tumors irradiated *in vivo*. We observed upregulated expression of DNA damage response markers days prior to changes in immune markers, whereas phosphorylation of the STAT1 transcription factor occurred concurrently with changes following RT.

Conclusion: This study highlights time-dependent, transcription-mediated changes in tumor immune susceptibility marker expression following RT. These findings may help in the design of strategies to optimize sequencing of RT and immunotherapy in translational and clinical studies.

Radiation therapy (RT) kills tumor cells through the induction of DNA damage. However, it has long been recognized that tumor sensitivity to RT may be impacted by host immunity, and conversely, RT may affect tumor immune susceptibility [1,2]. Prior studies have suggested that the mechanisms whereby RT may modulate tumor immune susceptibility include immediate local release of inflammatory cytokines, induction of immunogenic cell death, and temporary local eradication of suppressor (as well as effector) immune lineages [3]. In addition, multiple studies of individual tumor markers have demonstrated that RT may induce phenotypic changes in the plasma membrane expression of specific markers of immune susceptibility on tumor cells that survive RT [4]. Pre-clinical and early-phase clinical data from studies combining RT with immunotherapy suggest that, collectively, these mechanisms may result in enhanced antigen cross-presentation [5] and increased diversity of the anti-tumor T cell response following RT [6].

Prior studies have consistently demonstrated phenotypic upregulation of FAS and MHC-I following of RT [7–9], and recent studies have suggested non-cell autonomous mechanisms whereby RT may influence tumor expression of the checkpoint ligand, PD-L1 [10]. The time course, potentially shared underlying mechanisms, and the possibility of a broader impact of RT on expression of other phenotypic markers of tumor immune susceptibility remain to be clarified. Others and we have demonstrated the cooperative interaction of combination treatment with radiation and immunotherapy *in vivo*. Many of these studies demonstrate that the timing and sequencing of radiation and immunotherapy may be critical for optimizing cooperative anti-

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tumor activity [11,12]. Illustratively, in preclinical studies we recently demonstrated a cooperative interaction between RT and the antibody-dependent cell-mediated cytotoxicity (ADCC) response to tumor-specific monoclonal antibodies (mAb) in murine melanoma [11]. This effect was enhanced using a combination of RT and immunocytokine, a fusion of the tumor-specific antibody with IL2, resulting in elimination of 5-week established tumors in most mice and a memory T cell response. However, this effect was time-sensitive and observed when immunotherapy was delivered 6–10 days after RT but not with administration on days 1–5 or 11–15 [11]. Therefore, it is valuable to understand not only how RT may affect tumor cell susceptibility to immune response but also to define the time course and mechanism of such effects.

Here, we investigate the effect of RT on the expression of multiple immune susceptibility markers over time, at both transcriptional and post-translational levels. Our results suggest a broad and potentially coordinated pattern of immunomodulation in murine and human melanoma cells following RT and elucidate the time course over which these changes take place.

Materials and methods

Cell lines

The human melanoma A375 cell line was obtained from ATCC. Cells were grown in DMEM (Mediatech Inc.) and were supplemented with 10% fetal bovine serum (FBS), 1 μg/mL hydrocortisone, and 1% penicillin and streptomycin. The murine melanoma B78-D14 (B78) cell line is derived from B16 melanoma as previously described [13] and was obtained from Ralph Reisfeld (Scripps Research Institute) in 2002. B78 cells were grown in RPMI-1640w/ L-glutamine (Mediatech Inc.) and were supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell line authentication was performed per ATCC guidelines using morphology, growth curves, and *Mycoplasma* testing within 6 months of use. All chemicals were purchased from Sigma. Cell culture media and supplements were obtained from Life Technologies, Inc.

Radiation

All RT (*in vitro* and *in vivo*) was delivered in a single fraction. Delivery of radiation *in vitro* was performed using a ^{137}Cs-irradiator (JL Shepherd & Associates Model 109) or an X-ray biological cabinet irradiator X-RAD 320 (Precision X-ray, Inc). Delivery of RT *in vivo* was performed using an X-ray biological cabinet irradiator X-RAD 320 (Precision X-ray, Inc). The dose rate for RT delivery in all experiments was approximately 2 Gy/min. Dosimetric calibration and monthly quality assurance checks were performed on these irradiators by University of Wisconsin Medical Physics Staff. Unless otherwise indicated, the dose of RT delivered to B78 melanoma cells and tumors was 12 Gy and the dose delivered to A375 melanoma cells was 9 Gy. For B78 melanoma, selection of RT dose was based on our prior findings of a time-sensitive *in vivo* synergy between single fraction 12 Gy and intra-tumor injection of specific immunotherapies [11]; that observation provided initial rationale for this study. For A375, which exhibits a greater intrinsic sensitivity to RT, a comparable biologically effective dose (9 Gy) was chosen, as determined by clonogenic survival assays.

Cell culture

RT was delivered when cells were approximately 60–70% confluent. Cells were not synchronized prior to RT. Following RT, cell media was exchanged with fresh media every other day but cells were not trypsinized or re-plated. Many cells were observed to die and detach from the plate after RT. This cellular debris was discarded with medium changes. Only adherent cells were collected and analyzed for *in vitro* studies. Consequently, the great majority of cells analyzed at each time point in our *in vitro* assays were viable and in flow cytometry studies the viability of analyzed cells, as determined by dye exclusion, was comparable between control and radiated samples. In order to ensure enough material for analysis, for later time points (>3 days following RT) multiple plates were treated with RT and harvested together. Cells harvested from these synchronously treated plates generated clonogenic colonies (typically >50 per plate) and these cells were pooled for analyses. Untreated controls cells were passaged twice weekly to maintain a sub-confluent state during the experiment. In preparation for harvesting at the conclusion of a time course experiment, these cells were seeded at 60% confluence at 2.5 days prior to harvest and harvested at ~90% confluence. As an additional control, we also tested the effect of tissue culture conditions on non-radiated cells. For this, we plated ~100 A375 or B78 cells per plate and cultured these for up to 19 days like radiated cells by changing media every other day but not splitting or re-plating.

Quantification of mRNA expression

Relative mRNA levels of all targets were quantified via real-time PCR (RT-qPCR) using a Bio-Rad iQ5 RT-qPCR Detection System and Power SYBR Green PCR Master Mix (Life Technologies). All reactions were performed in technical and biological triplicates. *PGK* was used as an endogenous control. Fold changes after treatments were normalized to an untreated control sample. RNA isolation and reverse transcription procedures and a list of all targets and detailed primer information for each molecule are provided in the Supplementary Materials and Methods.

Flow cytometry analysis

Flow cytometry was performed as previously described [14] using a MacsQuant Analyzer (Miltenyi Biotec). Cells were labeled as indicated using anti-CD80 (EBiosciences, 16-10A1), anti-DR5 (EBiosciences, MDS-1 PE), anti-BrdU (Cell Signaling Technology, Bu20a), or anti-PD-L1 (EBiosciences, MIH5) fluorescent-conjugated monoclonal antibodies and/or DAPI. For all studies, cells were gated using forward- and side-scatter to identify single cells and DAPI or Ghost Red Dye 780 (Tonbo Biosciences) exclusion was used then to identify live cells. Analyses of immune susceptibility markers were performed only on live single cells. FlowJo Software was used for analysis.

Immunoblotting

Protein isolation, quantitation, and immunoblotting were performed as previously described [15]. Anti-DR5, anti-Fas, anti-PD-L1, anti-phospho-STAT1, anti-phospho-STAT3, anti-STAT1, and anti-STAT3 primary antibodies and HRP-linked secondary antibodies were obtained from Cell Signaling Technologies. The anti-alpha-Tubulin primary antibody was obtained from Calbiochem. All other primary antibodies were obtained from Santa Cruz Technologies.

shRNA knockdown of STAT1

STAT1 specific short hairpin RNA (shRNA), as well as non-target shRNA, were purchased from Origene and transfection was performed using Lipofectamine 3000 reagent (Invitrogen). Knockdown of STAT1 was confirmed by immunoblot. Procedures and constructs are described specifically in the Supplementary Materials and Methods.

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