Published in final edited form as:

Int J Radiat Oncol Biol Phys. 2013 November 15; 87(4): 769–776. doi:10.1016/j.ijrobp.2013.07.015.

Combined Treatment Effects of Radiation and Immunotherapy: Studies in an Autochthonous Prostate Cancer Model

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Abstract

Purpose—Optimize the combination of ionizing radiation and cellular immunotherapy using a pre-clinical autochthonous model of prostate cancer.

Methods and Materials—Transgenic mice expressing a model antigen under a prostate-specific promoter were treated using a platform that integrates cone-beam CT imaging with 3D conformal therapy. Using this technology we investigated the immunological and therapeutic effects of combining ionizing radiation with GM-CSF secreting cellular immunotherapy for prostate cancer in mice bearing autochthonous prostate tumors.

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Conflict of Interest: None.

Summary

Combining a cell-based cancer vaccine with radiation in a mouse model of prostate cancer results in improved local tumor control. Moreover, the combination of immunotherapy and radiation prevents metastatic disease and mitigates the progression of established micrometastatic disease.

Results—The combination of ionizing radiation and immunotherapy resulted in a significant decrease in pathologic tumor grade and gross tumor bulk that was not evident with either single modality therapy. Furthermore, combinatorial therapy resulted in improved overall survival in a preventive metastasis model and in the setting of established micrometastases. Mechanistically, combined therapy resulted in an increase of the ratio of effector-to-regulatory T cells for both CD4 and CD8 tumor infiltrating lymphocytes.

Conclusions—Our pre-clinical model establishes a potential role for the use of combined radiation-immunotherapy in locally advanced prostate cancer, which warrants further exploration in a clinical setting.

Keywords

Prostate cancer; immunotherapy; T cell; radiotherapy

Introduction

Although radiotherapy can be curative for men with high-risk prostate cancer, the median time to biochemical failure for such men is approximately 5 years, suggesting that combining primary radiotherapy with additional treatment modalities could be useful in extending relapse-free survival for such patients. Combining radiotherapy with immunotherapy (1–3) could prove especially attractive given the favorable side effect profile associated with most immunotherapy agents, particularly when compared to cytotoxic chemotherapy regimens (4). Indeed, a recent provocative publication demonstrated enhancement of systemic anti-tumor responses with the combination of CTLA-4 blockade and irradiation of a single metastatic focus (5). However, testing combination therapy regimens in men with aggressive prostate cancer is challenging because of the relatively long lead-time from initial treatment to biochemical relapse. Since the relative timing of immunotherapy and radiotherapy might prove critical (6), modeling combination approaches in animals would prove useful in guiding clinical development.

Such studies are compromised by the difficulties inherent in modeling the complex radiotherapy techniques used clinically. Indeed, the majority of animal studies have employed relatively simple, single-beam and/or single-fraction techniques. The Small Animal Radiation Research Platform (SARRP) was developed by members of our team to address these issues, and combines cone-beam computed tomography (CBCT) imaging with three-dimensional treatment planning and radiotherapy treatment (7). To apply this technology to a relevant pre-clinical model, we used a murine system based on the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse, in which prostate cancer spontaneously and uniformly arises, with progression to both metastatic and hormone refractory disease. By mating TRAMP mice with transgenic mice that express the model antigen hemagglutinin (HA) in a prostate-restricted manner (ProHA), we created a model to study the specific immune response to tissue/tumor-restricted HA as a function of radiation and/or immunotherapy administration (6, 8–11). Among the various immunotherapy strategies for prostate cancer that have been tested clinically, we chose to utilize cell-based immunotherapy using GM-CSF secreting cells (12). While well tolerated, prior studies have demonstrated that this approach is relatively poorly immunogenic in mice bearing

autochthonous tumors (12, 13), leading us to hypothesize that combinatorial approaches might be required to elicit an optimal anti-tumor immune response in tumor-bearing hosts.

Methods and Materials

Mice

ProHA transgenic mice express a secreted form of HA under control of the minimal rat Probasin promoter on the B10.D2 genetic background. Double transgenic (ProHA/TRAMP) mice were generated by backcrossing TRAMP mice onto the ProHA background > 10 generations. TRAMP mice have nearly complete penetrance for prostate cancer with approximately 90–95% of animals having in-situ disease by 12 weeks, 90–100% having invasive disease by 18 weeks, and 50% having metastatic disease by 20 weeks (14–16). Double transgenic animals develop autochthonous prostate tumors that express HA as a tissue/tumor-restricted antigen and disease development is indistinguishable from their TRAMP counterparts (8). Control B10.D2 (H-2^d) mice were purchased from the Jackson Laboratory. Clone 4 is a CD8 T cell Receptor (TCR) transgenic strain recognizing the K^d-restricted (MHC Class I) HA peptide in a K^d-restricted manner (17). 6.5 is a CD4 TCR transgenic mouse recognizing the I-E^d restricted (MHC Class II) HA peptide (18). Clone 4 and 6.5 mice were backcrossed onto a Thy1.1⁺ B10.D2 background. Animal care and experimental procedures were carried out in accordance with the Institutional Animal Care and Use Committee of XXX, under an approved protocol.

Cell lines

B78H1-GM is a GM-CSF secreting cell line utilized in bystander immunotherapy regimens (19). SWPC1 is a prostate cancer cell line established from a primary ProHA/TRAMP prostate tumor in our laboratory. These cells are of epithelial origin, and are androgen-insensitive. SWPC1 cells were maintained in RPMI 1640 supplemented with 10% heat inactivated FCS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, nonessential amino acids, 25 mmol/L HEPES buffer, and 50 µmol/L 2-mercaptoethanol.

Radiation

All treatments were performed using the SARRP, combining CBCT with ionizing radiation in a unified platform (7). Treatment was administered using a 3-mm focal spot with the X-ray tube maintained at 225 kVp. For all treatment studies, animals were anesthetized using 2,2,2-tribromoethanol (350 mg/Kg) via intraperitoneal injection. IV contrast material was administered via tail vein to visualize the urinary bladder. Prior to treatment, CBCT scan was performed to localize the prostate gland. For treatment, a parallel-opposed, lateral beam arrangement was applied with the central axis of each beam being localized to a point along a line joining the pelvic bone and the posterior surface of the bladder (as visualized on a sagittal CBCT cross section at mid-line). 5x10 mm radiation fields were utilized, assuring coverage of the prostate while sparing of the bladder and rectum, prescribing 6 Gy per field for a 12 Gy total.

Immunohistochemistry

Indicated organs were removed immediately after sacrifice, rinsed in phosphate-buffered saline, and snap-frozen in OCT. Frozen sections were stained as previously described (20), using a monoclonal anti-phospho-H2AX (Ser139) antibody (Millipore/Upstate). Briefly, slides were steamed for 40 minutes in DAKO Antigen Retrieval Solution, and sections were stained at a 1:16,000 dilution using the PowerVision+ Poly-HRP System (ImmunoVision Technologies). Staining was visualized using 3,3'-Diamino benzidine (DAB) and slides were counterstained with hematoxylin.

Adoptive T cell transfer

TCR transgenic donor mice were sacrificed. Spleens and lymph nodes were collected, homogenized, and RBCs lysed. CD8⁺ or CD4⁺ T cells were purified using Miltenyi beads. $2.5\text{--}5\times 10^6$ cells were injected via tail vein.

Cell-based immunotherapy

To model cell-based immunotherapy using bystander cells, 1×10^6 SWPC1 cells were admixed with 5×10^4 B78H1-GM cells and irradiated (5000 cGy). Cells (T-GVAX) were resuspended in a total volume of 200 μ l HBSS and administered by SQ injection into each hind limb.

Flow cytometry

Prostate glands, prostate-draining lymph nodes (DLN) and spleens were harvested on the indicated days post adoptive transfer, and single cell suspensions were prepared. Adoptively transferred TCR transgenic (6.5 and Clone 4) T cells were gated using Thy1.1. Reagents were purchased from BD Biosciences, with the exception of anti-FoxP3 (eBioscience). Intracellular cytokine analysis (ICS) was performed after a brief ex vivo stimulation with specific peptide as previously described (9). Data were analyzed using the FlowJo software package.

Combined therapy

ProHA/TRAMP mice were treated at 12–14 weeks of age. Immunotherapy was administered three times, with doses one week apart. Radiation was administered as detailed above. For analysis, mice were sacrificed at 22–24 weeks of age and urogenital tracts micro-dissected under a stereomicroscope and weighed. The ventral lobes of the prostate gland were removed from urogenital tracts and fixed in 10% neutral buffered formalin followed by 70% EtOH prior to embedding in paraffin. Four micron sections were cut using a cryostat and placed onto poly-lysine-coated slides. Tissues were processed and stained with H&E for histopathological analyses. Tumor histology was scored in a blinded manner by two individual pathologists as previously described (9, 11, 21). 0=benign; 1=PIN without cribriform formation; 2=PIN with cribriform formation; 3=intraductal carcinoma; 4=moderately differentiated carcinoma; 5=poorly differentiated or small cell carcinoma.

Metastasis models

10^6 SWPC1 cells were injected via tail-vein into tumor-bearing ProHA/TRAMP 20–22 weeks of age. For metastasis prevention studies, therapies were performed 3 weeks prior to tail-vein injection. In metastasis treatment studies, therapies were administered 3 days post tail-vein injection.

Results

The SARRP in an autochthonous prostate cancer model

Utilizing the SARRP, we were able to target the mouse prostate and limit the dose applied to the bladder and rectum. As shown in Figure 1A, administration of IV and intra-prostatic contrast media prior to 3-D image acquisition demonstrated that both lobes of the prostate gland could be treated with two spatial fields approximately 5x10 mm in size. (For treatment studies, intra-prostatic contrast material was not used; the gland was localized based on the relative bladder position and boney landmarks.) To confirm well-localized RT targeting, phosphorylated histone H2AX was stained following administration of 12 Gy, demonstrating diffuse γ H2AX throughout the prostate gland, with only minimal off-target effects noted in the rectum and bladder (Fig 1B, C).

Immunological effects of radiation combined with immunotherapy

We next examined whether radiation could alter immune recognition of the tumor-bearing prostate gland. Prior to treatment we adoptively transferred CD8⁺ T cells specific for prostate (Clone 4). In this model, adoptively transferred cells are not given as immunotherapy, but function as surrogates for specific recognition of prostate tumor (6, 8–11). These studies were performed in the absence of specific vaccination (no treatment or radiation alone), as well as in combination with vaccination. In terms of timing, for initial studies we co-administered immunotherapy and radiation (12 Gy) on the same day. As shown in Figure 2A, we found that the combination of radiation and immunotherapy resulted in a significant increase in the number of prostate-specific (Clone 4) CD8⁺ T cells in all sites examined, with the relative expansion/accumulation most profound in the prostate gland itself. Next, we performed a series of experiments to determine whether the relative timing of vaccination and radiation affected prostate-specific CD8⁺ T cell expansion (Fig 2B). This schema was designed so that the adoptively transferred prostate-specific CD8⁺ T cells remained in tumor-bearing hosts for a fixed time period prior to vaccination. As in our previous studies (6), we found that the relative timing of radiation and immunotherapy was critical, with maximal effector T cell expansion noted when immunotherapy was administered on the same day as radiation (Fig 2C). Using this optimized treatment sequence, we next examined whether higher or lower doses of radiation could prove more efficacious in terms of effector CD8 T cell prostate infiltration. Interestingly, a dose of 12 Gy (in combination with immunotherapy) appeared optimal in this regard (Fig 2D), with an approximate 4-fold relative T cell accumulation as compared to vaccination alone.

Pre-clinical effects of radiation combined with immunotherapy

We next sought to determine if combination therapy could mediate a more clinically relevant endpoint. To this end, tumor-bearing ProHA/TRAMP mice (12–14 weeks in age) were treated with a single dose of radiation (12 Gy) and/or 2 doses of immunotherapy (Fig 3A). Notably, these studies did not involve adoptive T cell transfers; therefore, only endogenous immune cells were responsible for any anti-tumor immunologic responses. As shown in Figure 3B, at this dose, radiation alone did not alter the wet weight of the urogenital tract, which represents a gross measure of tumor burden in this model (Fig 3B). Similarly, radiation alone did not affect the pathological tumor score (Figure 3C). Immunotherapy alone was similarly ineffectual in this regard. However, combining radiation with immunotherapy resulted in a significant treatment effect, consistent with the immunological effects noted earlier. This treatment effect was reflected in the urogenital tract weight, tumor score, and gross appearance upon dissection (Supplemental Figure 1). Perhaps not surprisingly, the above pathologic changes did not correspond to an increase in peripherally circulating endogenous anti-prostate T cells, which were quantified with tetramer staining 6 weeks post-combination therapy (Fig 3D).

Immunological mechanisms underlying an immunotherapy/radiation combination treatment effect

While CD8⁺ T cells are the cytotoxic cells ultimately responsible for cell-mediated immune killing, CD4⁺ T cells are immunologic potentiators important in modulating immune responses, including anti-tumor immune responses. Using adoptively transferred HA-specific CD4⁺ T cells as a readout of immune activation (not immunotherapy), we next examined the effect of combined treatment on tumor-specific CD4⁺ T cell expansion and accumulation. Similar to results obtained with CD8⁺ T cells (Fig 2–3), the combined treatment regimen elicited CD4 expansion most pronounced in the prostate gland itself (Fig 4A). In terms of CD4 phenotype, we noted an expanded T_H1 effector population (Fig 4B), which is thought to be the primary CD4 population responsible for anti-tumor immune responses. Only small percentages of prostate-specific T_H2 and T_H17 cells were detected, with no statistically significant changes mediated by RT, either alone or in combination with vaccination. As has been seen in other models (22), immunotherapy also mediated expansion of a regulatory T cell (T_{REG}) population (Fig 4C, left panel), both alone and in combination with radiation. T_{REG} expansion appeared to be relatively confined to antigen-specific adoptively transferred T cells, and was not noted when endogenous CD4⁺ T cells were analyzed (Fig 4C, right panel). In fact, combination therapy actually resulted in a significant decrease in the endogenous prostate infiltrating T_{REG} population. As previous studies demonstrated that the ratio of effector-to-regulatory T cells is a critical parameter in determining the outcome of anti-tumor immunity (1), we quantified this ratio for both HA-specific CD4⁺ and CD8⁺ T cells in the context of combinatorial treatment. As shown in Figure 4D, the combination of radiation and immunotherapy resulted in a significant increase in the T_{EFF}/T_{REG} ratio for both CD4⁺ and CD8⁺ T cells, consistent with the treatment effects shown in Figure 3.

Effects of combined immunotherapy/radiation on metastatic disease

Although definitive radiotherapy is typically administered to men with localized disease, a proportion of high-risk patients will either (A) fail treatment and ultimately develop metastatic disease, or (B) have occult micrometastatic disease at the time of treatment, which becomes clinically apparent post-treatment. Consequently, we examined whether combining radiation with immunotherapy could mediate a preventative or treatment effect for metastatic disease, particularly in the micrometastatic setting. To explore combined treatment in a metastatic model, SWPC1 cells were injected via tail vein into primary-tumor bearing ProHA/TRAMP mice. Immunologically, these mice are tolerant to their own primary tumors (8) as well as to implanted SWPC1 cells. As shown in Figure 5A, the combination of radiation and immunotherapy mediated a small but statistically significant treatment effect, modestly extending survival in this metastasis prevention model. Indeed combined therapy resulted in long-term survival in 25% of the animals. A similar benefit from combined therapy was noted in the setting of pre-established micrometastatic disease (Fig 5B).

Discussion

Our results are consistent with previous studies suggesting additive—or potentially synergistic—clinical and immunologic effects with combined radiotherapy and immunotherapy (1, 2, 6). Both timing and dosage of radiation were critical for immunologic endpoints; optimal accumulation/proliferation of cytotoxic T cells was observed at 12 Gy with concurrent vaccination. While 24–36 Gy doses are likely to have a clinical effect on the tumor, these higher doses were unable to potentiate tumor-specific lymphocyte infiltration. These data could reflect the suppressive effects of radiation on tumor-infiltrating antigen presenting cells, or effects of radiation on the tumor microenvironment and/or microvasculature. However, it is unlikely that radiation-mediated disruption of the tumor microenvironment, particularly fibrosis, could account for the low lymphocyte cellularity observed at higher doses of radiation, given the short time course of our dose escalation experiments.

In our immunological studies, combined therapy was associated with a dramatic increase in effector CD8⁺ T cells infiltrating the prostate gland and resident within prostate draining lymph nodes. Combined therapy also increased the effector-to-regulatory T cell ratio. Interestingly, radiation as a single modality did not have differential effects on regulatory versus effector T cells. Consistent with our previous work (22), vaccination of tumor-bearing mice increased the percentage of CD4⁺ T cells that express FoxP3. The significant increase in the effector-to-regulatory ratio of both CD4⁺ and CD8⁺ T cells provides a logical immunological mechanism to explain the pre-clinical effects of combined therapy. In multiple preclinical tumor models, radiation upregulates surface molecules responsible for anti-tumor immune responses (23, 24). In our studies, we observed an increase in tumor-specific cytotoxic T-cells in the spleen following combined therapy, which suggests enhancement of priming and proliferation. We similarly observed increased surface expression of MHC-I and ICAM-1 on TRAMP cells irradiated in vitro suggesting that radiation can potentiate the immune response through the combination of increased T-cell

priming (MHC-I) and homing (ICAM-1) (Supplemental Figure 2). Interestingly, a decrease in endogenous tumor-specific circulating cells was observed following combined therapy, which could be indicative of recruitment of endogenous anti-tumor T cells from circulation to the prostate.

Finally, we used a micrometastasis model to test whether combination therapy could mediate systemic anti-tumor immunity. Our findings were clear in demonstrating a modest, but significant, treatment effect when applied in both a preventive setting and in the setting of established micrometastatic disease. Relevant to this, a Phase III randomized control trial of GVAX (a cell-based immunotherapy against prostate cancer) versus docetaxel in the hormone-refractory metastatic setting, failed to demonstrate clinical benefit with cell-based immunotherapy (25). Our results suggest that combining radiation with immunotherapy might be maximally beneficial in locally aggressive disease where there is a likelihood of occult micrometastatic disease and distant failure. Our data define the timing for cell-based immunotherapy in conjunction with definitive radiation, and provide a pre-clinical rationale for future clinical studies in prostate cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

CGD is a Damon Runyon-Lilly Clinical Investigator. This work was also supported by National Institutes of Health R01 CA127153 (CGD), K08 CA096948 (CGD), CA108449 (JW) the Patrick C. Walsh Fund, the Prostate Cancer Foundation, the Koch Fund and the OneInSix Foundation. DMP is a Januey Scholar, holds the Seraph Chair for Cancer Research, and is supported in part by gifts from William and Betty Toperer, Dorothy Needle, and the Commonwealth Foundation.

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Figure 1. Focal radiation of autochthonous prostate tumors

A: Detection and targeting of the mouse prostate gland using the Cone-Beam Computed Tomography (CBCT) mode of the Small Animal Radiotherapy Research Platform (SARRP). Yellow rectangles represent treatment planning – a parallel-opposed, lateral beam arrangement was utilized with the central axis of each beam being localized to a point along the line that joined the pelvic bone and the posterior surface of the bladder. Yellow boxes indicate prostate situated 5.5-mm anterior to sacrum, and posterior to bladder.

B, C: H2AX Immunostaining. ProHA/TRAMP mice were irradiated using the SARRP and sacrificed 30 minutes post-therapy. Untreated, age-matched non-transgenic (B10.D2) or ProHA/TRAMP served as negative controls. (B) Ventral/Dorsal prostate. (C) Bladder/Rectum.

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Figure 2. Combined immunotherapy and radiation of autochthonous prostate tumors

A: Interaction between radiation and immunotherapy (T-GVAX). 12 Gy and/or immunotherapy were administered on the indicated days. Adoptively transferred, prostate-specific CD8⁺ T cells were administered on day -2, and harvested 9 days later in all groups. Prostate-specific CD8⁺ T cells were quantified from the indicated sites using flow cytometry.

B, C: Optimization of combined radiation and-immunotherapy. (B) Experimental design. 12 Gy and/or immunotherapy were administered on the indicated days. Adoptively transferred,

prostate-specific CD8⁺ T cells were administered on day -2, and harvested 9 days later in all groups. When co-administered on the same day, immunotherapy was given immediately following radiation. (C) Prostate-specific effector cells (lower panel), quantified using intracellular staining (ICS) for IFN- γ following ex vivo peptide stimulation (upper panel). D: Prostate infiltrating Clone 4 cells following titration of radiation dose. Radiation and immunotherapy administered on the same day.

Experiments had 3–5 mice per group and were repeated twice.

Figure 3. Anti-tumor efficacy of combined immunotherapy/radiation

A: Experimental design. 12–14 week ProHA/TRAMP mice were treated with radiation, immunotherapy (T-GVAX), or combined therapy. Animals receiving vaccination were given boost vaccinations days 7 and 14 post-radiation. Animals were harvested between 22–24 weeks of age.

B: Wet weight of the urogenital tract.

C: Pathologic tumor score, see methods for details.

D: Quantification of prostate-specific CD8⁺ T cells in the periphery. 4 weeks post-boost, HA tetramer positive CD8⁺ T cells and effector cells (CD62L^{low}, CD95⁺) were quantified. Experiments had 8–10 mice per group and were repeated twice.

Figure 4. Immunological mechanisms of combination radiation-immunotherapy

A: Prostate-specific CD4⁺ T cell expansion. Adoptively transferred CD4⁺ T cells from TCR transgenic donors (6.5) were harvested from indicated sites and quantified using FACS analysis.

B: CD4⁺ T cell subsets. After adoptive transfer, HA-specific CD4⁺ T cells were analyzed for IFN-γ, IL-4, and IL-17 secretion by intracellular staining after ex vivo peptide stimulation.

C: Regulatory T cells. Adoptively transferred prostate-specific (left panel) or endogenous (right panel) T_{REG} were quantified using ICS for FoxP3.

D: Effector/Regulatory ratio. Ratios were calculated using absolute cell numbers of IFN-γ secreting, HA-specific T cells to FoxP3⁺ T_{REG}.

Experiments had 5 mice per group and were repeated twice.

Figure 5. Effect of radiation and immunotherapy on metastatic disease

A: Prevention model. 20–22 week ProHA/TRAMP mice received 1×10^6 syngeneic SWPC1 prostate tumor cells IV. Immunotherapy and radiation were administered as shown.

B: Treatment model. As in panel A, with treatment initiated 3 days post tumor inoculation. Experiments had 5–8 mice per group and were repeated once.