The effect of hypofractionated radiation and magnetic nanoparticle hyperthermia on tumor immunogenicity and overall treatment response

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ABSTRACT

It is now known that many tumors develop molecular signals (immune checkpoint modulators) that inhibit an effective tumor immune response. New information also suggest that even well-known cancer treatment modalities such as radiation and hyperthermia generate potentially beneficial immune responses that have been blocked or mitigated by such immune checkpoints, or similar molecules. The cancer therapy challenge is to; a) identify these treatment-based immune signals (proteins, antigens, etc.); b) the treatment doses or regimens that produce them; and c) the mechanisms that block or have the potential to promote them.

The goal of this preliminary study, using the B6 mouse – B16 tumor model, clinically relevant radiation doses and fractionation schemes (including those used clinically in hypofractionated radiation therapy), magnetic nanoparticle hyperthermia (mNPH) and sophisticated protein, immune and tumor growth analysis techniques and modulators, is to determine the effect of specific radiation or hyperthermia alone and combined on overall treatment efficacy and immunologic response mechanisms.

Preliminary analysis suggests that radiation dose (10 Gy vs. 2 Gy) significantly alters the mechanism of cell death (apoptosis vs. mitosis vs. necrosis) and the resulting immunogenicity. Our hypothesis and data suggest this difference is protein/antigen and immune recognition-based. Similarly, our evidence suggest that radiation doses larger than the conventional 2 Gy dose and specific hyperthermia doses and techniques (including mNP hyperthermia treatment) can be immunologically different, and potentially superior to, the radiation and heat therapy regimens that are typically used in research and clinical practice.

Keywords: hypo-fractionated radiation, hyperthermia, magnetic nanoparticles, cancer therapy, abscopal effect

INTRODUCTION

Multimodality therapy is currently accepted as the optimal approach to treating cancer. This includes modalities such as chemotherapy, surgery, hyperthermia, radiation, molecular targeting, and immunotherapy. The latter is rapidly becoming one of the most promising and important approaches. How these modalities are combined to achieve the best outcome is a central question in translational cancer research. The ability of local cancer treatments to prevent metastasis is central to many cancer cures, however the ability of a local treatment to induce an effective systemic/metastatic cancer treatment is also possible, this is known as the abscopal effect. The abscopal effect of radiation therapy is a rare but a well-documented event. Although the mechanism is unclear most believe the abscopal effect to be immunologically based. The overall goal of this research is that specific radiation or hyperthermia doses/techniques have definable and reproducible antitumor immune responses that can be exploited through appropriate dosing or combination with immunoreactive agents. This approach is attractive because the cell stress and death caused by the radiation or hyperthermia may be capable of supporting a strong antitumor reaction that modifies the tumor microenvironment to reverse local immunosuppression and support local and systemic antitumor immune responses. [1-8]

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Radiation Therapy
The field of radiation therapy is becoming increasingly accepting of hypo-fractionated therapy. The primary reasons are four-fold: 1) few patient treatments, 2) larger fraction size is more cytotoxic to the tumor (but of course carries a greater risk of normal tissue damage), 3) greatly improved tumor imaging, treatment planning and beam management, and importantly 4) larger fraction sizes promoting a tumor cell killing mechanism that induces immune activation – a situation that is not appreciated with conventional small fraction size regimens.

The abscopal effect has recently been well reviewed in multiple places (Grass, 2016). The main concept is that the radiation injury has an immune effect that can be exploited. Clearly, radiation therapy (RT) by itself is generally not sufficient to create an effective and reliable antitumor immunity (Siva, 2015). Studies report that the damage of RT alone recruits M2 type tissue repair macrophages that suppress adaptive immunity (Crittenden, 2014). The crucial aspect appears to be if radiation can in addition to mitotic or apoptotic cell death, stimulate immunogenic cell death (ICD). ICD is characterized by a grouping of the following danger associated molecular signals (DAMPs): calreticulin expression on the cell surface, release of ATP, release of HMGB1 protein, and expression of type one interferons (IFN alpha and beta) (Grass, 2016). When the tumor environment is sufficiently immunogenic, tumor associated antigens (normal proteins expressed at the wrong level, wrong tissue or wrong time during development) and neoantigens (mutant proteins expressed by tumors due to accumulated mutations) are taken up by antigen presenting cells that go to the lymph nodes, present them to T cells and stimulate an adaptive immune response against tumor cells. That adaptive immune response not only impacts tumors near that lymph node but also can become a systemic response against the same tumor wherever it may occur. [9-12, 25]

Hyperthermia
Although less well known and appreciated there is also documentation of therapeutic heat/hyperthermia induced abscopal effect. In one recent paper (Wang et al, 2014) the abscopal effect it was demonstrated in a Walker-256 carcinoma using magnetic seed-mediated hyperthermia at a higher temperatures (42°C –46°C for 30 minutes and 50°C - 55°C for 10 minutes). The abscopal endpoint effect was regression of a non-heated tumor (contralateral) following heat treatment of one tumor in rat with bilateral tumor growth. It was also noted that the levels of $CD4^+$ and $CD8^+$ lymphocytes and IFN-γ and IL-2 cytokines were significantly increased in the blood of hyperthermia groups compared with those of the three control groups; the increase in $CD8^+$ cells was higher than that of the $CD4^+$ cells. Our group also demonstrated this situation, showing that a mild hyperthermia treatment, of an established tumor, can generate systemic resistance to rechallenge (Toraya-Brown, 2014). [13-24]

MATERIALS and METHODS

MTG-B / C3H and B6 / B16 Mouse Model
This research utilized two mouse models: MTG-B mammary adenocarcinoma in a sygeneic C3H mouse and a B16-F10 melanoma studied in syngeneic C57-BL6 mice. We grow the tumors intradermally because it is an orthotopic site and because we can then access the tumor readily to inject reagents for immune studies and because it provides ease of measurement these studies typically utilized tumor regrowth, tumor rechallenge and metastatic endpoints.

Iron Oxide Nanoparticle (IONP) / Alternating Magnetic Field (AMF) Technology and Treatment
In these studies, we used BNF iron oxide nanoparticles (product number 10-00-801 from Micromod Partikeltechnologie GmBH, Rostock, Germany). These iron oxide nanoparticles (mNP) are composed of a hematite core surrounded with crystals of average diameter of approximately 20 nm (total core diameter approximately 40 nm) and a hydroxyethyl starch or dextran shell to a final average hydrodynamic diameter of 117 nm. The mNP were concentrated to a final particle concentration of 44 mg/ml and iron concentration of 28 mg/ml. A cooled helical coil with an inner diameter of 4 cm and is composed of hollow copper tubing was used to generate AMF. The AMF coil (Fluxtrol Inc) was powered at variable 10 KW generator (Huttinger Elektronik GmbH, Freiburg, Germany) at a field of 150 kHz and 400 Oe. The AMF coil and generator were cooled by a chiller (Tek-Temp Instruments, Croydon PA.) operating at 20°C and four gallons per minute.

Figure 1 demonstrates the AMF treatment facility. Figure 2 demonstrates intratumoral injection of mNP into a flank MTG-B mammary tumor. Figure 3 demonstrates mNP biodistribution in an injected (tumor cut section) 10 minutes following injection. Figure 4 demonstrates a mouse positioned in the AMF coil.

Figure 1

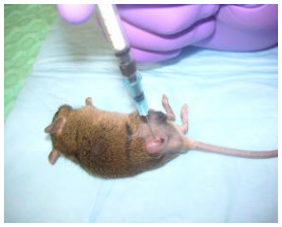


Figure 2

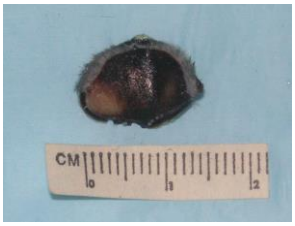


Figure 3

Figure 4

IONP Tumor Uptake

As an example below (Giustini et al. Figure 5) most cancer cells are programmed to endocytose specific sized material, such as mNP, at a relatively high and rapid level. The results show dextran-coated mNP are 90% intracellular at four (4) hours following direct mNP injection into a mouse mammary adenocarcinoma (MTGB). In spite of this situation, two significant mNP cancer therapy challenges are: 1) ensuring that the mNP distribute uniformly throughout the tumor (local and systemic delivery) and 2) improving the percentage of mNP that reach the tumor following systemic injection. Several factors, including differences in interstitial tumor pressure and tumor pathophysiology affect mNP uptake heterogeneity. In an attempt to address these issues, we gave a single low dose of radiation (15 Gy) before systemic mNP injection, to determine the potential for mNP tumor uptake enhancement. Pre-mNP radiation lowered interstitial tumor pressure and improved systemic mNP uptake significantly (Figure 6). Figure 7 demonstrates this situation in two tumors located on the same animal, one tumor was irradiated prior to systemc mNP delivery while the other was not. The single 15 Gy radiation dose, lowered the tumor interstitial pressure by 60% reduction at the 72-hr post-radiation time point (time of greatest mNP/Fe uptake advantage). Tumor irradiation resulted in a 2.5 fold increase in mNP/Fe uptake 72 hours post injection.

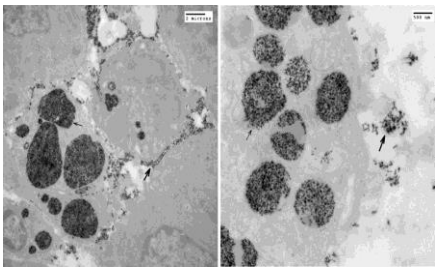
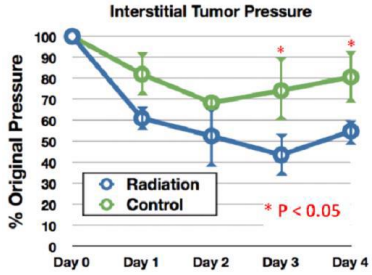


Figure 5



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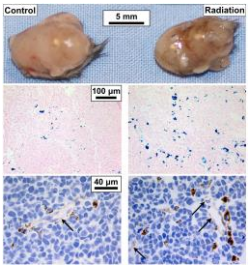
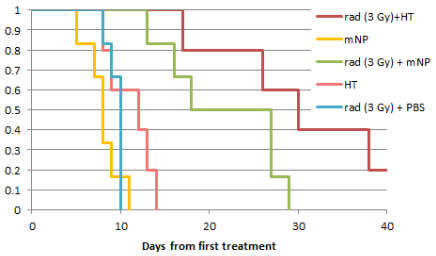


Figure 7

Radiation and mNP hyperthermia

All irradiated mice in this study were treated with either a single dose or 15 Gy or 3 x 5 Gy. For fractionated therapy radiation treatments are delivered three (3) times per week (M, W, F). Mice receiving mNP-AMF received two thermal doses of 43°C / 30 min (2 x CEM 30). The Kaplan-Meyer curve below (Figure 8) demonstrates the relative effectiveness (tumor regrowth analysis) of radiation alone (3 x 5 Gy), hyperthermia alone (2 x CEM 30) or combined. In this study radiation and hyperthermia provided a superior benefit. It also appears that irradiation with mNP (no heat) is beneficial. Ongoing studies are examining the immune effects of these therapies.

Figure 8



Hyperthermia Abscopal Effect

In this study BALB/c or C57BL/6 mice (6-8 weeks old) were injected intradermally (ID) on 1.25×10^5 B16F10 cells, to establish dermal tumors of about 5 mm × 6 mm on both flanks of the mice. The left side tumor was injected with approximately 300 ug mNP (140 ug Fe). The right side tumor received no mNP (no heat). Using the previously described AMF exposure, tumor were heated to 43 °C for 30 min. In support of the abscopal effect the Heated tumors on the left flank disappeared completely in 5 days while the non-heated right-flank tumors grew significantly slower than in the unheated group (Figure 9; Toraya-Brown S, Hoopes P.J., Fiering S.N. et al. "Local hyperthermia treatment of tumors induces CD8(+) T cell-mediated resistance against distal and secondary tumors". Nanomedicine: nanotechnology, biology, and medicine. 2014; 10 (6): 1273-85).

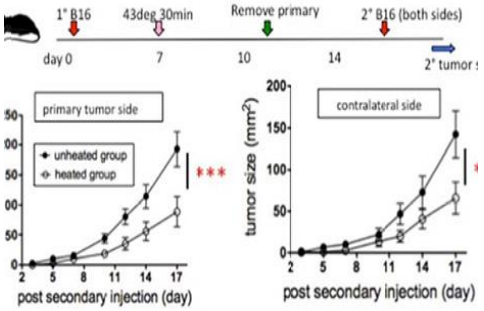


Figure 9

Pathology and Immunopathology

We have used a variety of anatomic (H&E) and immunohistochemical pathology stains and markers to identify the inflammatory and immune cells that infiltrate the tumor and prei-tumor region post treatment. Below are examples such tissues following hypofactionated radiation treatment. Figures 10 and 11 represents pro-apoptotic BAX and cleaved caspase in irradiated murine melanoma tissue. Figures 12 and 13 are high magnification H&E and IHC (CD11c) photomicrographs, for irradiated tissue, showing the preponderance of CD11c tagged lymphocytes (fig. 13). Morphologically, the lymphocyte population is compatible with an abundance of B-cell and a lesser number of T-cells. Although CD11c is typically viewed as a dendritic cell (DC) marker, new information suggests it is also a marker of activated lymphocytes in some immune situations.

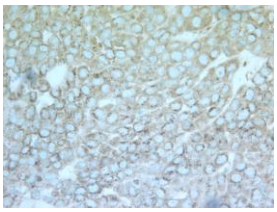
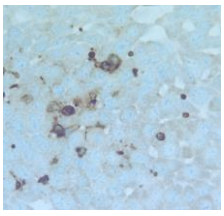


Figure 10



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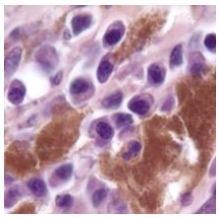

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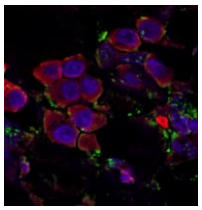

Figure 13

Assessment of Pathobiological and Therapeutic Markers

In addition to the qualitative and quantitative histopathological and immunohistochemistry assessment described above, western blot assessments of tumor and peri-tumor tissue, following radiation or mNP hyperthermia are used to identify and semi-quantify potentially important therapuetic and immune proteins. Preliminary results, Figure 8 and 9 below, suggests apoptosis markers (caspases), heat shock proteins, calreticulin, CD47 and MTA1 are potentially important proteins in the radiation- hyperthermia- abscopal/immune-pathway.

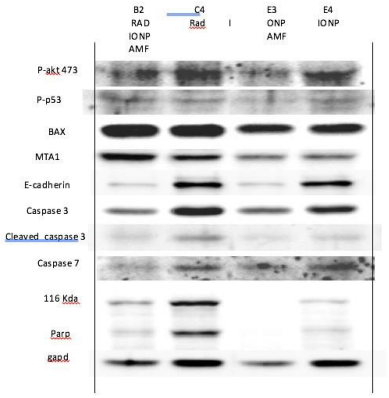

Figure 14

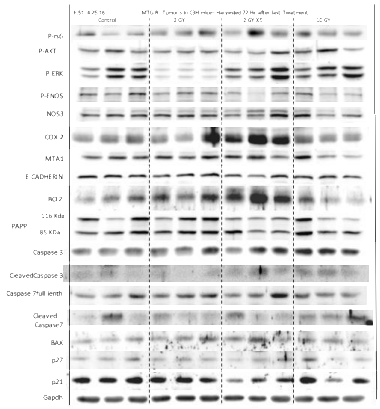

Figure 15

RESULTS

Hypo-fractionated radiation and magnetic hyperthermia treatment of murine tumors: Our studies have shown these modalities not only provide basic therapeutic benefit alone and in combination but also create anti-tumor immune based activity in the tumor tissue. This effect is demonstrated by the western blot/protein analysis exampled above. To date our primary cell death and immune targets include apoptosis markers (caspases), heat shock proteins, calreticulin, CD47 and MTA1.

Magnetic nanoparticle hyperthermia and the immune/abscopal effect: Our studies have demonstrated that even relatively low doses of mNP hyperthermia are capable of creating a systemic anti-tumor response.

Radiation and the immune /abscopal effect: To date we have not demonstrated a clinical/tumor response immune or abscopal effect using hypofractionated radiation. However, quantitative/qualitative conventional and immunohistochemistry pathology studies and protein analysis studies demonstrate the presence of post treatment immunogenic proteins in tumor and peri-tumor tissue.

CONCLUSIONS

In these preliminary study we have shown both specific hypofractionated radiation and magnetic nanoparticle hyperthermia in murine tumors have the ability to not only be clinically effective but to stimulate immunological activity. This study is too limited in size, scope, and statistics to make conclusive determinations regarding the treatment efficacy and abscopal effect contributions of each modality. However, it can be clearly stated that these cancer treatment modalities all have significant independent therapeutic responses, as proven by prolonged disease free survival, and that this effect is markedly enhanced through their combined use. The study also demonstrates that a clinical anti-tumor immune response, resulting from these treatment modalities, is likely to be a major contributor to overall therapeutic efficacy.

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