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Enhancing radiotherapy for lung cancer using immunoadjuvants delivered in-situ from new design radiotherapy biomaterials: a preclinical study

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Abstract

Studies show that radiotherapy of a primary tumor in combination with immunoadjuvants (IA) can result in increased survival or immune-mediated regression of metastasis outside the radiation field, a phenomenon known as abscopal effect. However, toxicities due to repeated systematic administration of IA have been shown to be a major obstacle in clinical trials. To minimize the toxicities and prime a more potent immune response, Ngwa et al. have proposed that inert radiotherapy biomaterials such as fiducials could be upgraded to multifunctional ones loaded with IA for in-situ delivery directly into the tumor sub-volume at no additional inconvenience to patients. In this preliminary study, the potential of such an approach is investigated for lung cancer using anti-CD40 antibody. First the benefit of using the anti-CD40 delivered in-situ to enhance radiotherapy was tested in mice with subcutaneous tumors generated with the Lewis Lung cancer cell line LL/2 (LLC-1). The tumors were implanted on both flanks of the mice to simulate metastasis. Tumors on one flank were treated with and without anti-CD40 and the survival benefits compared. An experimentally determined in-vivo diffusion coefficient for nanoparticles was then employed to estimate the time for achieving intratumoral distribution of the needed minimal concentrations of anti-CD40 nanoparticles if released from a multifunctional radiotherapy biomaterials. The studies show that the use of anti-CD40 significantly enhanced radiotherapy effect, slowing the growth of the treated and untreated tumors, and increasing survival. Meanwhile our calculations indicate that for a 2 to 4 cm tumor and 7 mg/g IA concentrations, it would take 4.4 to 17.4 days, respectively, following burst release, for the required concentration of IA nanoparticles to accumulate throughout the tumor during image-guided radiotherapy. The distribution of IA could be customized as a function of loading concentrations or nanoparticle size to fit current Stereotactic Body Radiotherapy schedules. Overall, the preliminary results support ongoing work in developing multifunctional radiotherapy biomaterials for in-situ delivery of immunoadjuvants such as anti-CD40 to leverage the abscopal effect, while minimizing systemic toxicities. The potential of extending such an approach to other cancer types is discussed.

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Keywords

Abscopal Effect; SBRT; Nanoparticles; Immunoadjuvants; Multifunctional Radiotherapy Biomaterials

Introduction

Radiotherapy is employed in the treatment of over 50% of cancer patients, either alone or in combination with other approaches. In 1953, Mole described the abscopal effect (Golden *et al.*, 2013; Tang *et al.*, 2014), whereby radiotherapy at one site may lead to regression of metastatic cancer at other sites, which were not irradiated. Recent studies indicate that the abscopal effect is mediated by the immune system (Demaria *et al.*, 2004); however, immune tolerance mechanisms hamper the development of therapeutically effective responses (Golden *et al.*, 2013). A combination of radiotherapy and immunoadjuvants (IA) could overcome immune-suppression and lead to a vigorous anti-tumor T cell responses (Golden *et al.*, 2013; Tang *et al.*, 2014). However, while such combinations are promising, their systemic/overlapping toxicities are a major obstacle reported in many studies (Tang *et al.*, 2014).

Towards overcoming these toxicity limitations, Ngwa et al. (Ngwa and Ngoma, 2016) have proposed a potential new approach whereby the IA is administered *in-situ* directly into the tumor sub-volume from multifunctional/smart radiotherapy biomaterials (e.g. fiducials) loaded with the IA. Such smart radiotherapy biomaterials have previously been investigated for *in-situ* delivery of radiosensitizers (Ngwa *et al.*, 2014; Cormack *et al.*, 2010). A similar approach could be adopted whereby radiotherapy biomaterials are coated with a biodegradable polymer incorporating a payload of the IA or IA nanoparticles in a biodegradable polymer matrix. Once in place, the new design radiotherapy biomaterials can be programmed to release the payload *in-situ*, directly into the tumor, as the polymer degrades. During the course of radiotherapy, antigens from the dying tumor cells will serve as an *in-situ* vaccine, which can work together with the released IA to potentiate the immune-mediated abscopal effect (Golden *et al.*, 2013; Tang *et al.*, 2014). This effect can enable killing of metastatic cells, distant from the irradiated site (Golden *et al.*, 2013). It could also lead to the generation of immunologic memory, to prevent cancer recurrence (Tang *et al.*, 2014).

A major advantage of the proposed approach employing new design smart radiotherapy biomaterials for *in-situ* administration of IA is that it will allow direct and sustainable delivery of the IA into the tumor sub-volume, significantly minimizing systemic/overlapping toxicities, which are currently a critical barrier/concern with competing approaches (Golden *et al.*, 2013; Tang *et al.*, 2014). This takes into account the fact that slow/sustainable *in-situ* release of IA like anti-CD40 antibody leads to minimal toxicities as shown recently (Fransen *et al.*, 2014; Sandin *et al.*, 2014a; Sandin *et al.*, 2014b). Since radiotherapy biomaterials are used as the delivery vehicle, this approach would also come at no additional inconvenience to the patient. In this work, we carried out theoretical investigations to determine the feasibility of this *in-situ* IA release approach customizable to radiotherapy schedules.

Preliminary in-vivo studies were performed in order to verify that in-situ delivery would enhance radiotherapy outcomes as anticipated, for lung cancer mouse models. Lung cancer is a leading cause of cancer death and a site where the smart radiotherapy biomaterials could replace currently used inert biomaterials such as fiducials.

Methods and Materials

Experimental

Mouse Lewis Lung cancer cell line LL/2 (LLC-1) were obtained from American Type Culture Collection (ATCC) and cultured with Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and 1% penicillin/streptomycin. Anti CD40 antibody (FGK 4.5) was purchased from BioXcell. B57BL/6NTac background wild type male mice were bought from Taconic Bioscience Inc. to generate a mouse lung cancer metastatic model, 10 weeks old mice were implanted by subcutaneous injection in both flanks with 1×10^5 mouse Lewis Lung cancer cell line, LL/2 (LLC-1) suspended in PBS. On day 22 (3 weeks after tumor challenge) mice were randomized into different cohorts. Four mice were used for each cohort. One cohort served as control, no treatment. A second cohort was treated with 6 Gy of radiation only, a third cohort was treated with only anti CD40 antibody one dose (20 μ g/tumor), a fourth cohort was treated with only anti CD40 antibody 3 doses on consecutive days (6.66×3 μ g/tumor). Finally, another cohort was treated with 6 Gy radiation + anti CD40 antibody 3 doses on consecutive days (6.66×3 μ g/tumor). In treatments, only tumor on the left flank of the mice were treated, while the right side tumors were not treated. A Small Animal Radiation and Research Platform (SARRP) was used for image-guided radiation therapy at 220 kVp and 13 mA. Tumor volume was measured 2 times/week and a survival assay was performed. All mice were maintained in the Dana Farber Cancer Institute Animal Core Facility following Institutional Animal Care and Use Committees (IACUC) approved protocol.

The SARRP employs an isocentric design for computed tomography (CT) imaging and irradiation (Armour *et al.*, 2010; Wong *et al.*, 2008) using the same source operating up to 225 kVp. The X-ray source is mounted on a gantry, with a source-to-isocenter distance of 35 cm. A robotic stage is used to position the small animal subject. CT is achieved by rotating the animal about the vertical axis between a stationary x-ray source at 90° gantry position and a two-dimensional 20×20 cm² (1024×1024 pixel) amorphous Si flat-panel detector. All irradiations were performed at 220 kVp, 13 mA with 0.15 mm Cu filter in place, while all CT imaging was done at 65 kVp, 0.5 mA and 1 mm Al filter.

Theoretical

Three fiducial markers are typically used during image-guided radiotherapy for lung cancer (Collins *et al.*, 2007). For simplicity, in this study, we assumed that one of those fiducials, would be a smart fiducial, loaded with IA as illustrated in Figure 1. A solid tumor was modeled as a spherical shape with tumor draining lymph nodes (TDLNs) located in the vicinity of the tumor outer surface. For simplicity we simulated a scenario where smart radiotherapy fiducial is implanted in the center of the tumor. In our model, TDLNs are

considered to be part of the antitumor immunological processes which will amplify the abscopal effect.

Assuming a burst release of the IA nanoparticles (NP) from the new design fiducial, the diffusion of the NPs, given by the concentration $C(x, t)$, as a function of distance (x) from the fiducial over time (t) can be calculated by applying the following equation employed in previous experimental studies of intratumoral diffusion of NPs (Wong *et al.*, 2011):

$$C(x, t) = C_i \left(1 - \operatorname{erf} \left(\frac{x}{2\sqrt{Dt}} \right) \right)$$

Equation above is a one-dimension solution to Fick's second law of diffusion under the given boundary conditions that the concentration of IA NPs is 0 mg/g at infinity prior to the release, whereas the concentration of NPs at the interface of the tumor and smart fiducial is C_i . Two sample nanoparticles concentrations of 7 mg/g and 18 mg/g NPs were investigated, because previous studies have employed these loading concentrations for other nanoparticles (Cho *et al.*, 2009; Hainfeld *et al.*, 2004; Hainfeld *et al.*, 2006; Ngwa *et al.*, 2010; Jain *et al.*, 2012). Here D is the isotropic diffusion coefficient. An experimentally determined in-vivo nanoparticle diffusion coefficient D , of $2.2 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for 10 nm nanoparticles (Wong *et al.*, 2011), was used in analytic calculations. As described in our recent study (Sinha *et al.*, 2015), Stokes-Einstein equation was employed to estimate D for other sizes of nanoparticle (2 and 6 nm). The consequences of the assumptions of isotropic diffusion and this model are discussed below in the discussion section.

The IA investigated here was anti-CD40 antibody (CP-870,893). In vitro data suggest that at least 3.5 µg/ml of anti-CD40 antibody is required to engender an effective immune response (Small *et al.*, 2007; Fan *et al.*, 2012). By diffusion throughout the tumor, the minimum concentration of anti-CD40 antibody is expected to reach antigen presenting cells (APCs) anywhere in the tumor volume. The APCs which take up the neo antigens from irradiated tumor cells can then be activated by the anti-CD40 antibody. Those activated APCs usually migrate to the draining lymph nodes via the immense network of lymph vessels. For different NPs concentrations and tumor sizes, we estimated the time for achieving the concentrations of anti-CD40 antibody that are needed to engender the abscopal effect during radiotherapy. Conversely, assuming a typical 2-3 weeks patients waiting time before the beginning of SBRT treatment (Ohta *et al.*, 2013), we investigated the minimal concentration of anti-CD40 antibody that should be loaded in the smart fiducial to ensure the minimal concentration of IA reaches the whole tumor. This was investigated as a function of tumor size.

Results

Experimental

The purpose of the experimental study was to first establish that in-situ delivered anti-CD40 would enhance the radiotherapy treatment outcomes. This then justifies further investigation of the innovative approach to potentially replace currently used radiotherapy fiducials with the smart multifunctional radiotherapy biomaterials. As anticipated, Figure 2 shows that use

of anti-CD40 significantly enhances radiotherapy resulting in the delay in tumor growth relative to when using radiotherapy alone. The untreated tumors on the right flank also showed slowed growth reminiscent of an abscopal effect. The slowed tumor growth results are consistent with the increased survival shown when using a combination. Altogether, the results suggest that in-situ delivery of IA e.g. from smart radiotherapy biomaterials would increase survival for lung cancer models.

Theoretical

The IA diffusion profiles for different nanoparticle sizes are shown in Figure 3. Our results (Figure 3 (a)) indicate that for a 2 to 4 cm tumor and 7 mg/g concentration of 2 nm nanoparticles it would take 4.4 to 17.4 days, respectively, following burst release, for the required concentration of IA nanoparticles to accumulate throughout the tumor during SBRT. If the loading concentration is increased to 18 mg/g (Figure 3 (b)), it will take 3.9 to 15.2 days for 2 to 4 cm tumor, respectively. As expected, as the nanoparticle size increases, significantly more time is needed as shown by the results for 6 nm and 10 nm NPs (Figures 3 (a) & 3 (b)). These findings highlight the ability to customize the IA distribution as a function of loading concentration and/or nanoparticle sizes when planning for the different tumor sizes at a given distance from a fiducial. The antibody concentration at certain distance away from fiducial is also observed to increase with increasing time of diffusion, consistent with expectations. Experimentally obtained diffusion coefficient prepared by (Pluen *et al.*, 2001; Netti *et al.*, 2000) using tumor implanted in mouse dorsal chambers: human colon adeno-carcinoma LS174T and human soft tissue sarcoma HSTS-26T were applied to verify the results. The diffusion coefficient as for HSTS26T for 10 nm particle agrees with the calculated value used in this paper.

Meanwhile, Figure 4 results indicate that for patients with a 2 to 4 cm tumor, who typically would begin SBRT 14 days after fiducial implantation, 0.07 to 33.10 mg/g of 2 nm NPs are needed at the interface of tumor and smart fiducial, respectively, for the required concentration of IA to cover the whole tumor during SBRT. For 21 days, 0.03 to 2.26 mg/g of NPs are needed. To achieve the minimal concentration throughout the tumor, the amount of IA that needs to be loaded in the smart fiducial decreases with increase of the therapeutic time window e.g. increasing from 14 days to 21 days. For increasing tumor sizes, the required amount of antibody to be loaded also increases.

Discussion

Overall, our results predict that the delivery of IA payloads from new design radiotherapy biomaterials or smart fiducials, could enhance radiotherapy at no additional inconvenience to cancer patients. The delivery could also be customized for sustained release to fit current radiotherapy schedules. Fransen and colleagues have previously investigated the biological effects of anti-CD40 antibody and anti-CTLA-4 antibody therapy in mice model without radiotherapy (Fransen *et al.*, 2013; Fransen *et al.*, 2011). Low dose of antibody and Montanide ISA 51 emulsions were injected close to the tumor, to achieve a slow release of the antibody and lower level of antibody in the blood compared to systemic injection. Their study showed that slow release of low doses of antibody can trigger a systemic anti-tumor

immune response and further eradicate tumor cells at a distant site, while limiting systemic toxicity.

Our approach innovatively considers the delivery of the IA such as anti-CD40 antibody using smart fiducials loaded with the IA e.g. by coating currently used fiducials with a biodegradable polymer like PLGA containing the payload of IA. It is also possible to load the IA along with gold nanoparticles (GNP). Because gold nanoparticles are biocompatible, can serve as radiosensitizing agent (Ngwa *et al.*, 2014; Hainfeld *et al.*, 2008; Hao *et al.*, 2015) and can be easily functionalized with IA (Cao-Milan and Liz-Marzan, 2014), the GNP could be used as carriers for IA in our model. Our previous studies have highlighted the feasibility of loading radiotherapy biomaterials with GNP (Ngwa *et al.*, 2014). The current study highlights the potential for also employing a combination of IA and GNP as the payload, delivered using smart fiducials for current radiotherapy schedules.

During radiotherapy, the released GNP could substantially enhance local tumor cell kill as shown in our recent studies (Ngwa *et al.*, 2014; Hao *et al.*, 2015). Such highly localized radiation boosting may be achieved while reducing the primary radiation dose to healthy tissue, hence minimizing normal tissue toxicity. Meanwhile the irradiated tumor cells from localized boosting would serve as an *in-situ* ‘vaccine’ working in conjunction with the IA to leverage the abscopal effect (Figure 1). Compared to competing approaches, the slow *in-situ* release would help minimize systemic toxicities and would come at no additional inconvenience to cancer patients. Altogether, the highly localized tumor cell kill from GNP with minimal toxicity to normal tissue, combined with the immune-mediated distant/metastatic cell kill (with minimal systemic toxicity) proffers a potentially transformative SBRT approach which could allow treatment of both local and metastatic disease.

The diffusion model used in this study assumes isotropic diffusion. Due to the complexity of tumor microenvironment (Jain and Stylianopoulos, 2010) and immune system, IA diffusion would likely not be isotropic. However, the current study serves as a useful first step or reference for further studies with models incorporating more complexity. Also some of the anti-CD40 antibody may be eliminated during diffusion e.g. via activation of the APCs which drive anti-tumor T cell-mediated immune responses (Vonderheide and Glennie, 2013; Khong *et al.*, 2012). Hence the distribution of available IA needs to further take this into consideration. Thirdly, in our model calculations, it is assumed that the viscosity coefficient of 10 nm NP is same as that for other sizes of NPs investigated. However, as highlighted in previous studies, for smaller size nanoparticles e.g. 2 nm, the consequence of this assumption is not significant (Thomas, 1965). For more perspective, we also investigated the diffusion using an instantaneous source solution model as described by Balluffi et al (Balluffi *et al.*, 2005). The results (not shown) using this alternative model are consistent with our findings that the delivery of IA is also feasible for current SBRT schedules.

The calculations in this work did not consider the time required post implantation before burst release of the IA. Previous studies (Ngwa *et al.*, 2014) have indicated that this can be customized e.g. by varying the PLGA polymer weighting. The current results provide perspective on the time window needed for customization or programming the burst release taking radiotherapy schedules into account.

In preclinical studies, successful results have already been obtained by combining radiotherapy with IA. Dewan and colleagues showed that anti-CTLA-4 hamster monoclonal antibody (9H10) in combination with radiotherapy could achieve enhanced tumor response at the irradiated site, and could also lead to a significant suppression of the tumor outside the radiation field (Dewan *et al.*, 2009). In their study, treatment with 9H10 as single modality had no effect on either primary or secondary tumors. Although, radiotherapy alone had substantially inhibited the growth of the primary tumor, it had no effect on secondary tumors. By combining radiotherapy and 9H10, significant secondary tumor response was achieved (Dewan *et al.*, 2009). Another study combining 9H10 with radiation conducted with a model of poorly immunogenic metastatic mouse mammary carcinoma 4T1 reported the similar results and suggested the combination treatment was inhibiting the metastases (Demaria *et al.*, 2005). Furthermore, clinical trials have reported cases of the abscopal effect in patients when combining IA and radiotherapy (Golden *et al.*, 2013; Postow *et al.*, 2012; Hiniker *et al.*, 2012; Kodama *et al.*, 2013; Slovin *et al.*, 2013). However, in these studies, rates of immune-related adverse events were high (Tang *et al.*, 2014), and therapeutic doses are limited by normal tissue toxicity. We expect that our approach using slow release of IA from smart fiducials will help minimize such adverse effects.

Systemic tumor specific immune response seems to rely on the radiation regimens. Optimal radiation or IA dose and the delivery schedule remain to be defined for different types of tumors. It has been shown that, hypofractionation radiotherapy like SBRT has more potential than conventional radiation therapy to induce the abscopal effect (Dewan *et al.*, 2009; Schaue *et al.*, 2012). For example, using a B16 mouse melanoma model, Lee et al. showed that a single dose of radiation (20 Gy) generated stronger antitumor T-cell response, while the fractionated radiation (5 Gy $\times$ 4 fractions over 2 weeks) showed less tumor growth inhibition (Lee *et al.*, 2009). However, in order to induce an effective immune response, more studies are needed to determine the optimal fractionation (Formenti and Demaria, 2013).

Conclusion

Overall, our results demonstrate potential for combining radiotherapy and immunotherapy using new design smart or multifunctional radiotherapy biomaterials. These new biomaterials could significantly enhance radiotherapy treatment of lung cancer or other sites where inert radiotherapy biomaterials (fiducials, beacons, etc.) are currently routinely used in the clinic. The results justify further investigations developing smart radiotherapy biomaterials towards clinical translation. If successful this has potential to transform radiotherapy practice, extending its use from mainly the treatment of localized tumors to include treatment of metastasis with minimal collateral damage, or systemic toxicities compared to current approaches.

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Figure 1 (a)

Figure 1 (b)

Figure. 1. (color online). Schematic diagram outlining the antitumor activity and abscopal effect in combining radiation with slowly/sustained released IA (Fig. 1 (a)). Radiation induces tumor cell death. And the dying tumor cells release tumor antigens. Antigen presenting cells (APCs) take up and process the antigens into peptides that can be loaded into the major histocompatibility complex (MHC) class. This is then recognized by T cells, and promote antigen specific T cells activation and proliferation. Activated CD8 T cells migrate to both irradiated tumor and off-field distant metastatic sites. Schematic of smart radiotherapy biomaterial loaded with IA nanoparticles (Fig. 1 (b)).

Figure. 2. (color online). The abscopal effect is induced in Lung tumor-bearing mice by single dose radiation in combination with the IA anti-CD40 antibody. Line graphs shows (Fig. 2(a)) tumor growth delay of primary treated tumors, (Fig. 2(b)) secondary treated tumors and (Fig. 2(c)) survival of mice. Dash line indicate starting of treatment, error bars indicate SEM., and **, $P < 0.01$, showing significance of the treatment of RT and anti-CD40 with the combinations.

Figure. 3.

(color online). Showing the results for antibody (Ab) concentrations as a function of diffusion time for three different kind of nanoparticle sizes for both 2 and 4 cm diameter tumor. (Fig. 3 (a)) with constant concentration of 7 mg/g at the interface of smart fiducial and tumor. (Fig. 3 (b)) with constant concentration of 18 mg/g (Fig. 3 (b)) and (Fig. 3 (c)) with experimentally obtained diffusion coefficient for comparable collagen content in two different type of tumors LS174T/MCAIV/U87cw and HSTS26T.

Figure. 4. (color online). For the purpose of achieving minimal Ab concentration to saturate APCs throughout different sizes of tumors after 14 days and 21 days, corresponding constant concentrations that needs to be kept at the interface of tumor and fiducial were calculated.