Radiation increases BTB permeability in a preclinical model of breast cancer brain metastasis.

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Research

Keywords: Dosimetry, Pharmacokinetics, Central Nervous System, Abscopal Effect, Metastases, Radiotherapy, Leaky, Gafchromic Film, Efflux, Preclinical

Posted Date: February 12th, 2020

DOI: <https://doi.org/10.21203/rs.2.23290/v1>

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Abstract

Background

Brain metastasis is a devastating stage of cancer progression, occurring in ~30% of metastatic breast cancer patients. Two-year survival rates for these patients is low, and most typically survive less than one year. Treatments for these women are limited by the blood-brain barrier, but include cytotoxic chemotherapy, surgical resection, and radiation therapy (whole-brain radiotherapy or stereotactic radiosurgery). Radiotherapy is considered to be capable of inducing disruption of the blood-brain barrier and eliciting an abscopal response to extracranial tumors.

Methods

A combination of ionization chamber and Gafchromic film dosimetry was used to commission and determine dose outputs for our experimental design. Dose deposition in-vivo was verified by immunohistochemistry. To evaluate the effects of ionizing radiation at the normal blood-brain barrier and the blood-tumor barrier, athymic nude and FVB mice were used. Athymic nude mice were injected with MDA-MB-231Br cells. Lesions were allowed to develop for ~28 days. Mice were then irradiated at the prescribed dose. Prior to tissue collection, mice were injected with Texas red, followed by a vascular washout with physiological buffer. Fluorescence in normal and diseased brain was quantified by fluorescent microscopy.

Results

Using a 10mmx10mm collimator, determined to have adequate field homogeneity as determined by Gafchromic film analysis, we were able to successfully treat a single hemisphere in mice. The blood-brain-barrier remained undisrupted in athymic nude mice at doses up to 12Gy compared to untreated brain and radiation naïve controls. Immune competent FVB mice treated with radiation showed significant blood-brain barrier disruption at a dose of 12Gy only. The blood-tumor barrier showed significant disruption at 24hrs following radiation treatment (6 or 12Gy).

Conclusion

Our study demonstrated that radiation therapy disrupts the blood-tumor barrier, but fails to disrupt the normal blood-brain barrier in athymic nude mice. However, in FVB immune competent mice, the blood-brain barrier was disrupted at a dose of 12Gy, suggesting an abscopal-like response impacts extent of barrier leakage.

Introduction

Breast cancer is the most common cancer diagnosis among women in the United States, affecting nearly one in every eight women, resulting in up to 270,000 new diagnoses each year (1). Of these women, up to 30% are at risk for development of brain metastases during their lifetime (2,3). After diagnosis with an

intracranial lesion survival is poor with only one in five women surviving longer than one year post diagnosis (4). In triple negative, or basal like, breast cancer (TNBC) up to 30% of women are likely to develop brain metastases at some point in their lifetime (5,6). Treatment typically includes a combination of radiation, chemotherapy and or surgical resection (7,8). In general drugs for TNBC are limited to cytotoxic chemotherapies, due to the lack of any receptor targets (estrogen, progesterone, and HER2 receptors) (9).

One reason for the overall treatment failure in patients with brain lesions is the presence of the blood-brain barrier (BBB) (10–12). The BBB is an anatomically unique, physicochemical vascular barrier which forms the interface between blood system and brain (13,14). Under normal physiological conditions the tight junction sealing of BBB endothelia precludes paracellular passive diffusion of most solutes into brain parenchyma. While lipophilic molecules may diffuse across the cell membranes, and generally do not rely on paracellular diffusion, active efflux transport pumps, including P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP; ABCG2), and multidrug resistance protein-1 (MRP1; ABCC1) (15–19) actively extrude solutes to the luminal side of the BBB. In the context of a brain tumor normal components that surround the BBB, such as astrocytes and neurons are displaced by cancer cells resulting in a leaky vascular barrier, known as the blood-tumor barrier (BTB). While paracellular diffusion is generally higher at the BTB, we have shown previously that it is compromised, the BTB still prevents numerous chemotherapeutics from reaching cytotoxic concentrations in 90% of all brain metastasis lesions (11).

Current standard of care for brain metastasis of breast cancer usually includes radiation therapy, which may be delivered differently depending on cancer progression and patient status. For a single solitary lesion, the tumor will be resected if operable, and a dose of radiation can be delivered to the resection cavity by (stereotactic radiosurgery) SRS or postoperatively via whole brain radiotherapy (WBRT) to reduce the risk of local and regional recurrence. For patients with a limited number of small intracranial masses (< 3 cm), SRS can be used (20). Some experts suggest the use of additional, or boost, WBRT following SRS. However, no differences in overall survival have been observed in the data reported in clinical trials comparing the two modalities (21–25). The use of SRS for 5 or more metastases has been investigated as a stand-alone approach or with the use of WBRT in addition to SRS (26–28). The results from this work are ongoing, but it appears that omitting WBRT may result in increased incidence of distant brain failure and recurrence. Despite the amount of research conducted regarding treatments involving radiation therapy, complications such as neurocognitive decline and local/distant recurrence are unsolved.

While these therapies provide efficacy and may reduce central tumor progression, it has been reported that it may also increase the permeability of the BBB (10,29). However, the timing and magnitude of the BBB and BTB permeability changes are not defined well and remain in some debate in the current literature (10,29). Several groups have reported permeability changes up to 24hrs following radiation therapy, while others suggest that any changes occur at later time points. Other reports have not been able to document increases in permeability following radiation treatments (30–38). Clinically,

neurological effects with radiation-induced BBB permeability changes have been segregated into two categories – acute (i.e., initial 24hrs), and those described thereafter, usually weeks to months (39–42).

Based upon the clinical relevance of the therapy, and the relative lack of clarity regarding the effects of radiation on the BBB, we developed a system for brain irradiation in a preclinical model of breast cancer brain metastasis using clinical radiotherapy protocols. Using this model, we quantified the pharmacokinetics of tracer accumulation across the BBB and BTB in a time and size dependent fashion. We observed increased permeability of the BTB at both 8 and 24hrs following radiation therapy in our immune-compromised preclinical metastasis model and immune competent model. While there was no BBB disruption in athymic Nu/Nu mice, we did observe increased permeability in immune competent mice. This data suggests that radiation increases the permeability of the BTB and normal BBB with a competent immune system and provides a platform for the study of the mechanism by which this increased permeability occurs.

Methods

Cell Culture

Brain tropic, human triple negative breast cancer cells, transfected to express firefly luciferase (MDA-MB-231Br-Luc), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). MDA-MB-231Br-Luc breast cancer cells were kindly provided by Dr. Patricia Steeg of the National Cancer Institute of Health, Center for Cancer Research.

Development and Optimization of a Half Brain Irradiation Protocol

To confirm the dose output given by the manufacturer's commissioning of our XenX small animal irradiator (Xtrahl, Suwanee, GA) a Farmer® ionization chamber was placed at a depth of 2 cm in a solid water commissioning phantom setup and irradiated at 220KeV and 13.0 mA for one minute for each of the various conditions required for correction factors as outlined in the Task Group 61 protocol released by the American Association of Physicists in Medicine (43). The dose output at isocenter, with a source to surface distance (SSD) of 33 cm and an open radiation field filtered with a 0.15 mm copper filter was 3.62 Gy/min. This dose rate was used as a reference to irradiate a set of EBT3 Gafchromic calibration films at doses ranging from 1 to 20 Gy at a depth of 2 cm in the same solid water phantom setup. These films were utilized to obtain a standard curve depicting the optical densities of known doses. To determine the dose rate, field homogeneity, and size of our radiation beam collimated with a 10 × 10 mm collimator using our custom 3D printed mouse restraint, EBT3 Gafchromic films were irradiated at 0.5 cm depth in solid water with an additional 1 cm of solid water below the film to allow for appropriate buildup and backscatter.

EBT3 Gafchromic Film Analysis

Films were scanned using an Epson (Suwa, Japan) Perfection 4870 flatbed photo scanner in professional mode without color correction at a resolution of 72dpi. Images were analyzed using the red channel on ImageJ software for all films. Blank, non-irradiated films were also scanned to minimize background for each set of films scanned. All films were scanned at least 24hrs following irradiation exposure (43). Optical density (OD) was defined as follows (44):

$$netOD = \log_{10} \frac{I_{unexp}}{I_{exp}} \text{ (Equation 1).}$$

To determine dose homogeneity in films irradiated using the 10 × 10 mm collimator, the line function was used to determine the dose at each point along the line. For each point OD was calculated.

Histological Confirmation of Dose Deposition and Absolute Positioning

Naïve female FVB mice were irradiated through the right cranial hemisphere with a single dose of 15.5 Gy at dose rate of 2.7 Gy/min. Mice receiving a total dose of 15.5 Gy in one fraction is similar to the biological effective dose (BED) of mice receiving a total dose of 30 Gy in 10 fractions of 3 Gy with an assumed α/β ratio of 10, accounting for the biological effect being mitotic catastrophe and cell death in MDA-MB-231 breast cancer cells. The equation defining BED can be found below:

$$BED = nd \left[1 + \frac{d}{\alpha/\beta} \right] \text{ (Equation 2)}$$

Following treatment mice were anesthetized with ketamine/xylazine (100 and 8 mg/kg respectively) before being transcardially perfused with ice-cold 4% PFA. Mice were decapitated, brains were harvested and then post-fixed overnight in 4% PFA at 4°C. Following fixation, brains were then incubated sequentially in 10%, 20%, and 30% sucrose each for 24hrs. Brains were then co-embedded in 15% gelatin matrix, 6 brains per matrix, for bulk sectioning. The gelatin matrix was then processed sequentially in 4% PFA for 24hrs, 15% Sucrose for 24hrs, and 30% Sucrose for 48Hrs. The block was then trimmed and placed at -80°C for 30 minutes. Brains were then sliced in the coronal plane at a thickness of 30 μ m on a sliding microtome (HM 450, ThermoFisher Scientific, Waltham, MA) equipped with a 3 × 3 freezing stage (BFS-40MPA, Physitemp, Clifton, NJ) at -20°C. Sections were collected and immuno-stained in 6-well plates containing 0.06% sodium azide in PBS (45).

Sections were immunostained using a standard free-floating section protocol as described (45,46). Briefly, sections were blocked with PBS, methanol, and 30% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) and incubated on a shaker for 15 min. Sections were then washed three times and permeabilized for 30 min on a shaker with 1.83% lysine (Fisher Scientific, Pittsburgh, PA) in 1% Triton (Sigma-Aldrich, St. Louis, MO), and 4% heat-inactivated horse serum (Sigma-Aldrich, St. Louis, MO). Sections were then incubated for 24 h with anti- γ H2AX (Ser139; 1-500) primary antibody (Cell Signalling

Technology, Boston, MA) at room temperature, followed by a 2 h incubation with the appropriate secondary antibody at room temperature.

Metastatic Brain Tumor Model of Breast Cancer

MDA-MB-231Br-Luc cells (1.75×10^5) were injected intracardially into the left cardiac ventricle and allowed to develop into metastatic brain lesions for 21 days. Presence of CNS metastases was confirmed by bioluminescent imaging (BLI) on day 21 using the IVIS Spectrum CT imaging system (PerkinElmer, Waltham, MA). D-luciferin potassium salt (150 mg/kg; PerkinElmer) was administered intraperitoneally and allowed to circulate for 15 minutes for mice with MDA-MB-231Br-Luc metastases before capturing BLI signal. Mice were allowed to progress until substantial tumor burden was observed as indicated by BLI intensity (approximately 4 to 5 weeks).

Radiation Treatments

Mice were irradiated through a single cranial hemisphere, as to provide the contralateral hemisphere as an internal control for each mouse. Mice received varying doses ranging from 3 to 30 Gy in fractionation and up to 20 Gy in a single fraction. All radiotherapy treatments were delivered at a dose rate of 3.01 Gy/min using a 10 mm x 10 mm collimator adjusted to target the right hemisphere. At 8 and 24hrs following the final irradiation treatments, mice were collected and brain tissue was harvested as described above. Mice were euthanized via exsanguination during the vascular washout period while under deep anesthesia with ketamine/Xylazine (100 mg/kg and 8 mg/kg respectively). Brain tissue was harvested and flash frozen in isopentane (-80°C) in < 60 s. Brains were sectioned and mounted on glass slides and stored at -20°C until analyzed via fluorescent microscopy.

Qualitative and Quantitative Fluorescence Imaging

For all image acquisition, an Upright MVX10 Stereomicroscope (Olympus, Center Valley, PA) equipped with Hamamatsu ORCA Flash4.0 v2 sCMOS camera for fluorescence imaging, a 2x PlanApo (0.5NA) objective, and a DAPI/FITC/RFP/Cy5/Cy7 filter set. The GFP (excitation/band λ 470/40 nm, emission/band λ 525/50 nm and dichromatic mirror at λ 495 nm) filter was used to acquire images confirming half-brain dose deposition with increased γH2AX signal. Texas Red accumulation in brain metastases was determined by Texas Red sum intensity (SI) per unit area of brain lesion using the RFP filter (excitation/band λ 545/25 nm, emission/band λ 605/70 nm and dichromatic mirror at λ 565 nm). CellSens image analysis software was used to analyze images and quantitate Texas red accumulation. (47,48)

Data Analysis

Differences in permeability between treated and untreated lesions were compared using a student T-test (GraphPad® Prism 7.0, San Diego, CA) and were considered statistically significant at $p < 0.05$.

Results

EBT3 Gafchromic Film Dose Response

The calibration curve for the Gafchromic Film model used is shown in Fig. 1B, and used as a source of reference for dose delivered in all other film analyses. The points correspond to the mean $\pm$ standard deviation determined by use of Eq. 1. In the same graph, corresponding error bars are drawn, but are not visible because they are smaller than the symbols in the figure. The points were fit with a non-linear regression with an R^2 value of 0.9987. Representative images of irradiated films are shown in Fig. 1C-J. As shown, the films have a change in color (or optical density) as the dose of radiation increases.

Half Brain Irradiation Protocol and Histological Verification

It is important to identify the dose rate of each experimental design in case there are instances of change of dose rate from isocenter under open field conditions. To determine the dose output of our experimental design, films were irradiated at a depth of 2 mm in solid water placed on our custom restraint with the Gafchromic film at isocenter. Images of the film were repeated in triplicate (data not shown). The irradiated field size was consistent with the intentional square field size of 10 mm x 10 mm measured with calipers (data not shown). The irradiated field was in good agreement with predicted doses and demonstrated both horizontal and vertical beam uniformity as depicted in Fig. 2. A penumbra of $\sim$ 0.850 mm was observed for this treatment field, as defined by the region where the dose drops from 80% of the max dose deposited to 20% of the max dose.

To ensure the 10 mm x 10 mm field size was accurate and precise for single hemisphere irradiations, individual radiograms were taken of each individual mouse alone and then again with the collimator in place. Images were overlayed using ImageJ at an opacity of 70% as seen in Fig. 3B. Radiograms were taken under the alignment conditions in Fig. 3A. Our custom 3D printed mouse restraint ensures the placement of the collimated beam for each mouse given the lasers are aligned on the outside border of the right eye (y-orientation) and at the base of the ear (x-orientation) for each mouse. Further confirming targeting of our in-vivo treatments, anti- γ H2AX immunofluorescence was used to identify regions exposed to radiation. Figure 3C demonstrates the ability to precisely target a single hemisphere in the brain.

Radiation Therapy Does Not Affect Normal BBB Permeability in Athymic Nu/Nu Mice

To understand the effects of radiation therapy on normal BBB integrity in our preclinical model of breast cancer brain metastasis, mice were irradiated through the right cranial hemisphere at 3-12Gy in fractionation. Mice were euthanized 24hrs following the last radiation exposure and the brains were collected, sliced, and analyzed for TxRd accumulation. Compared to untreated hemispheres in mice that were not exposed to radiation of any dose, no significant increase in TxRd accumulation was observed at any dose, indicating that the BBB in athymic Nu/Nu mice retains its integrity 24hrs after radiation therapy (Fig. 4A-B). The accumulation of TxRd is reported as sum intensity divided by the area of interest (mm^2)

for each area. For mice that did not receive radiation therapy, TxRd accumulation was 4.12 ± 0.24 and in mice that received radiation therapy, the contralateral untreated hemisphere had a value of 4.076 ± 0.045 . Mice treated to at 3, 6, 9, and 12 Gy had accumulations of 4.17 ± 0.02 , 4.15 ± 0.02 , 4.08 ± 0.03 , and 4.10 ± 0.01 respectively.

Radiation Therapy Induced BBB Permeability at Low Doses of Radiation Therapy in Immune Competent Mice

In some patients the immune system elicits an abscopal effect in some patients treated with both radiation therapy and immunotherapy leading to synergistic outcomes. To ascertain the effects of radiation therapy on naïve mice with intact immune function, female FVB mice were irradiated through the right cranial hemisphere at doses from 6-30Gy in fraction identical to the fractionation schedule that the Nu/Nu strain mice received. Significant disruption of physiologically normal BBB was observed in mice treated to a total dose of 12 Gy ($p < 0.05$) and, in mice treated to a total dose of 6 Gy, an obvious increase was observed, although it was not significant (Fig. 4C,D). At higher doses of 18 and 30 Gy, there was no statically significant accumulation of TxRd in irradiated hemispheres compared to the contralateral untreated hemispheres. Means and standard deviations for the contralateral hemispheres, and hemispheres receiving 0, 6, 12, 18, and 30 Gy were 3.99 ± 0.13 , 4.08 ± 0.10 , 4.21 ± 0.02 , 3.88 ± 0.02 , and 3.87 ± 0.01 , respectively.

Radiation Therapy Disrupts the BTB and Increases Permeability at 8 and 24hrs Post Insult

To understand the effect of radiation therapy on the BTB in our preclinical model of breast cancer brain metastasis, mice were injected with MDA-MB-231Br brain tropic TNBC cells. After substantial tumor burden was measured (~ 4–5 weeks) mice underwent radiation treatments to total doses of 6 and 12 Gy. Following treatment at 8 and 24hrs mice were injected with the small (625 Da) passive permeability tracer TxRd. After a ten minute circulation period mice were euthanized, brains harvested, and sliced before analysis with a fluorescent microscope. Tumors in the irradiated regions were compared to contralateral, untreated hemispheres for total accumulation of TxRd per lesion size, reported in sum intensity/mm². For mice receiving 6 Gy, untreated tumors at 8 and 24hrs following treatment had accumulation of 4.697 ± 0.272 and 4.409 ± 0.284 respectively, while their treated counterparts had total accumulations of 4.846 ± 0.600 and 4.963 ± 0.777 at 8 and 24hrs respectively (Fig. 5A). For both time points, treated tumors had statistically significant more accumulation of TxRd compared to their untreated counterparts ($p < 0.05$). At the 12 Gy dose at the 8 hour time point, untreated and treated lesions had values of 4.239 ± 0.192 and 4.389 ± 0.125 respectively. The data was not significant (Fig. 5B). At 24 h following radiation treatment, values of 4.558 ± 0.379 and 4.798 ± 0.5404 were determined (Fig. 5B). Tumors receiving radiation therapy had significantly more accumulation of TxRd at 24hrs following

treatment ($p < 0.05$). Representative images of an untreated lesion with low permeability to TxRd and a treated lesions with high permeability to TxRd are shown in Fig. 5C,D.

Discussion

Several studies have investigated the effects of radiation on the BBB or BTB, all reporting different results concerning permeability of brain barriers (49–51). Additional disparities are observed between reports owing to the non-uniform, clinically dissimilar dosing schemes. In this study we validate a new experimental design using the commercially available XenX Small Animal Irradiator and observed increased BBB permeability to TxRed 24hrs following a total dose of 12 Gy in immune competent animals only. Moreover, we also saw increased permeability of the BTB following low to moderate doses of radiation at 8 and 24hrs following radiation treatment.

In this work, first we validated our experimental design through small field radiation dosimetry using a combined ionization chamber and EBT3 Gafchromic® film approach. A similar approach using an equivalent radiation system has been used previously (52,53). Multiple groups have used dose rate measurements in solid water phantoms, cross calibrated with EBT3 films to gauge doses delivered for a particular experimental setup (54). Herein the dose rate for our small animal irradiator (SAI) at isocenter and an open field was determined to be 3.62 Gy/min, consistent with dose rates for similar field sizes (52). The irradiated field demonstrated quality beam uniformity (Fig. 2) in comparison with our intended field size and had a penumbra, where dose deposition falls from 80% of the max dose to 20% of the max dose, measuring 0.850 mm. Measurement and outcomes of beam uniformity and field penumbra for our experimental design are comparable, but vary slightly from others reporting a beam penumbra of 0.40–0.41 mm (55) using a $10 \times 10 \text{ mm}^2$ field. While the beam penumbra is critical in small scale irradiation methodology, the intent of this work was to study the effect of radiation on tumors in a large treatment field consisting of half of the brain. For this purpose, a beam penumbra of < 1 mm would not deliver substantial dose to the region outside the intended field, nor would it prevent the intended field from receiving a significantly lower dose.

To translate from a dosimetric evaluation of our SAI and its beam characteristics, we transitioned to an in-vivo system. Using naïve female FVB mice and immunostaining, we were able to histologically verify successful irradiation of a brain hemisphere by increased γH2AX signal in the treated hemisphere (Fig. 3C). The use of anti- γH2AX staining to ascertain radiation damage, specifically double stranded DNA breaks, and field sizes in in-vivo systems has been established (52,56,57).

In order to understand the effects of WBRT on the normal brain and brain tumor vasculature, we modeled clinical dosing patterns to treat and ablate brain metastases. Patients are commonly prescribed a total dose of 30 Gy over 10 fractions (58,59). When fractionation schemes are used, it is critical to understand their translational relevance. One group (60) studied the effects of fractionated radiotherapy on the BBB and BTB in rats. While the dosimetry was well executed, the doses and fractionation patterns do not appear to match what is typically used in patients in the clinic. In a similar study (31), mice were treated

with a single fraction of 10 Gy. Interestingly, Zarghami et al. (56) limited doses to single fractions, but incorporated the use of a BED equation to demonstrate equivalence to clinical dosing parameters. Of note, changes in fractionation have shown little impact on tumor progression and survival (59).

However, when examining the effects of a treatment on the blood brain barrier, it is important to follow clinical parameters and understand the intent of the treatments. Our experiments were poised to examine the events following a radiation treatment intended to treat brain tumors. Doses outside of what are typically used in patients are not necessarily as translationally plausible as studies using methods employed in the clinic. Our findings are presented at low and moderate doses, but were given in the same 3 Gy fractions that would be continued to 30 Gy in the clinic.

In non-tumor bearing, healthy female Nu/Nu mice, the BBB was unaffected by radiation therapy at doses from 0-12Gy in fractions of 3 Gy at 24hrs following treatment (Fig. 4A). Contrary to our results, Wilson et al (30) demonstrated increased normal BBB permeability to a 4.4 kDa FITC dextran at 24 and 48hrs following radiation. However, this result was following a single exposure to a relatively large, 20 Gy dose of radiation. Using the BED equation, this equates to an effective dose that is greater than 1.5 times that of a total dose of 30 Gy over 10 fractions (61). Another study using a single dose of 20 Gy that used various sized FITC dextran molecules observed increased permeability peaking at 24hrs post-treatment. However, they observed no increases in normal BBB permeability following a dose of 5 Gy, which is much closer to the single fraction dose we used in our work (37). The differences in reported measurement of BBB permeability alterations following radiation therapy can be partially attributed to the large heterogeneity in the way the dose was delivered, i.e. high dose vs low dose or single vs multiple fractions.

While our results using athymic nude mice may conflict with reported data, experiments with mice bearing an intact immune system had a different outcome. When immune competent female FVB mice were used in the same experiment, we observed a significant increase in normal BBB permeability to TxRed 24hrs following a dose of 12 Gy, as well as an increased, albeit not significant, permeability change 24hrs following a dose of 6 Gy (Fig. 4C). It should be noted that in the previously discussed experiments, immune-competent rodent models were used (30,37). These results suggest an active role of the peripheral and CNS immune system in BBB regulation following radiation therapy. Increased cytokine expression has been observed following treatment with radiation (62–64). Specifically, TNF α , IL1 β , and IL6 have increased expression, similar to acute periods after neuro-immunological insults (65,66). Additionally, at a cerebral blood flow rate of 2 mL/min/g (67), immune cells traversing the cerebrovascular network will be exposed to a substantial dose of radiation, more than likely perturbing an inflammatory response. The damage associated molecular patterns released and innate immune cell cytokine production following radiation therapy could potentially amplify this immune response (10,68,69). All of the underlying inflammatory events following radiation treatments may result in a potential mechanism for BBB disruption in immune competent subjects.

Lastly we set out to determine the effects of WBRT on the vascular system within metastatic brain tumors. Our data indicated increased BTB permeability at both 8 and 24hrs following treatment with 6 Gy

of radiation in 2 fractions, while after 24hrs we saw increased BTB permeability following a dose of 12 Gy in 4 fractions (Fig. 5). This data is consistent with increased K_{trans} values (BBB permeability measured clinically) seen in quantitative DCE MRI in irradiated tumors at 24hrs post-irradiation (70). Broad beam radiotherapy also displayed increased BTB permeability in treated lesions (71). Tumor vasculature response has also been studied clinically. In 30 patients and 64 total lesions receiving WBRT or SRS, treatment with radiation increased permeability in initially low leaky tumors (72). However, in tumors that were already highly permeable, there were no significant increases in permeability. In opposition to what we have observed in this study, there have been observations of no permeability changes measured by MRI gadolinium enhancement (51), though a dose of 20 Gy over two fractions was given. While this is different from our study in terms of single fraction dose and fraction number, the BED is similar to that of a completed 30 Gy in ten fractions. For a better visualization of how our results align with concluded studies, pertinent data available in the literature for both preclinical and clinical experiments are organized in table 1.

Conclusions

In summary, this study was able to provide a means of commissioning for our SAI similar to that detailed by previous work. Additionally we were able to provide a method for targeted, reliable, and reproducible brain irradiation without the need for expensive onboard CT equipment. Finally we evaluated permeability at both the BBB and the BTB following radiation therapy with doses of clinical importance. Moving forward, this platform will serve for continued evaluation of brain barriers and their pathophysiology following irradiation, but also to be used as a therapeutic tool in preclinical cancer approaches. Moreover, the difference in normal BBB integrity in different strains of mice with or without an intact immune suggests an abscopal-like response to radiation.

Abbreviations

- BBB
- Blood-brain barrier
- BTB
- Blood-tumor barrier
- WBRT
- Whole-brain radiotherapy
- SRS
- Stereotactic radiosurgery
- TNBC
- Triple negative breast cancer
- HER2
- Human epidermal growth factor receptor 2
- P-gp

P-glycoprotein
BCRP
Breast cancer resistance protein
MRP1
Multidrug resistance protein 1
SAI
Small animal irradiator
OD
Optical density
BED
Biological effective dose
TxRd
Texas red

Declarations

Ethics Approval and Consent to Participate

All animal handling and procedures were approved by Institutional Animal Care and Use Committee at West Virginia University in Morgantown, West Virginia (Protocol number 16404001894).

Consent for Publication

Not applicable.

Availability of Data and Material

Upon reasonable request, the interpreted and analyzed data in this manuscript are available from Dr. Paul Lockman.

Competing Interests

The authors declare that they have no competing interests.

Funding

Study design, experimental followthrough, and data collection, analysis, and interpretation for this manuscript were funded by a grant from the National Institute of General Medical Sciences (P20GM121322) and by the Mylan Chair Endowment Fund. Microscopy imaging and analysis were further supported by another grant from NIGMS (P20GM103434).

Author's Contributions

SAS conception and design, experimental work, analysis and interpretation of data, writing, and review and approval of manuscript. TAA experimental work and review and approval of manuscript. BNK experimental work, and review and approval of manuscript. ARS analysis and interpretation of data, writing, and review and approval of manuscript. PRL Conception and design, analysis and interpretation of data, writing and review and approval of manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

We would like to thank the WVU HSC Microscope Imaging and the Animal Modeling Imaging Facilities, as well as the Office of Lab Animal Resources, specifically Ethan Galand.

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Table

Due to technical limitations, Table 1 is provided in the Supplementary Files section.

Table Caption

Table 1: Comparison of dose, BED, and permeability changes among literature reports investigating the BBB and radiation therapy.

Figures

Figure 1

Calibration curve at isocenter generated using WVU HSC's Xstrahl Small Animal Irradiator (SAI). (A) Farmer® chamber calibration of WVU HSC's SAI. (B) Calibration curve of Gafchromic EBT3 film generated at isocenter. (C-J) Representative images of film irradiated to doses from 0-2000cGy.

Figure 2

Dose homogeneity output of a 10x10mm field size irradiated to a target dose of 5.4Gy. The irradiated 10x10mm field was uniform in both the horizontal and vertical directions. The penumbra, or the distance between 80% and 20% of the max dose was determined to be 0.850mm.

Figure 3

Histological verification of half-brain irradiation in an in-vivo system. (A) Representative photographic image of laser alignment on mouse providing placement for collimator and (B) dual overlaid radiograms. (C) Representative image of irradiation of FVB mice with a single dose of 15.5Gy through the right cranial hemisphere. Nuclei (Blue) were stained with DAPI. Double stranded DNA breaks (green) are indicated by enhanced γ H2AX signal.

Nu/Nu

FVB

Figure 4

The BBB remains intact in athymic Nu/Nu mice but is disrupted at an intermediate dose in immune competent FVB mice. (A) Athymic nude mice treated with daily fractions of 3Gy showed no significant difference in normal BBB permeability to Texas Red at 24 hours following radiotherapy. (B) Representative image of a Nu/Nu mouse treated with radiotherapy through the right cranial hemisphere. (C) Immune competent FVB mice showed no significant difference in BBB permeability to Texas Red,

except following a total dose of 12Gy given in 4 fractions. (D) Representative image of a FVB mouse treated with radiotherapy through the right cranial hemisphere.

Figure 5

Permeability of metastatic brain lesions increases in a time and dose dependent manner following half-brain irradiation. (A) BTB permeability is significantly increased at both 8 and 24 hours following 6Gy ($p<0.05$, $n=13$) in metastatic tumors in the portion of the brain receiving radiation treatment. In the mice

treated with 12Gy of radiation a significant increase in BTB permeability to Texas Red was only seen at 24 hours post treatment ($p < 0.05$, $n = 12-18$). (C-D) Representative images of an untreated metastatic brain lesion and a lesion that was in the radiation field.

Supplementary Files

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- [Table.1.xlsx](#)