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Breast Cancer Brain Metastasis Response to Radiation After Microbubble Oxygen Delivery in a Murine Model

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Abstract

Objectives—Hypoxic cancer cells have been shown to be more resistant to radiation therapy than normoxic cells. Hence, this study investigated whether ultrasound (US)-induced rupture of oxygen-carrying microbubbles (MBs) would enhance the response of breast cancer metastases to radiation.

Methods—Nude mice ($n = 15$) received stereotactic injections of brain-seeking MDA-MB-231 breast cancer cells into the right hemisphere. Animals were randomly assigned into 1 of 5 treatment groups: no intervention, 10 Gy radiation using a small-animal radiation research platform, nitrogen-carrying MBs combined with US-mediated MB rupture immediately before 10 Gy radiation, oxygen-carrying MBs immediately before 10 Gy radiation, and oxygen-carrying MBs with US-mediated MB rupture immediately before 10 Gy radiation. Tumor progression was monitored with 3-dimensional US, and overall survival was noted.

Results—All groups except those treated with oxygen-carrying MB rupture and radiation had continued rapid tumor growth after treatment. Tumors treated with radiation alone showed a mean increase in volume $\pm$ SD of $337\% \pm 214\%$ during the week after treatment. Tumors treated with oxygen-carrying MBs and radiation without MB rupture showed an increase in volume of $383\% \pm 226\%$. Tumors treated with radiation immediately after rupture of oxygen-carrying MBs showed an increase in volume of only $41\% \pm 1\%$ ($P = 0.045$), and this group also showed a 1 week increase in survival time.

Conclusions—Adding US-ruptured oxygen-carrying MBs to radiation therapy appears to delay tumor progression and improve survival in a murine model of metastatic breast cancer.

Keywords

contrast-enhanced ultrasound; hypoxia; oxygen delivery; radiosensitivity

Breast cancer is the most common of all cancers among women, projected to affect more than 12% of women in the United States.¹ Although several treatment options are available for early-stage breast cancer, they are more limited once the tumor becomes metastatic. This is particularly critical for metastasis to the brain, for which focused radiation therapy is the main treatment method.² It has been documented that metastasis is linked to tumor angiogenesis, especially in aggressive breast cancers.^{3–5} The rapid and chaotic process of tumor angiogenesis leads to the development of hypoxic conditions within the tumor (≈ 2 –18 mmHg partial pressure of oxygen [pO_2] compared to 50–60 mmHg pO_2 in healthy tissues). Tumor hypoxia is due to inadequate oxygen delivery at the cellular level compared to the rate of oxygen consumption.^{6–10} This chronic hypoxia has been identified as a major barrier to radiation therapy in breast cancer, as cells under hypoxic conditions are inherently more resistant to death from exposure to radiation.^{10,11} Even small increases of pO_2 in the hypoxic environment (> 10 mmHg pO_2) have been shown to rapidly and significantly sensitize tumor cells to radiation therapy.^{8,12}

Since hypoxia-related resistance to radiation can be overcome almost immediately after oxygenation,^{8,12} a platform that could locally increase pO_2 should substantially improve radiation therapy. Researchers have investigated various systemic approaches to delivering oxygen to hypoxic tumors before radiotherapy, including hyperbaric chambers and pharmacologic agents, but with only limited clinical success, primarily because of the body's natural ability to regulate oxygen.^{13–15} Current research efforts focus on more targeted or localized delivery approaches, such as oxygen scavengers to inhibit oxygen consumption within the tumor and angiogenesis inhibitors.^{16–18} Investigations of angiogenesis inhibitors led to the finding that disruption of the tumor vasculature also improves radiosensitivity, but clinical translation to date has failed.^{16,19}

In response to these limitations, other approaches for disruption of tumor vasculature and localized therapeutic delivery have been studied, including the use of ultrasound contrast agents (UCAs). Briefly, UCAs are intravenously injected, gas-filled microbubbles (MBs) ranging from 1 to 8 μm in diameter that can permeate into the tumor microvasculature. Previously, these agents have been used to visualize angiogenic vessels.^{20,21} At increased acoustic pressures, these MBs undergo inertial cavitation, causing rupture of the UCA shell and localized endothelial cell death within the vasculature.^{20,22} Such disruption to the endothelial lining has been shown to affect the vasculature within the entire tumor, surpassing the limited efficacy of other angiogenesis inhibitors.^{16,19,22} UCA cavitation has also been widely studied as a method for localized delivery of therapeutic agents to the tumor vasculature, with some success.^{23–30} UCA cavitation for localized delivery of gases, specifically oxygen, is less studied but represents a potentially viable delivery platform. Groups have proposed using nano- and micro-sized UCAs stabilized with chitosan, dextran, protein, and lipid-based shells to deliver oxygen.^{30–37}

Our group previously developed ultrasound (US)-sensitive, surfactant-stabilized MBs that encapsulate an oxygen core for localized gas delivery to hypoxic tumors.^{38–40} These agents are stable on intravenous injection and able to increase the average pO_2 in solid tumors to as high as 20 mmHg and provide substantial radiation sensitization.^{38–40} This study investigated whether US-induced rupture of oxygen-carrying MBs would enhance the tumor response to radiotherapy in a murine model of metastatic breast cancer in the brain.

Materials and Methods

All animal studies were performed ethically under approval from the Institutional Animal Care and Use Committee at Thomas Jefferson University.

Microbubble Development

We developed an optimized US-sensitive MB platform that can be used to locally deliver gases, such as oxygen, to the tumor environment.³⁹ These are surfactant-stabilized, gas-filled MBs with an average diameter of approximately 3 μm , comparable in size to red blood cells (6 μm diameter in mice⁴¹). For this experiment, some MBs were loaded with oxygen, whereas others were loaded with nitrogen as a control group. Briefly, Span 60 (Sigma-Aldrich, St Louis, MO) and water-soluble vitamin E (Eastman Chemical, Kingsport, TN) were dissolved in 50 mL of phosphate-buffered saline (PBS; Sigma-Aldrich) and autoclaved for 30 minutes. The mixture was then cooled under rapid stirring, purged with a steady stream of octafluoropropane, and sonicated for 3 minutes at 110 W using a 1.27 cm probe horn (Misonix, Farmingdale, NY). Microbubbles were extracted from the solution by gravity separation and washed every 90 to 120 minutes with cold PBS for 3 washes. The resulting MB solution was then aliquoted into lyophilization vials and supplemented with equal parts of 200 mM glucose (Sigma-Aldrich) in PBS as a lyoprotectant. Aliquots were frozen in a -20°C bath and then freeze dried under a vacuum for 24 hours (Virtis, Gardiner, NY). The gas of choice (oxygen or nitrogen; Airgas, Radnor, PA) was introduced through the vial stoppers using a syringe needle at a rate of 50 mL/min for 5 seconds and then 20 mL/min for 1 minute to ensure filling. Immediately before use in the study, MBs were resuspended by injecting 4 mL of sterile deionized water into the vials.³⁹

Cell Line Development and Tumor Inoculation

Brain-seeking metastatic human breast adenocarcinoma cells (MDA-MB-231-BR) were kindly provided by Patricia S. Steeg, PhD (National Cancer Institute, Bethesda, MD), and maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA). The culture medium was supplemented with 10% fetal bovine serum (Thermo Fisher), 2.5% horse serum (Thermo Scientific), and 1% penicillin-streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% carbon dioxide. MDA-MB-231-BR cells (1×10^5 cells in 5 μL of sterile PBS) were injected stereotactically into the right hemisphere of the brains of 15 immunodeficient female nude mice (Charles River Laboratories, Wilmington, MA) under an Institutional Animal Care and Use Committee–approved protocol following National Institutes of Health guidelines for animal care and use. After inoculation, animals were monitored twice per week with a high-frequency US scanner equipped with a 32 MHz transducer (Vevo 2100; FujiFilm VisualSonics, Toronto, Ontario, Canada) to monitor tumor

growth. Mice were anesthetized with 1.5% isoflurane and oxygen during imaging. Before any treatment, baseline tumor volumes were measured with the Vevo 2100 system equipped with a 3-dimensional (3D) stepper motor.

Tumor Treatment

Animals were randomly assigned into 1 of 5 treatment groups ($n = 3$ per group). The first group received no intervention, only monitoring, whereas the second group received 10 Gy low-linear energy transfer radiation using a small-animal radiation research platform (Xstrahl, Camberley, England) with cone beam computed tomographic guidance to focus the 3D conformal radiotherapy to the right hemisphere. Tumors were irradiated by using 4 confocal beams fractionated at 2.5 Gy per approach using a 5×5 mm square fixed collimator at a rate of 242 cGy/min (225 kV [peak]), as shown in Figure 1.

All animals receiving radiotherapy were anesthetized with a combination of ketamine (75 mg/kg subcutaneously; Zoetis, Parsippany, NJ) and acepromazine (1 mg/kg subcutaneously; Zoetis). Unlike our prior work, which used 5 Gy radiation for control of subcutaneous MDA-MB-231 tumors,^{38,40} 10 Gy radiation was used to treat this more-aggressive tumor model. The higher dose of radiation was well tolerated by all animals in the study. The third group received an injection of nitrogen-carrying MBs combined with US-triggered MB rupture immediately before 10 Gy radiation (N_2 MBs + US + 10 Gy). Microbubbles were destroyed with 75 seconds of flash-replenishment sequences using an S3000 scanner equipped with a 9L4 transducer (Siemens Healthineers, Mountain View, CA) at 4 MHz, 100% output power, and a 1.39 mechanical index. The imaging plane was maintained at the midline of the tumor, and sequences were timed to allow adequate reperfusion of the vasculature for the maximum effect. The fourth experimental group received an injection of oxygen-carrying MBs immediately before 10 Gy radiation (O_2 MBs + 10 Gy), and the final group received oxygen-carrying MBs and flash-replenishment insonation immediately before radiotherapy (O_2 MBs + US + 10 Gy).

Tumor Evaluation

The tumor response to treatment was quantified twice weekly until euthanasia with 3D US using the Vevo 2100 system, as described for the baseline measurements. Euthanasia criteria were defined by our Institutional Animal Care and Use Committee as a mass size of greater than 1.5 cm in any direction, an animal with a loss of body mass of 20% or greater, or an animal showing signs of distress. Tumor volumes were calculated for each time point by 3D B-mode imaging and offline Vevo 3D mapping software (FujiFilm VisualSonics).³⁸ Researchers were blinded to experimental groupings during the 3D tumor mapping to prevent bias, and mapping was performed under the supervision of an experienced sonographer (with 9 years of experience).

Statistical Analyses

Results were collected in triplicate, and means are reported with standard deviations. Tumor volumes were normalized to the baseline volume for each mouse. Survival was calculated from the day of inoculation until the day each animal was euthanized by Kaplan-Meier curves. Statistical differences between groups were determined by one-way analysis of

variance tests, whereas significance between individual groups was determined by paired *t*-tests. All statistical analyses were performed with Prism 7 software (GraphPad Software, La Jolla, CA) at a significance level of $\alpha = .05$.

Results

An example of oxygen-loaded MBs visualized in the periphery of the murine skull is shown in Figure 2. Microbubbles were seen perfusing into the skull periphery after injection (Figure 2A), were successfully destroyed at a higher mechanical index during the destructive pulse (Figure 2B), and then reperfused into the skull after the destructive pulse (Figure 2C). Visualization of the MBs with the lower-mechanical index imaging mode was limited because of attenuation of the skull, but they can still be seen entering the skull periphery.

Tumor growth was quantified by blinded 3D US rendering, examples of which are shown in Figure 3 for the O₂ MB + 10 Gy and O₂ MB + US + 10 Gy groups. All groups except the one receiving O₂ MBs + US + 10 Gy had rapid tumor growth after treatment. Mice receiving only 10 Gy radiation showed a mean tumor growth of $337\% \pm 214\%$ of the original size in the 7 days after treatment, and tumor volumes in the group receiving O₂ MBs + 10 Gy increased $383\% \pm 226\%$ of their original size in this time. With the addition of flash-replenishment US sequences, O₂ MBs + US + 10 Gy showed significantly reduced tumor growth after treatment compared to O₂ MBs + 10 Gy without insonation, with tumor volumes increasing only $41\% \pm 1\%$ their original size over the 7 days after treatment ($P = 0.045$). Ultrasound-triggered local delivery of oxygen immediately before radiation therapy (O₂ MBs + US + 10 Gy) also reduced tumor growth compared to radiation alone (10 Gy; $P = 0.025$). Tumor growth increased by $80\% \pm 33\%$ over 7 days in the N₂ MB + US + 10 Gy group, indicating some background cavitation effect on radiation sensitivity ($P = 0.08$ compared to O₂ MBs + US + 10 Gy). Treatment with oxygen-loaded MB cavitation in combination with 10 Gy radiation slows tumor growth, but ultimately the animals still succumb to the disease. Normalized tumor growth is summarized in Figure 4.

In terms of disease progression, the control group (receiving no intervention) had significantly less longevity than all interventional groups, with median survival of 2.9 weeks ($P = 0.0003$). Additionally, there was no significant difference in survival between the group receiving only 10 Gy (median survival of 3.9 weeks) and the group receiving O₂ MBs + 10 Gy (median survival of 3.7 weeks; $P = 0.32$), supporting the concept of tumor resistance to radiation treatment. Although survival was statistically similar to the O₂ MB + 10 Gy group ($P = 0.18$), the mice in the experimental O₂ MB + US + 10 Gy treatment group had slight increases in longevity by at least 1 week, with median survival of 4.0 weeks. Additionally, mice in the O₂ MB + US + 10 Gy group had increased survival compared to those treated with N₂ MBs + US + 10 Gy (median survival of 3.1 weeks; $P = 0.025$), suggesting that local delivery of oxygen does sensitize the tumor to radiation and not just MB cavitation effect.

Discussion

Overcoming tumor hypoxia for effective radiation therapy remains a critical need in oncology.¹¹ This study demonstrated the feasibility of using US-induced rupture of oxygen-loaded MBs to sensitize hypoxic tumors to radiation therapy in a murine model of metastatic

breast cancer within the brain. We have shown that injection of oxygen-carrying MBs followed by insonation sensitized these metastatic breast tumors to radiation therapy and improved survival in this murine model. This work expands on our previous studies by demonstrating treatment efficacy in a metastatic *in vivo* model.^{38–40} The tumor progression measurements shown in Figure 4 show significant improvement in tumor control (only 40% growth in the tumor volume over 12 days) in response to combination oxygenation and radiation therapy (O₂ MBs + US + 10 Gy), albeit in a small sample size. Additionally, we have built on our previous findings that cavitation of nitrogen-loaded MBs (N₂ MBs + US + 10 Gy) does not alter po₂ levels in hypoxic tumors³⁹ by also demonstrating that this cavitation does not significantly sensitize these tumors to radiation therapy (80% growth in tumor volume) compared to treatment with US-ruptured, oxygen-carrying MBs (40% growth). Nonetheless, we observed some modest improvement in tumor control in response to cavitation of nitrogen-loaded MBs compared to noninsonated controls, consistent with other studies in the literature.^{22,42–44} Microbubble cavitation has been shown to induce temporary disruption of tumor vasculature and endothelial cell death,^{22,43} which most likely contributed to the observed modest tumor control in these groups.

Although the study findings are encouraging, several limitations must be discussed. An important limitation to this study was the small sample size, which underpowered the otherwise encouraging results. The goal of this pilot study was to establish the feasibility of the delivery technique. Additionally, survival data were somewhat artificial as a result of animal euthanasia in accordance with ethical guidelines for humane handling. As shown in Figure 4, tumor control was highly variable in all experimental groups, most of which can be attributed to natural variation within *in vivo* models. Although we have normalized the data to account for the differences in baseline tumor size, variations in tumor aggressiveness and the subsequent need to euthanize animals due to distress were still present between the subjects. Finally, although the duration of tumor oxygenation (≈ 2 –3 minutes⁴⁰) in these studies appears to be sufficient for this small animal model, this may not be sufficient for clinical translation. Future work will include investigating methods of increasing the oxygen payload and duration of tissue oxygenation.

Another potential hurdle to the clinical translation of this therapy is the need for transcranial penetration with the US beam to induce MB cavitation, which is another possible explanation for the high variability encountered in this study. However, Hynynen and Jolesz⁴⁵ recently demonstrated that high-intensity focused US can effectively penetrate an intact skull and induce therapeutic changes in brain tissue. This group also demonstrated that acoustic parameters can be modified to allow temporary interruptions in the blood-brain barrier, therefore allowing entry of UCAs into the target tissue.^{46–48} We expect to be able to harness these capabilities to effectively rupture oxygen-carrying MBs in the brain for localized delivery and radiosensitization. A great advantage in this type of study would be to confirm and quantify oxygen delivery to the tumor, and in future studies, we will investigate transfecting our brain-seeking MDA-MB-231 cancer cells with hypoxia reporters to visualize changes in the tumor environment.^{49,50} Finally, conclusions from human tumor xenografts in nude animal models are inherently limited.^{51,52} Future work will explore syngeneic tumor models, as well as investigations of the tumor immune response in these more clinically relevant models.

In conclusion, this study demonstrates the feasibility of using UCA cavitation of oxygen-loaded MBs to sensitize hypoxic tumors to radiation therapy in a murine model of metastatic breast cancer. Although based on a small sample size, preliminary findings demonstrate that pretreatment with oxygen-carrying MBs and US immediately before radiation therapy may delay tumor progression and improve patient outcomes.

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Abbreviations

MB	microbubble
PBS	phosphate-buffered saline
pO₂	partial pressure of oxygen
3D	3-dimensional
UCA	ultrasound contrast agent
US	ultrasound

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Figure 1.
Treatment planning for 10 Gy radiation to the selected region of interest using the small-animal radiation research platform (SARRP).

Figure 2.

Examples of US imaging of O₂ MB + US + 10 Gy therapy to an MDA-MB-231 metastatic brain tumor within the murine skull. In all panels, the image on the right is the anatomic B-mode grayscale image, whereas the image on the left is the contrast mode image optimized to visualize the MBs within the tumor. **A**, Microbubbles were visualized along the periphery of the tumor on injection, as denoted by arrows. **B**, Destructive pulse to induce MB cavitation. **C**, Reperfusion of MBs after destructive pulses, with MB visualization shown with arrows.

Figure 3.

Examples of 3D US imaging of brain tumors with user-defined renderings. **A**, 3D tumor volume from the O₂ MB +10 Gy group on the day of treatment. **B**, 3D tumor volume from the O₂ MB +10 Gy group 7 days after treatment. **C**, 3D tumor volume from the O₂ MB + US + 10 Gy group on the day of treatment. **D**, 3D tumor volume from the O₂ MB + US + 10 Gy group 7 days after treatment.

Figure 4. Normalized tumor volume plotted against the time of observation after treatment ($n = 3$). Error bars indicate standard error. The line length corresponds to animal survival, where the end of each line represents the death of the last subject in that group or the last data point collected.