Antitumor effects of combining tumor radiation with the antivascular action of ultrasound stimulated microbubbles

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Abstract: Objective: More and more evidence indicates tumor vasculature plays an important role in tumor radiation response. In this study, we investigated ultrasound stimulated microbubbles to enhance the effects of radiation. Methods: Human bladder cancer HT-1376 xenografts in severe combined immuno-deficient mice were used. High-frequency (25 MHz) ultrasound was used to image tumor responses caused by ultrasound-stimulated microbubbles in combination with radiation. Human bladder xenografts grown in severe combined immunodeficiency (SCID) mice were treated using microbubbles stimulated with ultrasound at 250, 570, or 750 kPa, and exposed to 0, 2, or 8 Gy of radiation. Tumors were imaged prior to treatment and 24 hours after treatment. Spectral analysis of images acquired from treated tumors revealed overall increases in ultrasound backscatter intensity and the spectral intercept parameter. Results: There existed a synergistic effect in vivo with combined single treatments of ultrasound-stimulated microbubble vascular perturbation and radiation inducing an over 10-fold greater cell kill with combined treatments. We further demonstrate that induction of ceramide-related endothelial cell apoptosis, leading to vascular disruption, is a causative mechanism. In vivo experiments with ultrasound and bubbles permit radiation doses to be decreased significantly for comparable effect. Conclusion: We envisage this unique combined ultrasound-based vascular perturbation and radiation treatment method being used to enhance the effects of radiation in a tumor, leading to greater tumor eradication.

Keywords: Ultrasound stimulated microbubbles, radiation, antivascular action, tumor therapy

Introduction

The meshwork of blood vessels within the tumor microenvironment is constituted in part by endothelial cells that proliferate as part of the recruitment of new vessels to the tumor [1]. These cells are critical in response to both intracellular and extracellular signals that initiate neovascularization [2]. Anti-vascular agents that target endothelial cells are primarily focused on inhibition of angio-regulators such as vascular endothelial growth factor (VEGF), angiogenin and thrombin [3]. Antivascular pharmacological agents have been successful in preclinical trials although there are several limitations to their application as a mono-therapeutic approach. In the present study, we use ultrasound-mediated microbubbles as a novel treatment to perturb endothelial cells within the vascular framework in vivo. Microbubble microspheres are coated with a lipid, protein or biopolymer, and are heavily used in medical applications of ultrasound [4]. These microspheres encapsulate a specific gas that give them characteristic echogenicity and are currently used as contrast agents in ultrasound diagnostic imaging [5]. Evidence that endothelial cells can be damaged during ultrasound-mediated microbubble exposure have been reported and demonstrated that endothelial cells were susceptible bystanders to injury from cavitation [6]. A major effect caused by ultrasound interactions with microbubbles can be cavitation. Cavitation is described as rapid oscillating movements and morphological transformations that can result in outer-shell disruption of microbubbles and gas release [7]. In cardiovascular medicine ultrasound-driven microbubbles can disrupt red blood clots, making it an effective therapy for the treatment of
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intravascular thrombin. It was reported that ultrasound-driven microbubbles could irreparably damage kidneys locally. Previous studies have reported ultrasound-mediated microbubbles exposure to be efficacious in perturbing endothelial cells [8-10].

We demonstrate here that low mechanical index ultrasound-mediated excitation of microbubbles can enhance the effects of radiation in vitro and supra-additively in vivo using histological and functional assays of cell death and tumor growth delay experiments. Data obtained from experiments in vitro indicate that, under these ultrasound-exposure conditions, ceramide formation is induced by microbubble interactions with cells and associated with endothelial cell apoptosis. This is a known mechanism for radiation-based, ceramide-related endothelial cell death. Endothelial cell death in vivo caused by microbubble perturbation of tumor microvasculature leads to a pronounced vascular disruption and a 10-fold enhancement of tumor cell death when combined with single radiation treatments. Experiments indicate that single 2-Gy doses of radiation can lead to more than 40% tumor volume kill. Treatments with multiple fractions of the combined modalities demonstrate that ineffective doses of radiation can be made more effective in terms of tumor growth delay and mouse survival. We propose that such combined treatments lead to vascular cell death, which then secondarily induces tumor cell kill, and that such treatments can be used to increase the efficacy of cancer treatments.

Material and methods

Cell culture

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas VA, USA). Human bladder carcinoma HT-1376 cell lines were cultured in Eagle’s minimum essential medium (ATCC) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), 1% penicillin/streptomycin (Sigma-Aldrich) and exposed to 5% CO₂, hepa-filtered air at 37°C. Cells were cultured to 80% confluence and collected using 0.25% trypsin, 0.02% EDTA solution at room temperature. Cell pellets were isolated and re-suspended in 100 ul D-PBS (Mg, Ca-) per 1.0×10⁶ cells in preparation for inducing tumors in mice.

Animals

Animal research was conducted in accordance with the guidelines by the Canadian Council on Animal Care. CB-17 white-haired severe combined immuno-deficient (SCID) male mice were obtained from Charles River Inc. (Wilmington, MA, USA). A total cell volume of 1.0×10⁶ cells suspended in 100 ul of D-PBS (Mg, Ca-) was injected subcutaneously to the lower right hind leg of the mouse and tumors were allowed to develop over a period of 2-3 weeks in order to reach a diameter of 5-7 mm for experiments.

In vivo studies

Microbubbles and ultrasound activation Definity Perflutren lipid microspheres (Lantheus Medical Imaging, North Billerica, MA, USA) were shaken using a Lantheus device for 45 s at 3000 r.p.m. Low (1% v/v) and high (3% v/v) microbubble concentrations were calculated according to total mouse blood volume estimated by animal weight. The microbubbles were diluted in sterile normal saline and injected via the tail vein. A secondary injection (0.1 cc) of normal saline was used to flush the tail vein before treatment. Mice were mounted onto a custom stage and partially immersed into a 37°C water bath for ultrasound exposures. The ultrasound therapy system involved a micro-positioning system, waveform generator (AWG520, Tektronix, Beaverton, OR, USA), power amplifier with pulser/receiver (RPR4000, Ritec) and a digital acquisition system (Acquiris CC103, Agilent Technologies, Monroe, NY, USA). Animals were exposed within the half maximum peak of the acoustic signal 16-cycles tone burst at 500 kHz centre frequency using a 2.85-cm unfocused planar ultrasound transducer (ValPey Fisher Inc., Hopkinton, MA, USA) and at 3 kHz pulse repetition frequency for 50 ms at a time with a peak negative pressure set to 570 kPa, corresponding to a mechanical index of 0.8. An intermittent 1950-ms period between sonification was employed to minimize biological heating in the tissue during ultrasound exposures. The total insonification time was 750 ms over 5 min.

Irradiation

The tumours were X-irradiated 5 min after ultrasound treatment using an irradiation cabinet device (Faxitron, Wheeling, IL, USA). Doses of 0,
2 and 8 Gy were administered at a dose rate of 200 cGy min\(^{-1}\), 160 kVp energy and a source-skin distance of 30 cm as per the specifications of the device. Corporal lead sheet shielding was used with a circular aperture to expose only the tumour.

**Timing and exposure experiment**

For timing experiments, a delay of 0, 3, 6, 12, and 24 h was introduced between the low concentration microbubble-stimulated ultrasound treatments and the 0- and 8-Gy radiation treatment. Micro-Doppler measurements were also carried. For treatments with ultrasound-stimulated microbubbles, mice were killed at 0, 3, 6, 12, and 24 h. For experiments in which radiation was administered, mice were killed 24 h after irradiation. For exposure experiments, 0, 0.01 low, 0.1 low, the low and high bubble concentrations, were used with no delay between the treatments. There were four mice per group.

**Multiple-fraction experiments**

For multiple-fraction experiments, mice were exposed to no treatment, ultrasound microbubble stimulation (high concentration) twice weekly on Monday and Thursday for 3 wk, and the same microbubble exposure combined with 2 Gy four times weekly, Monday to Thursday [24 Gy, BED\((10)\) = 28.8 Gy]. Radiation was administered immediately after ultrasound treatments. For comparison, another group of mice received 3 Gy a day, five times weekly for 3 wk [45 Gy, BED\((10)\) = 58.5 Gy] and the same 45-Gy fractionated radiation dose with ultrasound treatments given twice weekly as above. Tumor sizes were measured for up to 28 d after treatment completion, with a doubling of tumor size to 2.0-cm diameter taken as a survival endpoint in addition to standard animal care endpoints. There were n = 5 mice per group.

**Ceramide and Sphingosine-1-Phosphate experiments**

In order to test if ultrasound-stimulated microbubbles in combination with ultrasound could stimulate ceramide formation, experiments were carried out using an additional cohort of 75 animals, with five mice per group. Mice were treated as above with no microbubbles, low, and high bubble concentrations in the presence of ultrasound and combined with 0-, 2-, and 8-Gy radiation doses given in single fractions as above for nine cohorts of n = 5 animals. In addition, 0-, 2-, and 8-Gy conditions with and without high-concentration microbubble exposure in the presence of ultrasound were carried out in the presence of S1P using modified protocols. This used six cohorts of n = 5 mice. For S1P treatments, 4 μg/g of S1P in 0.2 uL of PET (5% polyethylene glycol, 2.5% ethanol, and 0.8% Tween-80) was injected intravenously in mice 30 min prior to and 5 min after irradiation or after microbubble exposure with ultrasound and irradiation.

**Histopathology**

Tumors were fixed in 10% acetate buffered formalin (Fisher Scientific Canada, Ottawa, Ontario, Canada) following excision at 24 h (for acute studies) and 21-28 days (for long-term studies) after treatment. Tumors were fixed at room temperature for 4 h and then transferred to 4°C for 24 h before processing using a Leica ASP300 smart tissue processor (Leica Microsystems, Richmond Hill, Ontario, Canada). Tissues were embedded in paraffin (Leica EG 1160, Leica Microsystems) and prepared as 5 mm sections onto slides with standard hematoxylin and eosin (H&E) staining techniques used for both acute and longitudinal studies. Staining using TdT-mediated dUTP-biotin nick-end-labelling (TUNEL) was used in acute studies to visualise apoptotic regions. Cluster of differentiation-31 (CD31) staining was implemented to count and quantify endothelial cells within the tumor relative to the presence of intact and disrupted vessels. A normalised CD31 VI was calculated using 10 randomly selected ROIs per tumor slice from five slices per animal tumor. The VI was calculated as the ratio of the sum of intact luminal vessel number/area measured to the total vessel number/area measured (including intact luminal vessels and vessels which had been ruptured or collapsed by microbubble exposure). Vessels stained with CD31, in addition to areas of cell death, and all other microscopy measures were quantified in histology and immunohistochemistry tumour sections assisted by the use of Image-J (NIH, Bethesda, MD, USA).

**Micro-Ultrasound doppler imaging**

Ultrasound imaging to detect blood in tumors before and after treatment was carried out.
using a VEVO-770 (Visualsonics) in power Doppler mode and a VEVO RMV transducer with a central frequency of 20 MHz. Power Doppler imaging was carried out using a step size of 0.2 mm, a wall filter of 2.5 mm/s, a scan speed of 2.5 mm/s, and a 20-dB gain setting. Doppler data were analyzed to determine vascularization index defined as the relative volume occupied by Doppler signal within a tumor volume (30) using MATLAB. Mice were anesthetized as for therapy during imaging.

**Immunostaining**

Immunostaining was performed using a Histostain-Plus kit (broad spectrum; Invitrogen). For ISEL and von Willebrand factor, staining was used with horseradish peroxidase-conjugated streptavidin, to bind to biotinylated secondary antibody/primary antibody and an AEC (3-amino-9-ethylcarbazole) chromagen. For more detailed staining, slides from samples were triple stained with DAPI staining for nuclear positions, CY3-conjugated CD31 for vascular delineation, and CY2-conjugated TUNEL for apoptotic DNA fragmentation, and visualized with UV, 490-nm, and 550-nm light illumination, respectively. Primary antibodies were obtained from Abcam, Alexis Biochemicals, and Life Technology for anticeramide antibodies.

**Statistical analysis**

A detailed analysis was conducted using GRAPHPAD INSTAT (GraphPad Software Inc., La Jolla, CA, USA) and a statistician was consulted to review the most appropriate statistical method. One-way analysis of variance statistical analysis with Dunnett’s test or Tukey’s multiple comparison’s test was performed.

**Results**

**Increases in radiation dose, microbubble dose, or rarefractional ultrasound pressure cause increases in ultrasound backscatter intensity from tumor:** Results demonstrated that higher radiation doses caused increased cell death, resulting in higher backscatter intensity. In the B-mode images increases in intensity, indicative of higher backscatter, were visible within tumors receiving higher radiation doses. This was verified quantitatively in tumors treated with higher radiation doses which demonstrated spectra with greater overall signal amplitudes (Figure 1A). Tumors irradiated with 2 Gy and 8 Gy showed approximately 0.9 ± 2.0 dB and 3.7 ± 1.8 dB increases in mid-band fit values compared to the control, respectively. Tumors treated with higher dose of ultrasound-stimulated microbubbles similarly demonstrated greater backscatter intensity (Figure 1B and 1C). Mid-band fit values indicated 3.4 ± 2.3 dB and 7.0 ± 3.6 dB increases for 8 uL/kg, for 80 uL/kg and 1000 uL/kg microbubble concentrations, respectively (Figure 1B). Likewise, increases in rarefractional ultrasound pressure for microbubble stimulation resulted in specimens in vivo with elevated backscatter intensity. The increases ranged from 1.4 ± 3.3 dB to 3.4 ± 5.0 dB when comparing 250 kPa to 750 kPa, respectively (Figure 1C). A “plateau” of the backscatter intensity was observed at a pressure of 570 kPa and higher.

**Spectral parameters of ultrasound signal show sensitivity to changes in radiation Dose, microbubble concentration, and rarefractional ultrasound pressure:** Results demonstrated that mid-band fit, slope, and 0-MHz intercept spectral parameters were strongly correlated with increases in radiation dose, microbubble concentration, and ultrasound pressure (P < 0.0001). Overall, the average change in mid-band fit increased as radiation dose, microbubble concentration, or microbubble-stimulating ultrasound responses were increased (Figure 1D). The trend for every parameter was nearly linear for the lowest ultrasound pressure, 250 kPa, whereas with the highest pressure of 750 kPa, results appeared to plateau, especially where the highest change in mid-band fit values was 7.0 ± 4.1 dB, resulting from the combined treatment of 1000 uL/kg of microbubbles stimulated at 750 kPa and a radiation dose of 8 Gy. However, the treatment condition using the near clinically-utilized (for imaging) microbubble concentration (8 uL/kg of microbubbles, combined with 750 kPa, 2 Gy radiation) resulted in a 3.5 ± 2.4 dB increase in mid-band fit. The average change in 0-MHz intercept, linked to the concentration of scatterers, demonstrated trends analogous to the mid-band fit (Figure 1E). The 0-MHz parameter displayed increases when radiation dose, microbubble concentration, or ultrasound pressure were increased. In conditions where the highest microbubble concentration was administered, the 0-MHz intercept spectral parameter
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A

0 Gy  2 Gy  8 Gy

B

8 μL/kg/750 kPa/8 Gy  80 μL/kg/750 kPa/8 Gy  1000 μL/kg/750 kPa/8 Gy

C

8 μL/kg/250 kPa/8 Gy  8 μL/kg/570 kPa/8 Gy  8 μL/kg/750 kPa/8 Gy

D

250 kPa  570 kPa  750 kPa
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Figure 1. High-frequency ultrasound B-mode images of HT-1376 xenografts. B-Mode (left) and on the right side of each row, respective representative normalized power spectra are shown. A. Tumors treated with varying radiation doses (0-8 Gy) without ultrasound-microbubble treatment. B. Tumors treated with varying microbubble concentrations (8-1000 μL/kg) combined with 750 kPa of ultrasound pulse and 8 Gy-radiation. C. Tumors treated with varying ultrasound pressures (250-750 kPa) combined with 8 μL/kg of microbubbles and 8 Gy-radiation. The scale bar represents 2 mm. D. Average changes in midband-fit parameter. Each bar represents the mean of midband-fit values of five mouse-borne tumors (n = 5). The error bar indicates the standard error within the sample size. Statistical testing using 2-way ANOVA indicates the effects caused by the changes in both microbubble concentration and dose of radiation to be very significant for every graph (P < 0.0001). E. Average changes in 0-MHz intercept parameter. Each bar represents the mean of 0-MHz intercept values of five mouse-borne tumors (n = 5). The error bar indicates the standard error within the sample size. Statistical testing using 2-way ANOVA indicates the effects caused by the changes in both microbubble concentration and dose of radiation to be very significant for every graph (P < 0.0001). F. Average changes in slope parameter. Each bar represents the mean of slope values of five mouse-borne tumors (n = 5). The error bar indicates the standard error within the sample size. A statistical test using 2-way ANOVA indicates the effects caused by the changes in both microbubble concentration and dose of radiation to be very significant for every graph (P < 0.0001). Each graph shows the average changes in slope for varied microbubble concentration and radiation doses at a fixed ultrasound pressure: 250 kPa, 570 kPa, and 750 kPa.
was high regardless of the applied ultrasound pressure. The "highest-dose" treatment condition had an average change in 0-MHz intercept of 15.4 ± 2.5 dB, while the treatment condition using the lowest microbubble concentration increased by 9.2 ± 3.4 dB, relative to control. However, the average change in slope, which can be linked to the size of the scatterers appeared inversely correlated with increasing treatment dose (Figure 1F). It also exhibited an apparent plateau when increasing microbubble dose under the highest ultrasound pressure.

Ultrasound imaging and high-magnification immunohistochemical data for radiation and ultrasound treatments

In order to investigate the mechanism behind this enhancement of cell death we utilized non-invasive imaging techniques to track effects on the vasculature as well as immunohistochemical histology methods. Power Doppler micro-ultrasound imaging was carried out in a separate cohort of mice under the same experimental conditions (n = 36). Selected representative results are presented in Figure 2. Doppler data demonstrated moderate vascular disruption with ultrasound and microbubbles, and with 8-Gy radiation doses (20 ± 21% and 20 ± 32% decrease in Doppler vascular index, respectively). Significant reductions in blood flow at 24 h for the combined ultrasound-activated microbubble and radiation treatments were observed, suggestive of vascular disruption (65 ± 8% decrease in Doppler vascular index). The combination with ultrasound-stimulated microbubbles and radiation was significantly better in flow diminishment compared to the single treatments (P < 0.001) (Figure 2A). The effect of the combination treatments was more consistent with a smaller standard error compared to individual treatments. Corresponding immunohistochemistry under high-power microscopy indicated that ultrasound-activated microbubble treatments resulted in microscopic localized appearances consistent with endothelial cell apoptosis, whereas combined ultrasound-activated microbubble and radiation treatments resulted in near-total cell death of endothelial cells and tumor cells that was not apparent at the other experimental conditions (Figure 2A). Analysis (ANOVA) indicated that in situ end-labeling (ISEL) staining levels for ultrasound-stimulated microbubble treatment in combination with radiation (70 ± 8%) were significantly different in comparison to radiation alone (4 ± 2%) or ultrasound-stimulated microbubble exposure alone (36 ± 12%) (both P < 0.001) 24 h after treatment for the higher microbubble concentration. Immunohistochemical staining of von Willebrand factor revealed enhanced leakage from the vasculature with the combined ultrasound-activated microbubble and radiation treatments, further suggestive of vascular disruption (Figure 2A). In order to investigate the mode of endothelial cell death being induced by the ultrasound treatments in the presence of microbubbles, confocal microscopy of triple immunohistochemically stained sections of ultrasound-activated microbubble-treated xenograft tumors sections confirmed the induction of apoptosis in endothelial cells in tumors treated with ultrasound and microbubbles (Figure 2B).

Exposure experiments

In order to investigate the effect of ultrasound-stimulated microbubble exposure, experiments were conducted in which the concentration of microbubbles was varied (Figure 3A). For experiments, the microbubble concentration was varied from nil, to 0.01, and 0.1 of the low concentration, the low concentration, and to the high concentration. These concentrations were combined with 0, 2 and 8 Gy. Statistical analysis using ANOVA indicated an interaction accounting for 11% of the total observed effect (P < 0.001). Analysis with ANOVA indicated that radiation dose accounted for 10% of the effect (P < 0.001) and microbubble dose accounted for 70% of the observed effect (P < 0.001) (n = 4 for all groups). Treatment effects were present at 0.01 of the low concentration (approximate clinical imaging concentration) of microbubbles but increased at the higher concentrations. With the 2-Gy doses, exposure to the low and high microbubble concentrations produced equivalent results with better results at the higher concentration of bubbles combined with 8-Gy radiation treatment.

Timing experiments

Effects of ultrasound-stimulated microbubble exposure and resultant effects on cell death and micro-Doppler-detected blood flow were investigated. This modality was investigated alone and with a sequence of a time delay introduced with subsequent radiation treatment (0,
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Figure 2. Power Doppler ultrasound imaging and high-magnification immunohistochemical data for radiation and ultrasound treatments. A. Rows show power Doppler images (PD), ISEL high-magnifications data (ISEL), and von Willebrand factor staining (VWF) for vasculature. Columns indicate data for no treatment (Nil), ultrasound-activated microbubbles (MB), 8-Gy radiation (XRT), and microbubble and radiation treatments combined (MB XRT). Power Doppler data obtained at 20 MHz indicate blood flow disruption with treatments (20 ± 37% decrease with microbubble ultrasound treatment, 18 ± 22% decrease with radiation alone, and 65 ± 8% decrease with the combined treatments; mean ± standard error). The difference between the single treatments and the combined ultrasound-stimulated microbubble treatment was significant (P < 0.05). Scale bar, 2 mm. Staining with ISEL demonstrates a stranding suggestive of vascular distributions with microbubble treatments. Combined treatments result in what appears to be complete ISEL t cellular staining. Scale bar, 60 microns. Staining with von Willebrand factor suggests vascular disruption with bland smearing from leaking serum in the combined treatment specimen. Scale bar, 60 microns. B. Triple staining for endothelial cell apoptosis with TUNEL t apoptotic nuclei, CD31 vascular delineation, and DAPI for nuclear positions for tumor samples treated with the higher concentration of microbubbles. Images (Merge) indicate the presence of apoptotic nuclei (cyan) associated with vascular endothelial cells (red). Scale bar, 20 microns.
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Figure 3. Quantitative analysis of cell death. (A) Quantitative analysis of cell death in response to microbubble exposure with different radiation doses. Percentage ISEL staining from four tumors per group is shown with different microbubble concentrations administered to mice. For microbubble concentrations: Nil indicated no treatment; 0.01 L and 0.1 L indicate dilutions of the low microbubble concentration (L); and (H) indicates the high microbubble concentration used. Different radiation doses include 0, 2, and 8 Gy, as labeled. (B) Quantitative analysis of cell death in response to timing between microbubble exposure and radiation treatment. Decrease in micro-power Doppler data measured vascular index with microbubbles and combined treatment. Nil, no treatment imaged before and 24 h later; MB, treatment with microbubbles only (low concentration) and killing of mice at the indicated times after microbubble exposure (0, 3, 6, 12, and 24 h); MBXRT, treatment with microbubbles and interval time as indicated between subsequent radiation treatment (8 Gy). (C) Resulting ISEL + cell death corresponding to treatments as described in (A). For treatments with microbubbles alone, note the maximal effect on blood flow at 6 h and cell death after 6 h. Combined treatments follow a similar trend.

3, 6, 12, and 24 h (n = 4 for all groups). Treatment with ultrasound-stimulated microbubbles indicated maximal cell death, detected using ISEL staining when the two treatments were separated by 6 h, which coincided with a maximal decrease in detected micro-Doppler blood-flow signal. Radiation at that time resulted in a maximal effect 24 h later, in terms of ISEL-detected cell death and disruption of blood flow-linked micro-Doppler-detected signal (Figure 3B and 3C). The data imply a 9-h window
for radiation therapy after microbubble exposure with no statistically significant difference between results from 3 to 12 h. For time interval experiments ANOVA indicated a statistically significant radiation effect \((P < 0.0001)\), a statistically significant microbubble effect \((P < 0.0001)\), and an interaction between the two treatments \((P < 0.0001)\) for cell death and blood flow disruption each.

**Multiple-fraction growth delay**

Analysis of associated growth and survival curves and Ki-67 activity (as a marker of cellular proliferation) is presented in **Figure 4**. Analysis of survival curves to mouse death or modified human endpoint or 2-cm tumor size indicated that they were significantly different \((P < 0.05)\) with mean survivals of 10 ± 1, 19 ± 1, 20 ± 3, 25 ± 3, and 28 ± 0 d (mean ± standard error) for mice receiving no treatment, and treatment with the 2-Gy fractionation scheme [biological effective dose \((\alpha/\beta = 10)\), BED\((10) = 28.8\) Gy], the ultrasound-stimulated microbubble regimen, the 3-Gy fractionation scheme \([BED(10) = 58.5\) Gy], and the combined ultrasound-stimulated microbubble and 2-Gy radiation fractionation regimen. Growth delay data indicated there was no significant difference between 2 Gy combined with ultrasound-stimulated microbubbles compared to the 3-Gy regimen. For analyses of growth delay at day 21, the 2-Gy regimen combined with ultrasound-stimulated microbubbles was significantly dif-
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A

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![Graph showing % Ceramide Staining](image)

C

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D

![Graph showing % Ceramide Staining](image)
Figure 5. In vivo ceramide staining data. (A) Representative ceramide staining of sections of PC3 bladder tumors treated with radiation and/or ultrasound activated microbubbles. Columns represent 0, 2 and 8 Gy of radiation exposure from left to right. Rows indicate no (Nil), low-concentration microbubble exposure (LMB), and high microbubble exposure (HMB) from top to bottom, respectively. Exposure to radiation alone (Top) shows no appreciable ceramide formation except at the 8-Gy dose (brown staining). Microbubble-alone exposure demonstrated minor ceramide staining at the low concentration but, at the higher concentration, appeared to have a more prominent effect. The addition of radiation to the microbubble treatments led to detectable ceramide staining, greatest in the high microbubble concentration when combined with 8 Gy (diffuse brown staining). Scale bar, 200 microns. (B) Quantification of ceramide immunohistochemistry staining. Samples were assessed with respect to ceramide staining, Brown-stained cells were identified by their brown:blue ratio. Data indicate increases with respect to background staining. Labels indicate non (Nil), low (Low), and high (High) microbubble exposure and radiation doses (0, 2, or 8 Gy). Treatment with 8 Gy caused a small increase above background staining. Treatments in the presence of low and high concentrations of bubbles at all radiation doses caused increases in ceramide staining. (C) Representative ceramide staining of sections of PC3 bladder tumors treated with radiation and/or ultrasound-activated microbubbles. Labeling is as in (A), with the exception that sphingosine-1-phosphate (S1P, ceramide cell death inhibitor) has been added. Experiments were conducted at the higher (HMB) concentration with the addition of 0, 2, and 8 Gy of radiation. Note the lack of ceramide staining in the presence of S1P with the exposure to microbubbles and ultrasound (HMB) and 0, 2, and 8 Gy. Scale bar, 300 microns. (D) Quantification of ceramide immunohistochemistry staining for experiments with S1P. Data indicate an inhibition of ceramide staining in response to S1P when tumors in vivo were exposed to radiation in the presence of bubbles in comparison to data in (A).

Discussion

In this study, we used high frequency Doppler ultrasound in conjunction with biological methods to monitor tumor response to ultrasound-driven microbubbles and radiation in a bladder cancer xenograft model. We tested the hypothesis that ultrasound-mediated microbubble treatment of tumors can be an effective vascular targeting agent that can also potentiate the effects of radiation therapy in bladder cancer xenografts in vivo. The study demonstrated that ultrasound-stimulated microbubbles in combination with radiation can induce rapid...
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![Image of histological slides showing different irradiation conditions and their effects on tumor morphology. The images illustrate the impact of radiation on tumor cells, with variations in cell structure and density observed under different irradiation conditions.]
hematopoietic disruption, which presents as tumor cell death within the tumor centre in both short and long-term studies.

In the study here, treatment with ultrasound-stimulated microbubbles combined with radiation was used as the primary treatment and has been identified to cause rapid cell death, in terms of structural changes within cells (24 hours). Here, destruction of tumor vasculature leading to secondary tumor cell death was the mechanism for cellular changes. In these treatments, low doses (2 Gy) of radiation combined with ultrasound-stimulated microbubbles have exhibited comparable efficiency as high doses of radiation only (5 Gy), linked to ceramide-mediated apoptosis in endothelial cells within tumor vasculature [11-13]. As expected, more intense treatments (higher radiation dose, microbubble concentration, and ultrasound pressure) in this study resulted in greater amounts of cell death leading to ultrasound backscatter changes. For all treatment conditions, increases in average changes in spectral parameters such as mid-band fit and 0-MHz intercept were observed. These corresponded with increases in average backscatter intensity and were linked to increases in dead appearing or apoptotic appearing cells, respectively. The trends were in agreement with the histological results here, as assessed in more depth previously [14, 15]. The use of the pressure of 750 kPa caused rapid increases in cell death when combined with increasing microbubble concentration, which eventually appeared to plateau. As previously suggested, this implies a potential saturation effect due to pressure level (570 kPa) leading to microbubble collapse [16, 17]. The size of ultrasound scatterers has been previously reported to be correlated with changes in spectral slope [18-20]. Changes in spectral slope were also observed here, where higher treatment doses resulted in a more negative slope. Although the condensed and fragmented structures derived from apoptosis are generally much smaller in size than nuclei from normal intact cells and in homogenously responding samples slope may increase, we have demonstrated previously that in heterogeneous samples with patches of response, or responses with large amounts of necrosis, similar to that here, slope decreases can occur [21]. In a previous investigation that assessed spectral parameters at different times (0, 4, 12, 24, 48 h) after chemotherapy exposure, changes in the trend in spectral slopes were observed over time [22]. Results demonstrated increasing slope leading up to a 12-hour point, then a horizontal slope at the 12-hour-point, and a decreasing slope between the 12-hour-point to 48-hour point. This was correlated with the development of necrosis after apoptosis and mixed modes of cell death. The results are complex and affected not only by the size of individual ultrasound scatterers which cannot be inferred from the data but also their compressibility and density, number density of scatterers, and in the case of ultrasound for these wavelengths, where the wavelength is...
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3-4 times the size of a cell, the spatial distribution of the scatterers.

Radiation treatments present several limitations in achieving optimal therapeutic outcomes because of tumor cell heterogeneity [23]. As a result, many regimens require varying doses of radiation in order to control tumor growth and also to make a significant effect on the tumor vasculature. Our results indicated such a complication in the delivery of only 2 Gy of radiation to the tumor, demonstrating that tumor control at low doses may have weak effects on controlling the tumor, but that it can also induce pro-vascular effects through angiogenic signaling [24]. We suspect that this induction of vascularisation through low dose radiation alone is caused by the MAP-K dependent pathway [25]. Interestingly, when 2 Gy radiation was combined with low and high concentrations of ultrasound-driven microbubbles, vessel disruption was observed in tumors. Vascular disrupting agents serve as a feasible model in combination with cytotoxic agents such as radiation because targeting vasculature is independent of tumor type and can overcome the obstacles that cytotoxic drugs have in combating cell heterogeneity [26]. Furthermore, tumor cells are more likely to have cytotoxic therapies because of their genetic instability [27], and thus targeting the endothelial cells can be beneficial because of its relative accessibility to vascular targeting agents, its low potential to disrupt normal tissue and its ability to influence death on tumor clonogens that are dependent on vessels [28]. Ultrasound-driven microbubbles used as vascular disrupting agents can have many advantages to molecular-based targeting. For instance, VEGF-antibody based agents could potentially have unwanted effects on other biological pathways as VEGF expression is also found in certain types of hematopoietic and stromal cells. Therefore, the added advantage to using ultrasound-driven microbubbles to perturb endothelial cells is its disposition to be locally targeted, thereby minimising the influence on other biochemical pathways found in distant physiological processes.

Vascular targeting agents can be an effective partnering modality in eradicating the tumor stroma. The current study suggests that ultrasound-mediated microbubbles can be successfully used as a vascular targeting agent and can enhance radiosensitivity in bladder cancer carcinoma. Endothelial cell disruptions and direct tumor cell kill are suspected to be implicated in both processes that render the tumor necrotic. Furthermore, this study emphasises the importance of considering multi-modality approaches in optimising the therapeutic ratio and draws attention to the importance of vascularity and endothelial cell interactions with radiosensitivity.

Disclosure of conflict of interest
None.

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