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Inhibition of glioma growth by microbubble activation in a subcutaneous model using low duty cycle ultrasound without significant heating:

Laboratory investigation

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Abstract

Object—In this study, the authors sought determine whether microbubble (MB) destruction with pulsed low duty cycle ultrasound can be used to reduce brain tumor perfusion and growth through nonthermal microvascular ablation.

Methods—Studies using C57BLJ6/Rag-1 mice inoculated subcutaneously with C6 glioma cells were approved by the institutional animal care and use committee. Microbubbles were injected intravenously, and 1 MHz ultrasound was applied with varying duty cycles to the tumor every 5 seconds for 60 minutes. During treatment, tumor heating was quantified. Following treatment, tumor growth, hemodynamics, necrosis, and apoptosis were measured.

Results—Tumor blood flow was significantly reduced immediately after treatment, with posttreatment flow ranging from 36% (0.00002 duty cycle) to 4% (0.01 duty cycle) of pretreatment flow. Seven days after treatment, tumor necrosis and apoptosis were significantly increased in all treatment groups, while treatment with ultrasound duty cycles of 0.005 and 0.01 inhibited tumor growth by 63% and 75%, respectively, compared with untreated tumors. While a modest duty cycle–dependent increase in intratumor temperature was observed, it is unlikely that thermal tissue ablation occurred.

Conclusions—In a subcutaneous C6 glioma model, MB destruction with low–duty cycle 1-MHz ultrasound can be used to markedly inhibit growth, without substantial tumor tissue heating.

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Disclosure

Author contributions to the study and manuscript preparation include the following. Conception and design: Price. Acquisition of data: Burke. Analysis and interpretation of data: Burke. Drafting the article: Price, Burke. Critically revising the article: all authors. Reviewed final version of the manuscript and approved it for submission: all authors. Statistical analysis: Price. Administrative/technical/material support: Klibanov. Study supervision: Price.

These results may have a bearing on the development of transcranial high-intensity focused ultrasound treatments for brain tumors that are not amenable to thermal ablation.

Keywords

ultrasound; microbubble; microvascular ablation; contrast-enhanced perfusion; glioma

HIGH-INTENSITY focused ultrasound for thermal tissue ablation is emerging as a noninvasive alternative to many surgical procedures^{16,25} and a treatment method for a variety of tumors.^{2,8,33,34} The key to success of HIFU as a clinical treatment for brain tumors via transcranial thermal ablation will be the capacity to localize the delivery of acoustic energy to a well-delineated region. The ability to deliver acoustic energy through the skull for purposes of transcranial thermal ablation is complicated by phase and power,³² which have been demonstrated to contribute to prefocal heating⁸ and cavitation in healthy tissues.

While the bony skull has historically been a significant impediment to transcranial HIFU, advances in medical imaging technology have enabled HIFU to overcome many obstacles.^{6,13} As a result, clinical brain tumor trials have been approved. However, preliminary results from these clinical trials have indicated that treatment can sometimes result in cranial heating, even in cases in which focal heating is subablative.¹⁸ In these cases, sonication length or sonications per treatment may be increased to achieve ablative focal heating.¹³ In addition to the risks of vital tissue damage associated with thermal accumulation in the skull, longer treatment time has the potential to increase the incidence of adverse effects. It is also important to note that the treatment of tumors close to the skull will result in greater heating on the brain surface due to a reduced area of ultrasound transmission, thereby possibly excluding patients with vertex or skull base tumors from current transcranial HIFU treatment options.^{7,12,18}

In attempts to minimize thermal accumulation and phase aberrations, other techniques to reduce the acoustic power required by HIFU for lesion formation have been tested.^{3,5} One such approach is to use an ultrasound contrast agent, MB, to generate mechanical damage at reduced acoustic power levels.^{9,14,15,20,21,37} Although the precise mechanism is unclear, it is widely accepted that inertial cavitation, in which an MB expands and then collapses in the presence of an acoustic field, allows for a range of therapeutic effects (mechanical damage to thermal ablation) to be achieved at lower acoustic intensities.²⁰ In ablative treatments, the additional mechanical damage produced by MBs at lower intensity levels may reduce the risk of off-target thermal accumulation. By reducing nonspecific heat accumulation through the use of MBs, cooling periods between successive sonications may be shortened and the treatment of brain tissue adjacent to the skull may be feasible, overcoming 2 major hurdles currently associated with transcranial HIFU.¹⁸

The aim of this study was to investigate the relationship between ultrasound duty cycle, intratumor temperature, and brain tumor treatment efficacy. Previous studies have demonstrated that tissue damage resulting from the interaction of MBs and ultrasound is strongly dependent on acoustic parameters such as acoustic power,^{1,22–24} pressure, pulse duration, pulse repetition frequency,³⁵ and MB concentration.³⁰ Our results indicate that

significant therapeutic gains can be achieved through the use of MBs and low duty cycle ultrasound, even in the absence of significant heat accumulation.

Methods

Preparation of MBs

To prepare albumin MBs, a 1% solution of serum albumin in normal saline was placed in a flask with a blanket of gas (octafluoropropane) above the aqueous phase. The solution was briefly sonicated (30 seconds) with an ultrasound disintegrator equipped with an extended 0.5-in titanium probe. This formulation is similar to Optison (GE Healthcare), which is provided in a concentration range of $5.0\text{--}8.0 \times 10^8$ MBs/ml. The mean MB diameter, as determined using a Multisizer Coulter Counter, was $2.01 \pm 1.29 \mu\text{m}$. To prepare lipid MBs, an aqueous dispersion of 2 mg/ μl polyethylene glycol stearate (Sigma Chemical Co.) and 2 mg/ μl phosphatidylcholine (Avanti Polar Lipids) was sonicated as previously described with decafluorobutane gas. Large MBs were removed from the preparation by flotation in a vertically positioned syringe. After purification, MBs were placed in glass vials, stoppered, and sealed under perfluorocarbon atmosphere.

Tumor Model

Animal studies were approved by the institutional animal care and use committee. The C6 glioma rat tumor cell line was maintained in F-12 K Nutrient Mixture (Gibco) supplemented with 16% horse serum (Sigma), 3% fetal bovine serum (Sigma), and 1% pen-strep (Gibco) at 37°C and 5% CO₂. Tumors were generated in vivo by injecting 10^6 tumor cells suspended in 300 μl of phosphate-buffered saline subcutaneously into the left hindlimb of C57BLJ6/Rag-1 mice. Tumors were allowed to grow for 12 days, reaching a diameter of 8–10 mm before treatment.

In Vivo Ultrasound Application

On the day of treatment, animals were anesthetized with an intraperitoneal combination injection of ketamine hydrochloride (1.56 ml/kg body weight), xylazine (0.52 ml/kg body weight), and sterilized water (3.12 ml/kg body weight). The tail vein of each animal was cannulated for intravenous administration of an MB solution. A water-based ultrasound gel (Aquasonic 100, Parker Laboratories, Inc.) was applied to the skin above the flank tumor, and a 0.75-in-diameter 1-MHz unfocused transducer (A314S, Panametrics) was coupled to the skin as shown in Fig. 1. The MBs (10^5 MBs/g body weight in 0.3 ml of 0.9% saline) were continuously infused intravenously with a pump (PHD 2000, Harvard Apparatus) for the duration of the experiment. Four pulse sequences were investigated (Fig. 2). Pulse sequences varied in the number of consecutive 1-MHz sinusoids each of 1-V peak-to-peak amplitude from a waveform generator (AFG-310, Tektronix, Inc.). The waveform signal was amplified by a 55-dB radiofrequency power amplifier (ENI 3100LA, Electronic Navigation Industries). The maximum peak negative pressure at the focus of the transducer, as measured using a needle hydrophone (PVDF-Z44-0400, Specialty Engineering Associates) was 1.0–1.2 MPa. The temperature profile in the tumor was measured during treatment by inserting a 30-gauge needle thermocouple probe (Omega T-type, HYP1-30-1/2-T-G-60-SMPW-M) into the tumor. Temperature output voltage was registered in an

electronically compensated isothermal terminal block (TC-2190, National Instrument) and recorded every 5 minutes.

Tumor Perfusion Quantification

Cadence CPS tumor perfusion measurements were made using an Acuson Sequoia 512 ultrasonography system (Siemens Medical Solutions) equipped with an 8–15-MHz linear 15L8 probe and nonharmonic imaging software. Prior to imaging, a lipid MB solution (10^8 MBs/g body weight in 0.3 ml of 0.9% saline) was infused intravenously at a rate of 15 μ l/minute. Tumors were first scanned using B-Mode ultrasonography to obtain the best imaging plane. In cadence CPS mode, tumors were scanned with a mechanical index of 0.25. A CPS video clip was acquired 5 seconds prior to and 20 seconds after a high-amplitude pulse at a frame rate of 13 Hz. Therapeutic low-frequency treatment was initiated 10 minutes after the pretreatment scan to allow lipid MBs to clear from the circulation. Perfusion measurements were repeated 10 minutes after treatment to allow albumin MBs to clear from the circulation. Perfusion recovery curves of the form $y = A(1 - e^{-\beta t}) + C$ were fit to CPS data.^{4,28,36} A relative measure of red blood cell velocity (b) was evaluated pretreatment and posttreatment.

Data generated from the CPS video files were broken into frame-by-frame image sequences to determine perfused area. Compaq Visual Fortran was used to generate a time-averaged image for image sequences between the destructive pulse and 8 seconds after the destructive pulse. The area of tumor containing intravascular contrast agent was determined by applying a threshold of 247 to the 8-bit (ImageJ) time-averaged CPS image and quantifying the percentage of enhanced pixels within the tumor volume. This method provided a quantitative measurement closely related to blood volume. The percentage of perfused area for a minimum of 4 CPS clips was averaged to obtain the final perfused area. Relative tumor blood flow was defined as the product of the percentage of perfused area and blood velocity.

Tumor Processing and Analysis

Seven days after treatment, animals were euthanized, and the left ventricle was cannulated. Blood was exsanguinated with 10 ml of 1% heparinized Tris buffered saline, followed by 10 ml of Tris buffered saline, each for 10 minutes at 100 mm Hg. Following blood removal, tumors were perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline (4°C) for 60 minutes. Specimens were excised, embedded in paraffin, and cut into 5- μ m sections. Standard H & E staining was performed to evaluate histological changes that may have occurred as a result of the ultrasound exposures. The detection of apoptotic cells was performed using an TUNEL assay (ApoptTag kit, Intergen Co.).

Tumor Growth Rate

Tumor volume (V) was evaluated by taking daily measurements with digital calipers. The volume was calculated using an ellipsoid approximation: $V = 1/6 \pi abc$, where a, b, and c are the maximum diameters of the tumor measured in 3 orthogonal planes.

Statistical Analysis

Data in Figs. 3, 5, and 6 were analyzed by 2-way repeated-measures ANOVA followed by pairwise comparisons with the Holm-Sidak method. Data in Fig. 4 were analyzed by 1-way ANOVA, followed by comparisons with the Holm-Sidak method. Significance was assessed at $p < 0.05$. The numbers of animals per group are provided in the figure legends.

Results

Ultrasonic MB Destruction Reduces Tumor Perfusion

Changes in blood velocity, perfused area, and fractional blood flow were quantified after ultrasonic MB treatment with the pulsing sequences described in Fig. 2. At all duty cycles tested (0.00002, 0.0001, 0.005 and 0.01), significant decreases in all 3 hemodynamic variables were observed after treatment (Fig. 3). In particular, treatments with duty cycles of 0.005 and 0.01 ultrasound elicited 44- and 86-fold decreases in tumor blood flow, respectively.

Ultrasonic MB Destruction Elicits Tumor Necrosis and Apoptosis

Tumors harvested 7 days after treatment were analyzed using H & E and TUNEL staining (Fig. 4). Staining with H & E (Fig. 4A and B) indicated that significantly more necrotic tissue was present in tumors treated with ultrasound at duty cycles of 0.0001, 0.005, and 0.01 than in untreated control tumors (Fig. 4C). In TUNEL assays, apoptotic cells were stained brown, and nuclei were counterstained using methyl green (Fig. 4D and E). Untreated tumors showed predominantly healthy cells with only limited areas staining positive for apoptosis (Fig. 4D), while ultrasonic MB-treated tumor sections exhibited more extensive apoptosis (Fig. 4E). When compared with untreated control tumors, the number of apoptotic cells per high power field of view increased significantly in all treatment groups 7 days after treatment (Fig. 4F).

Ultrasonic MB Destruction Inhibits Tumor Growth in a Duty Cycle Dependent Manner

Tumors were measured using calipers on a daily basis to assess their growth. Tumor growth curves following treatment with ultrasound and MBs are shown in Fig. 5. Treating tumors with ultrasound at a duty cycle of 0.0001 had no significant effect on tumor growth compared with untreated controls. However, treatments at relatively higher duty cycles (that is, 0.005 and 0.01) significantly inhibited tumor growth. In particular, 7 days after treatment with the 0.005 and 0.01 duty cycle pulsing protocols, 3.02 ± 1.52 -fold and 2.17 ± 0.63 -fold increases in tumor volume were observed, respectively, compared with an 8.72 ± 5.16 -fold increase in tumor volume for untreated controls.

Intratumor Temperature Increases Moderately With Duty Cycle

Intratumor temperature was monitored throughout treatment for groups treated with duty cycles of 0.0001, 0.005, and 0.01. Temperature did not change appreciably in the group treated with a duty cycle of 0.0001; however, temperatures in groups receiving ultrasound at duty cycles of 0.005 and 0.01 steadily increased and became significantly greater 15 minutes into treatment than the temperature in the group treated with a duty cycle of 0.0001 (Fig.

6A). Nonetheless, absolute maximum temperature increases were only moderate, reaching $2.9^{\circ}\text{C} \pm 0.89^{\circ}\text{C}$ and $5.4^{\circ}\text{C} \pm 1.2^{\circ}\text{C}$ for the groups treated with duty cycles of 0.005 and 0.01, respectively, at 60 minutes. Immediately after treatment, the gross appearance of the skin over the tumor was completely normal, with no signs of thermal damage. Furthermore, H & E staining confirmed this gross observation, as no histological changes in the overlying skin were evident at the microscopic level between ultrasound-treated and untreated tumors (Fig. 6B and C).

Discussion

Mechanical Brain Tumor Ablation With Ultrasonic MB Destruction: Rationale and Potential Limitations

The application of transcranial HIFU for the thermal ablation of a targeted mass remains difficult due to the potential for nonspecific damage in overlying or surrounding tissue.¹⁸ The MBs may be used to overcome such issues because they can lower the acoustic power required by HIFU for tissue ablation, suggesting that lesions may be generated with a lower associated temperature elevation.^{11,20,29} Reducing the threshold for lesion formation in turn lowers the probability of heat accumulation in off-target tissues, such as the bony skull and/or the brain surface. In addition, MBs may also be used to create mechanical ablation (that is, nonthermal effects) at reduced time-averaged acoustic intensities that are well below those levels required for MB enhanced thermal ablation. Although the exact mechanisms of mechanical ablation are not entirely clear and are dependent on many variables, it has been demonstrated that localized stresses and pressures resulting from MB cavitation can mechanically fragment and homogenize tissue, resulting in cellular destruction without significant thermal accumulation.²⁷ Since circulating MBs are present in microvessel lumens, it is thought that mechanical stresses imposed on the targeted tissue by MB cavitation can specifically be used to damage the microvasculature.^{20,24,30} In our study, we observed substantial decreases in tumor perfusion posttreatment and a trend toward greater reductions in tumor perfusion with increased duty cycle (Fig. 3), consistent with the hypothesis that the hydraulic resistance of the tumor microcirculation was increased due to widespread microvessel ablation.

However, it is important to note that, at least with these particular pulsing protocols, we were unable to completely inhibit tumor perfusion. It is likely that incomplete perfusion blockade occurred, at least in part, because some tumor capillaries were too large in diameter to be mechanically impacted by MB oscillation and/or destruction. Indeed, unlike normal tissues in which capillary diameters are typically small (that is, ~ 5 μm) and relatively uniform, tumor capillaries are often grossly dilated. While this feature of the tumor microcirculation could be an impediment to the eventual development of this method for clinical use, it is also possible that MB dimensions and/or ultrasound frequency could be tuned to achieve ablation in dilated tumor capillaries. By the same token, brain tumors exhibiting smaller capillary diameters on average may be more sensitive to treatment. Clearly, future studies are needed to determine whether such relationships between tumor capillary diameter, posttreatment perfusion, and brain tumor growth exist. Also, future

studies could explore the coupling of MBs with antitumoral agents to further induce growth inhibition of malignant brain tumors.

Potential Mechanisms of Tumor Growth Inhibition by Ultrasonic MB Destruction

As previously noted, we postulate that microvascular damage due to ultrasonic MB destruction leads to a sequential reduction in blood flow, tissue oxygenation and/or the delivery of nutrients, and the number of viable tumor cells. In support of this hypothesis, we note that correlations were observed between duty cycle, posttreatment blood flow, and tumor growth inhibition. For example, although not achieving statistical significance, we observed trends toward reduced posttreatment flows (Fig. 3C) and perfused areas (Fig. 3B) with higher ultrasound duty cycles. Furthermore, an apparent trend toward increased necrotic area with higher duty cycles appears to exist (Fig. 4C), and tumor growth inhibition was clearly enhanced at higher duty cycles (0.01 and 0.005) when compared with the lowest duty cycle (0.0001) (Fig. 5). Thus, at each duty cycle, we can directly relate posttreatment perfused area and blood flow to tumor growth inhibition. Based on these relationships, we contend that greater reduction in flow and the perfused area correlate with enhanced growth inhibition.

However, it is also important to consider that ultrasonic MB destruction may have caused growth inhibition through one or more alternative mechanisms. Three such potential alternative mechanisms are discussed here. First, it is possible that tumor cell damage resulted directly from intravascular MB cavitation, including gas jet streaming and enhanced shear stress.³¹ Second, it is possible that apoptosis could be initiated by the generation of oxygen free radicals,²⁶ which could occur through an “ischemia-reperfusion” response created by the eventual restoration of perfusion to some regions of the tumor after treatment. Third, it is possible that MB destruction triggered the release of endogenous “danger signals” that elicited a systemic antitumor response.¹⁰ In studies by other investigators, it has been reported that the release of such signals from tumors treated with mechanical effects are greater than those released after thermal ablation, leading to systemic antitumor immune responses.¹⁰ In the animal model used in this study, mice were T- and B-cell deficient due to deletion of the *RAG-1* gene; however, these mice did have nonspecific immune cells (that is, neutrophils and natural killer cells), and it is possible that these cell types played a role in regressing tumor growth in this particular model. Furthermore, it is reasonable to assume that degree of cellular damage, and thus the extent of the nonspecific immune response, would increase with treatment at higher duty cycles. Certainly, further analysis would need to be performed to determine the degree of immune response following treatment.

While it is possible that tumor growth was inhibited through multiple mechanisms, our results suggest that intratumor temperature changes during treatment were unlikely to have significantly contributed to growth inhibition. As shown in Fig. 6A, treatment with the 0.005- and 0.01-duty cycle pulsing protocols resulted in maximum tumor core temperature increases of $2.9^{\circ}\text{C} \pm 0.89^{\circ}\text{C}$ and $5.4^{\circ}\text{C} \pm 1.2^{\circ}\text{C}$, respectively. For the group treated with a duty cycle of 0.01, during the 60-minute duration of the experiment, the mean intratumor temperature was 3.0°C above ambient, yielding a thermal total dose of $180^{\circ}\text{C} \times 1$ minute. In

comparison, it has been shown that there is a 50% chance of damage when brain tissue is heated to 6°C above ambient for 17.5 equivalent minutes, which is a thermal dose of 105°C × 1 minute.¹⁹ While the thermal dose was higher in our study, there is an important caveat. Specifically, we observed that cooling due to the ultrasound coupling gel over the subcutaneous tumor reduced the initial ambient intratumor temperature to 30.4°C ± 1.4 °C. Therefore, with a maximum temperature increase of 5.4°C, absolute tumor temperature never rose higher than 37°C in this particular study, making it highly unlikely that bulk thermal effects led to tumor growth inhibition.

Nonetheless, even though bulk tumor temperature was only slightly increased, MB cavitation is known to induce localized “hot spots” within tissue, indicating that volumetric temperature measurements might be much lower than instantaneous temperature elevations occurring around individual MBs in the microvasculature during insonication.¹⁷ Based on these results, it is reasonable to suggest that thermal contributions of treatment, while presumably minimal compared with the mechanical effects, cannot be ruled out, and are most likely greatest surrounding the vasculature.

Conclusions

The central finding of this study is that MB destruction with low-duty cycle (0.005 and 0.01) 1-MHz ultrasound can be used to markedly inhibit brain tumor growth, with treated tumors exhibiting approximately 3–4-fold reductions in tumor volume at 7 days after treatment. Examination of hemodynamic parameters by contrast-enhanced ultrasonound revealed that, immediately posttreatment, blood velocity, perfused area, and blood flow were all significantly reduced. In addition, at 7 days after treatment, tumor apoptosis and necrosis were significantly enhanced. These therapeutic effects were generated in the presence of slight temperature increases; however, they were unlikely to have significantly affected tumor tissue. Based on these results, we postulate that the therapeutic effect created by ultrasonic MB destruction was due to microvascular ablation and the subsequent blockade of tumor perfusion. In turn, this lack of blood flow led to growth inhibition through enhanced tumor apoptosis and necrosis. Because clinical HIFU treatments of superficially located brain tumors require that ultrasound be transmitted through small regions of the skull, thermal ablation with HIFU and long duty cycles may create unacceptable brain surface heating. Our results suggest that, in these cases, the use of very short duty cycles in combination with MBs may be a feasible alternative approach for inhibiting brain tumor growth.

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Abbreviations used in this paper

CPS	contrast pulse sequencing
HIFU	high-intensity focused ultrasound

MB	microbubble
TUNEL	terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling

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Fig. 1.
Schematic illustration of the mouse tumor model with ultrasound exposure.

Fig. 2. Schematic illustration of the ultrasound pulse sequences. Note that, apart from the number of bursts per pulse and the number of sinusoids per burst, other acoustic variables were kept constant. The *asterisks* indicate 50 msec between consecutive bursts.

Fig. 3. Microbubble insonation reduces tumor blood velocity, perfused territory, and blood flow. Bar graphs of perfused area (A), blood velocity (β) (B), and tumor blood flow (C) before and after treatment for duty cycles of 0.00002 (8 animals), 0.0001 (9 animals), 0.005 (8 animals), and 0.01 (8 animals). *Significantly different from pretreatment ($p < 0.05$).

Fig. 4.

Microbubble insonation increases tumor necrosis and apoptosis. **A and B:**

Photomicrographs obtained in control tumors (**A**) and in tumors harvested 7 days after treatment with MBs and the 0.005 duty cycle pulsing protocol (**B**). **C:** Bar graph showing the percentage of necrotic tumor area for control tumors (6 mice) and tumors treated with ultrasound duty cycles of 0.00002 (5 mice), 0.0001 (5 mice), 0.005 (8 mice) and 0.01 (8 mice). **D and E:** Photomicrographs from control tumors (**D**) and tumors harvested 7 days after treatment with an ultrasound duty cycle of 0.005 (**E**). Arrows denote apoptotic cells (brown). **F:** Bar graph of apoptotic cells per 50× field of view (F.O.V.) for control tumors (6 mice) and tumors treated with duty cycles of 0.00002 (5 mice), 0.0001 (6 mice), 0.005 (8 mice), and 0.01 (8 mice). *Significantly different from control (p < 0.05). H & E (**A and B**); TUNEL (**D and E**). N = necrotic tissue; V = viable tissue.

Fig. 5.

Microbubble insonation inhibits tumor growth. Line graph depicting tumor growth (fold-change over Day 1) as a function of time posttreatment for untreated control tumors (16 mice) and tumors treated with duty cycles of 0.0001 (5 mice), 0.005 (8 mice), and 0.01 (8 mice). *Significantly different from the untreated control group and the 0.0001 duty cycle group at the same time point ($p < 0.05$). **Significantly different from untreated the control group at the same time point ($p < 0.05$).

Fig. 6.

A: Tumor temperature increases moderately as duty cycle increases. Line graph depicting changes in temperature increasing above ambient as a function of time during 60-minute treatments for ultrasound duty cycles of 0.0001 (4 mice), 0.005 (7 mice), and 0.01 (4 mice). *Significantly different from all other groups at the same time point ($p < 0.05$). **B and C:** Photomicrographs of the skin overlying ultrasound-treated (**B**) and untreated (**C**) tumors. The skin appears unchanged histologically, illustrating that no apparent thermal damage occurred in this tissue layer. H & E.