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Enhancement in blood-tumor barrier permeability and delivery of liposomal doxorubicin using focused ultrasound and microbubbles: evaluation during tumor progression in a rat glioma model

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Abstract

Effective drug delivery to brain tumors is often challenging because of the heterogeneous permeability of the “blood tumor barrier” (BTB) along with other factors such as increased interstitial pressure and drug efflux pumps. Focused ultrasound (FUS) combined with microbubbles can enhance the permeability of the BTB in brain tumors, as well as the blood-brain barrier in the surrounding tissue. In this study, dynamic contrast-enhanced MRI (DCE-MRI) was used to characterize the FUS-induced permeability changes of the BTB in a rat glioma model at different times after implantation. 9L gliosarcoma cells were implanted in both hemispheres in male rats. At day 9, 14, or 17 days after implantation, FUS-induced BTB disruption using 690 kHz ultrasound and Definity microbubbles was performed in one tumor in each animal. Before FUS, liposomal doxorubicin was administered at a dose of 5.67 mg/kg. This chemotherapy agent was shown previously to improve survival in animal glioma models. The transfer coefficient K_{trans} describing extravasation of the MRI contrast agent Gd-DTPA was measured via DCE-MRI before and after sonication. We found that tumor doxorubicin concentrations increased monotonically (823 ± 600 , 1817 ± 732 and 2432 ± 448 ng/g) in the control tumors at 9, 14 and 17 days. With FUS-induced BTB disruption, the doxorubicin concentrations were enhanced significantly ($P < 0.05$, $P < 0.01$, and $P < 0.0001$ at days 9, 14, and 17, respectively) and were greater than the control tumors by a factor of two or more (2222 ± 784 , 3687 ± 796 and 5658 ± 821 ng/g) regardless of the stage of tumor growth. The transfer coefficient K_{trans} was significantly ($p < 0.05$) enhanced compared to control tumors only at day 9 but not at day 14 or 17. These results suggest that FUS-induced enhancements in tumor drug delivery are relatively consistent over time, at least in this tumor model. These results are encouraging for the use of large drug carriers, as they suggest that even large/late-stage tumors can benefit from FUS-induced drug enhancement. Corresponding enhancements in K_{trans} were found variable in large/late-stage tumors and not significantly

different than controls, perhaps reflecting the size mismatch between the liposomal drug (~100 nm) and Gd-DTPA (molecular weight: 938 Da; hydrodynamic diameter: ~2 nm). It may be necessary to use a larger MRI contrast agent to effectively evaluate the sonication-induced enhanced permeabilization in large/late-stage tumors when a large drug carrier such as a liposome is used.

Keywords

Focused ultrasound; brain drug delivery; blood brain barrier; blood tumor barrier; dynamic contrast enhanced MRI; pharmacokinetic model

Introduction

The blood-brain barrier (BBB) is a major limitation to the use of drugs in the brain (Pardridge 2002) because it restricts the delivery of most therapeutic agents with molecular weight greater than 400 Da. This limitation has hindered the development of effective drugs for a wide range of disorders, including Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), neuro-AIDS, stroke, brain or spinal cord trauma, autism, lysosomal storage disorders, fragile X syndrome, inherited ataxias, and blindness. Tumors, particularly those in the brain, also have challenges for effective drug delivery. While the blood vessels in most brain tumors, including metastases, often do not have a fully intact BBB and are somewhat permeable, infiltrating cancer cells at the tumor margins and small metastatic seeds may be protected by the BBB of the surrounding normal and intact tissue (Eichler *et al* 2011). Furthermore, it is known that the tumor vasculature permeability is heterogeneous, and there are additional barriers to drug delivery such as increased interstitial pressures (Fukumura and Jain 2007) and drug efflux pumps that contribute to resistance of brain tumors to chemotherapy. Techniques that can deliver enhance drug delivery across both the "blood-tumor barrier" (BTB) and the BBB in the surrounding brain could enable the use of a wide range of anticancer agents in neurooncology.

In order to overcome these limitations, it is necessary to either bypass these vascular barriers altogether, or to facilitate passage across them via controlled exploitation of endogenous transport mechanisms. Different methods have been explored to bypass the BBB (Groothuis 2000, Pardridge 2007, Blumling Iii and Silva 2012). While these methods are promising, they also have limitations in that they are invasive or non-localized, which lengthens recovery and endangers normal healthy tissue. Over a decade ago, Hynynen *et al.* discovered that if short ultrasound bursts are preceded by an injection of a microbubble ultrasound contrast agent, the BBB can be consistently opened without the production of lesions or apparent neuronal damage (Hynynen *et al* 2001). Since 2001, it is becoming an emerging technology for brain drug delivery in a non-invasive, repeatable and targeted manner (Aryal *et al* 2014). The microbubbles, which are constrained to the vasculature, interact strongly with even low-intensity ultrasound, producing mechanical forces on the endothelium that result in transient disassembly of tight junctional complexes and the induction of active transport processes (Shang *et al* 2011, Sheikov *et al* 2004). No significant adverse effects have been observed resulting from this temporary BBB disruption (Tung *et al* 2010,

McDannold *et al* 2012, 2005). The barrier is restored in a few hours (Hynynen *et al* 2001), providing a time-window to deliver even long-circulating agents. Prior work in animals has demonstrated the delivery of a wide range of imaging and therapeutic agents, even large agents such as antibodies, nanoparticles, and liposomally-encapsulated drugs (Jordão *et al* 2010, Kinoshita *et al* 2006, Liu *et al* 2010b, Treat *et al* 2007). The method can also increase the delivery of agents to brain tumors (Liu *et al* 2010a, Mei *et al* 2009), and several studies have shown that it can improve outcomes in animal brain tumor models (Wei *et al* 2013, Aryal *et al* 2013, Liu *et al* 2010a, Chen *et al* 2010, Park *et al* 2012a, Treat *et al* 2012). While in some cases the response has been modest, multiple treatments were effective in achieving a pronounced improvement (Aryal *et al* 2013).

While delivery of these agents is promising, it would be desirable to demonstrate that the amount of drug delivered and its penetration into the brain parenchyma is sufficient to produce a therapeutic response. Ideally, this would be performed for each patient. Post-treatment imaging could provide this assurance by labeling the drug with a contrast agent for MRI or other imaging modality (Fan *et al* 2013). It might also be possible to use a standard contrast agent as a surrogate measurement, which would be advantageous since it could be used with existing drugs. A number of studies have related signal intensity changes in contrast-enhanced MRI after BBB disruption with tissue drug concentrations (Treat *et al* 2007, Kinoshita *et al* 2006, Yang *et al* 2010). More quantitative and repeatable techniques, such as estimating contrast agent concentrations via T1-mapping (Marty *et al* 2012, Chu *et al* 2013) or vascular transfer coefficients via analysis of dynamic contrast-enhanced MRI (DCE-MRI) (Vlachos *et al* 2010a) have been used to perform spatial and temporal characterization of BBB permeability after FUS. DCE-MRI can also been investigated to predict the resulting payload of drugs to the brain (Park *et al* 2012b) and in tumors (Chu *et al* 2013).

Despite these promising findings, one needs to be careful when expanding to different patient populations or if different drugs are used. The relationship between the delivery of a contrast agent and a therapeutic can be complicated. For example, a relationship found in the normal brain may not translate to patients with CNS disorders. This challenge could be particularly difficult in tumors where the vascular properties can change over time (Jain *et al* 1997). Further complications may arise when the therapeutic has a different size or other chemical property than the contrast agent. The purpose of this work was to investigate permeability changes induced by FUS and microbubbles for an MRI contrast agent with a relatively low molecular weight in a rat glioma model at different time points. These changes were then compared to concentrations of the drug doxorubicin, which was encapsulated in liposomes and administered immediately before the ultrasound exposures.

Materials and Methods

Sonication System

An air-backed, single element, 690 kHz focused piezoelectric transducer (diameter/radius of curvature: 100/80 mm) generated the ultrasound field. It was driven by an arbitrary waveform generator (model 395, Wavetek) and RF amplifier (240L, ENI); electric power was measured with a power meter (E4419B, Agilent,) and dual-directional coupler

(C5948-10, Werlatone). Reported exposure levels are absolute peak negative pressure amplitudes measured in water with a membrane hydrophone (Marconi; 0.5 mm diameter). Attenuation by the brain and rat skull is expected to reduce the pressure amplitude by ~30% at this frequency with additional uncertainty arising from standing waves within the skull and increases in skull thickness as the animal ages (O'Reilly *et al* 2011). The pressure distribution of the transducer was mapped using a 0.2 mm needle hydrophone (Onda, Sunnyvale, CA); its half-maximum diameter and length were 2.3 and 12 mm, respectively. The transducer efficiency was measured using a radiation force-balance.

Experimental Setup

The sonication system was operated within a clinical 3T MRI scanner (Signa; GE Healthcare). The transducer was immersed in a small tank of degassed, deionized water and attached to an MRI-compatible, manually-operated positioning system (Fig. 1). The animal was laid supine on a tray above this tank, with a water bag providing an acoustic path to the dorsal surface of the head. Images were obtained with a 7.5 cm-diameter transmit/receive MRI surface coil. The animal's body temperature was maintained with a heated water pad. Before treatment, we visualized heating in a silicone phantom using temperature-sensitive MRI to localize the acoustic focal point in the MRI space. Accurate targeting *in vivo* was confirmed by sonicating a spot in the brain near the tumor and checking that the resulting MRI contrast extravasation was at the desired target.

Animals

The experiments were approved by our institutional animal committee. Tests were performed in 17 Male Sprague-Dawley rats (Charles River Laboratories; ~250 g, ~ 8 weeks old). Before each procedure, the animals were anesthetized via intraperitoneal injections of Ketamine (80 ml/kg/h) and Xylazine (10 ml/kg/h). A catheter was placed in the tail vein, and the hair on the scalp was removed with clippers and depilatory cream. 9L rat gliosarcoma cells (passage number 3, obtained from the Neurosurgery Tissue Bank at UCSF) were grown in Minimum Essential Medium (1×) with Earle's salts, supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% MEM nonessential amino acids, and 0.1% gentamicin in a 5% CO₂ chamber held at 37°C. In order to minimize the number of rats used, tumors were implanted in both hemispheres. After preparing the skin, a 5-mm skin incision was made and 1-mm diameter burr holes were drilled into the skull 1 mm anterior to the bregma and 2 mm lateral to the midline in both hemispheres. A 4 µl volume of cell suspension (1×10^5 cells) was injected into the right and left caudate putamen at a depth of 3.5 mm relative to the dural surface using a 10 µl gas-tight syringe (Hamilton). Cells were injected over five minutes; two minutes later the needle was retracted slowly over another 5 minutes, and the skin was sutured. The sutures were removed 5 days later, and tumors on both hemispheres were observed in MRI on day 7 or 8. Animals were included in the study if the diameter of both tumors were both between 1–3 mm on day 7 or 8. The animals were euthanized if they exhibited severely impaired activity, weight loss exceeding 20% within one week, or tumor dimensions exceeding 10–11 mm.

Sonifications

The rats were randomly assigned to one of three groups where FUS was applied on day 9, day 14, or day 17 after implantation (N=5 for each group). After determining the coordinates of the focal point within the MRI space, the focal region was positioned in the tumor in the right hemisphere. The left tumors served as controls. Ultrasound bursts were then applied at multiple points in and around the tumor at pressures amplitudes ranging from 0.55–0.81 MPa (burst length: 10ms, pulse repetition frequency: 1 Hz, duration: 60s). The pressure amplitude was initially set based on a prior study in rats with this device (Pilatou *et al* 2008). It was increased based on the age and weight of the animals to achieve a consistent level of BBB disruption. This observation was made in our initial treatments and is similar to previous reports (O'Reilly *et al* 2011). Before each sonication, ultrasonic contrast agent (Definity, Lantheus Medical Imaging; 0.01 mL/kg) was administered intravenously. To facilitate the injections of such a small volume, the agent was diluted to 0.1× normal concentration in PBS. It was injected as a bolus approximately 9 s before each sonication, followed by a 0.2 ml saline flush. The sonications were delivered at 5 min intervals in a grid pattern (spacing: 1.5 mm) to 5–20 targets, depending on the tumor volume. We aimed to permeabilize the BTB in the entire tumor and the BBB in a surrounding rim of at least 1 mm.

For drug delivery, 5.67 mg/kg of doxorubicin hydrochloride encapsulated in long-circulating pegylated liposomes: Lipo-Dox (TTY Biopharm) was administered over multiple slow injections intravenously just before each sonication. This dosage was selected based on prior work that tested this agent in rats (Arnold *et al* 2005). This scheme aimed to ensure a high drug concentration in the blood stream during every sonication. Each injection was followed by a 0.2 ml saline flush.

Magnetic resonance imaging

MRI was used to plan the sonications, to evaluate the tumor appearance and growth over time, to evaluate the BTB/BBB permeabilization, and to detect hemorrhagic areas. Two-dimensional T2-weighted fast spin-echo (FSE) images were acquired in three orthogonal planes to determine the size and location of the tumor (parameters: repetition time (TR): 2000 ms; echo time (TE): 79.7 ms; echo train length (ETL): 8; matrix size: 256×256; slice thickness/spacing: 1 mm/interleaved; field of view (FOV): 8 cm; number of excitations/averages (NEX): 2; bandwidth: 15.63 kHz). Axial three-dimensional T2*-weighted fast spoiled gradient echo images were also acquired (TR/TE: 33.3/19 ms; matrix size: 256×256×28; slice thickness: 0.8 mm; FOV: 8 cm; NEX: 1; flip angle: 15°; bandwidth: 15.63 kHz). These images are sensitive to small susceptibility changes and were used for detecting petechiae caused by excessive ultrasound exposure levels (Hynynen *et al* 2001) and hemorrhagic regions within the tumor.

Before and after the sonications, DCE imaging (Tofts 1997, Tofts *et al* 1999) was performed using methods based on a prior study on non-tumor bearing rats (Park *et al* 2012b). Before contrast was injected, maps of the initial relaxation time T_1 were obtained using a spoiled gradient echo (SPGR) sequence (TR/TE: 9.9/4.8 ms; matrix size: 256×256; slice thickness: 2 mm; FOV: 8 cm; scan time: 15.3s; bandwidth: 31.25 kHz) acquired at multiple flip angles

(30/20/15/10/2°) (Wang *et al* 1987). For the DCE imaging, eight pre-contrast sets of SPGR images (TR/TE: 9.9/4.8 ms; matrix size: 256×256; slice thickness: 2 mm; FOV: 8 cm; flip angle: 35°; bandwidth: 31.25 kHz) were acquired. Then, a bolus of the MRI contrast agent gadopentatate dimeglumine (Magnevist Gd-DTPA; Bayer Healthcare; 0.5 mL/kg) was administered IV, and an additional 62 sets of SPGR images were acquired with a temporal resolution of 7.6 s for 8 min. This contrast agent (molecular weight: 938 Da, hydrodynamic diameter: 1.8 nm (Zhang *et al* 2014), plasma half-life in rats: 13 min (Vexler *et al* 1994)) does not normally cross the BBB. A double dose of Gd-DTPA was used to secure the detection of the bolus peak in the vascular unit (ophthalmic artery or transverse sinus artery) to calculate the arterial input function and to improve the signal to noise ratio. Identical slice locations were used for T1 mapping and DCE-MRI. The time interval between the two DEC-MRI measurements was at least an hour to avoid excessive Gd-DTPA accumulation. Due to concerns about the animals' health and their ability to tolerate a long experiment, pre-FUS DCE-MRI was not obtained in 3 animals. After DCE-MRI was obtained, axial T1-weighted FSE images were acquired for additional contrast imaging (TR/TE: 500/13 ms; ETL: 4; matrix size: 256×256; slice thickness/spacing: 1 mm/interleaved; FOV: 8 cm; NEX: 4; flip angle: 90°; bandwidth: 15.63 kHz).

Fluorometric assay and Doxorubicin Extraction

After the completion of the sonication and imaging session, Trypan Blue (Aldrich Chemical Company, Inc. Milwaukee, WI, USA; 2.5ml/kg) was administered through the tail vein to mark the regions where the BBB was disrupted and to improve visualization of the tumors. Animals were deeply anaesthetized with ketamine/xylazine and sacrificed about two hours after the last sonication. After transcardial perfusion with normal saline (0.9% NaCl, 250 mL) to flush unabsorbed liposomal doxorubicin from the cerebral vasculature, the brain was removed. Small tissue volumes (approximately 30 mg) from both tumor sites which were identified by its Trypan blue stain were harvested. Samples were homogenized in a tissue blender (Next Advance, Averill Park, NY) with acidified ethanol (50% ethanol in 0.3 N HCl), refrigerated for 24 hours at 4°C, and centrifuged at $16,000 \times g$ for 25 min. at 4°C. The fluorescent intensity of the extracted supernatant was measured using a benchtop fluorometer (VersaFluor; Bio-Rad Laboratories, Hercules, CA; Ex/Em: 480/590 nm). The concentration of doxorubicin was quantified by linear regression and a standard curve derived from eight serial concentrations. The concentration in each tissue sample was determined by taking the average of at least of three readings. Measurements were normalized to those obtained from a control animal that did not receive doxorubicin to correct for autofluorescence.

Data analysis

Tumor boundaries were manually segmented in axial T2-weighted images using ImageJ; the volume was calculated by the sum of the areas multiplied by the image thickness. On day 9, day 14 and day 17, tumors appeared as clearly circumscribed volumes which were sometimes surrounded by hyperintense regions (presumably edema).

The DCE MRI data were fit with the TOPPCAT software package (Barboriak 2004) to the modified Tofts-Kermode model (Tofts and Kermode 1991). This two-compartment, three-

parameter model uses the following equation to estimate the tissue concentration $C_t(t)$ of an MRI contrast agent vs. time:

$$C_t(t) = K_{trans} \int_0^t C_p(t') e^{\frac{K_{trans}}{v_e}(t-t')} dt' + v_p C_p(t),$$

where t is the time, $C_p(t)$ is the contrast agent concentration in plasma, K_{trans} is the influx forward volume transfer constant (describing transfer from the extravascular extracellular space (EES) to plasma), and v_e is the fractional volume of EES per unit volume of tissue. The software excludes the exponential term since either the back diffusion rate K_{trans}/v_e is small, $t-t'$ is small, or both (Barboriak 2004). It uses Patlak analysis to estimate maps of K_{trans} and v_e . Gd-DTPA concentrations were estimated using the signal intensity equation for the SPGR sequence, the initial relaxation time T_1 of the tissue, and assumed a relaxivity of $4.39 \text{ s}^{-1}\text{mM}^{-1}$ for Gd-DTPA (Li et al 2000). $C_p(t)$ was chosen from a region of interest in a vascular unit (ophthalmic artery or transverse sinus) and was adjusted for a 45% capillary hematocrit level (Barboriak 2004).

The entire tumor was manually segmented in the K_{trans} maps in Matlab (MathWorks), and the mean value was calculated. Rats that had regions within either tumor with little or no signal enhancement after Gd-DTPA administration were excluded, as these regions were assumed to be necrotic. Tumor volumes, measurements of K_{trans} and v_p , and doxorubicin concentrations for the sonicated and non-sonicated tumors were compared using unpaired, two-tailed t-tests. Comparisons of K_{trans} and v_p before and after sonication were made using a paired t-test. Other analysis included linear least-squares regression and calculation of correlation coefficients. Data analysis was performed using Matlab and Microsoft Excel.

Histology

Two additional animals, one animal with tumors at day 9 and one at day 14, were sacrificed 4 hours after the last sonication via transcardial perfusion with 0.9% NaCl solution followed by 10% phosphate-buffered formalin while under deep anesthesia with ketamine and xylazine. Brains were harvested and fixed in formalin. Tissue blocks containing the tumor were embedded in paraffin and cut into 4–6 μm serial axial sections. The section with biggest tumor was stained with hemotoxylin and eosin (H&E) for examination of light microscopy. Lipo-Dox and trypan blue was not administered in the animals used for the histology study.

Results

Bilateral tumor progression and BBB/BTB disruption

The MRI appearance of the bilateral tumors on day 9, 14, and 17 were monitored in MRI (Fig. 2A) where they grew rapidly over time (Fig. 2B), resulting in significant midline shift and compression of the lateral ventricles. The tumor volumes in the right hemisphere were 14.5, 113.8 and 151.4 mm^3 at days 9, 14, and 17 respectively. The corresponding volumes of the left tumors were 16.0, 110.8, and 143.8 mm^3 , and were not significantly different ($P=0.9$) from those on the right. A higher pressure amplitude (0.81 vs 0.55 MPa) was needed

at day 17 to induce consistent BBB/BTB permeabilization, presumably because of an increase in skull or dura thickness as the rats grew (O'Reilly *et al* 2011). The sonication pattern was similar with our previous study (Aryal *et al* 2013), where 5–20 targets were applied in a grid (spacing: 1.5 mm) to cover the entire tumor and a rim of surrounding brain tissue. Post-FUS T2*-weighted images were used to detect small petechiae which would appear in this imaging as hypointense spots.

Doxorubicin concentration and kinetics of BBB/BTB permeability changes

Liposomal doxorubicin was administered in fractions immediately before each sonication, and its concentration in the tumors was measured about two hours later. The doxorubicin concentration measured post mortem via fluorometry increased monotonically as a function of tumor volume in both the left (non-sonicated) and right (sonicated) tumors. A good correlation ($R^2 = 0.58$ and 0.51 for the left and right tumors, respectively) was observed between doxorubicin concentration and tumor volume (Fig. 2C). The concentration in the sonicated tumor (2222 ± 784 , 3687 ± 796 and 5658 ± 821 ng/g) was generally greater than non-sonicated tumor (823 ± 600 , 1817 ± 732 and 2432 ± 448 ng/g).

In most animals, DCE-MRI was obtained before and after FUS-induced BBB/BTB to calculate the transfer coefficient K_{trans} for Gd-DTPA. Example maps of K_{trans} and changes of signal intensity before and after sonication are shown in Fig. 3. After FUS, the signal intensity on sonicated tumor was increased significantly ($P < 0.05$), at day 9 but not at day 14 or 17 compared with before FUS (Fig. 3A–C). In Figure 3D, the red curves represent the ROI used to extract K_{trans} values in the tumors before and after FUS. The ROI was enlarged in the sonicated tumor (indicated by the arrow). Before FUS, the K_{trans} values for the both tumors appeared similar on days 9, 14 and 17. They increased, particularly at day 9, for the sonicated tumors (shown by arrow in Fig. 3D). Comparisons of mean K_{trans} before and after sonication are shown in Fig. 4. In the non-sonicated tumor, the mean K_{trans} values ($\pm$ S.D) before FUS were 0.0068 ± 0.0027 , 0.0088 ± 0.0026 and $0.012 \pm 0.0086 \text{ min}^{-1}$ at day 9, 14 and 17, respectively (Fig. 4A); after the sonications these values were 0.0044 ± 0.0018 , 0.0103 ± 0.0047 and $0.0115 \pm 0.0015 \text{ min}^{-1}$. In the sonicated tumors, the mean K_{trans} values before FUS were 0.0077 ± 0.0053 , 0.0082 ± 0.0025 , and 0.01006 ± 0.0019 at day 9, 14, and 17, respectively. After FUS they were 0.0129 ± 0.0064 , 0.0128 ± 0.0062 and 0.0116 ± 0.0018 (Fig. 4B). No significant difference ($P > 0.05$) was observed in the mean K_{trans} between the two DCE-MRI acquisition for the non-sonicated tumor at any day. However, for the sonicated tumor, K_{trans} was significantly increased ($P < 0.05$) at day 9 but not at day 14 or 17 (Fig. 4B).

Fig. 5 shows the comparison of K_{trans} and doxorubicin concentration between the sonicated and non-sonicated tumors obtained in DCE-MRI and includes the three animals that did not have a K_{trans} measurement before sonication. Similar to the results shown in Fig. 4, K_{trans} was significantly greater ($P < 0.05$) at day 9, but not at days 14 or 17. The mean ratio of K_{trans} between the sonicated and non-sonicated tumors was 2.9 ± 3.0 , 1.3 ± 1.2 , and 0.95 ± 1.0 , respectively, at days 9, 14, and 17 (Fig. 5A). In contrast, the doxorubicin concentration was enhanced significantly at all days ($P < 0.05$ at day 9, $P < 0.01$ at day 14 and $P < 0.0001$ at day 17) (Fig. 5B). The ratio of doxorubicin concentration between the sonicated and non-

sonicated tumors was 2.7 ± 1.3 , 2.03 ± 1.08 , and 2.33 ± 1.8 for days 9, 14, and 17 respectively. Other than a moderate correlation ($R^2 = 0.44$) at day 9, there was no clear relationship between doxorubicin concentration and K_{trans} after FUS-induced BBB disruption. No significant difference ($P > 0.05$) was observed in v_p between measurements made before and after FUS, between the sonicated and non-sonicated tumors, or between tumors at different times after implantation.

Histological findings

Histological examination was performed on two rats after sonication at day 9 and 14. Tumors in both groups appeared as solid masses that replaced large amounts of brain tissue (Fig. 6A–B). The tumor bulk consisted of viable and rapidly dividing spindle-shaped cells. Tiny necrotic sites were scattered on day 14 tumor but not on day 9 (yellow square in Fig. 6B). Those sites were comparatively smaller than the rest of the tumor mass. The sonicated tumors appeared unaffected in the H&E stained sections. The only effect of note was a small number of tiny clusters of extravasated erythrocytes (petechiae) that were scattered throughout the sonicated tumor regions (Fig. 6C–D, magnified view of red squares).

Discussion

This study investigated the pharmacokinetic characteristics of small and large-molecule agents at different stages of tumor growth after BBB/BBB permeabilization with microbubble-enhanced FUS. Gd-DTPA (938 Da; hydrodynamic diameter: ~ 2 nm) and DCE-MRI were used to noninvasively provide semiquantitative measures of transport across these vascular barriers, and the concentration of doxorubicin, which was administered in ~ 100 nm liposomes, was measured in the tumor post mortem. Microbubble-enhanced FUS succeeded in increasing doxorubicin concentrations by a factor of two or more regardless of the stage of tumor growth, but the enhanced delivery of Gd-DTPA, reflected in transport coefficient K_{trans} measured in DCE-MRI, was significantly enhanced only at the early stage of tumor growth. K_{trans} was slightly elevated compared to the non-sonicated tumors at later time points, but there was substantial variation and the increases were not statistically significant. That finding is consistent with the results of a study by Chu et al., who also found that microbubble-enhanced sonication could increase delivery of Gd-DTPA in early, but not in late-stage tumors (Chu *et al* 2013).

If this finding that K_{trans} for Gd-DTPA of late-stage tumors cannot be significantly increased is correct, it suggests that there could be a maximum level enhancement in BTB permeability that can be achieved by FUS and microbubbles. Vlachos et al. found a plateau above which K_{trans} could not be increased in the normal brain (Vlachos et al 2010), and it is possible such a plateau exists in tumors as well. If such a plateau exists, these data suggest that BTB permeability might depend on the size or stage of the tumor. Since enhancement in doxorubicin delivery was observed at days 14 and 17 as well as day 9, they also might suggest that it depends on drug size as well. However, experimental details and other factors may explain our findings. For example, it is possible that we missed the peak K_{trans} in the 14 and 17 week tumors since they required more time to sonicate. Also, doxorubicin concentrations were obtained approximately two hours after sonication compared to 8

minutes for K_{trans} . With Lipo-DOX's long circulation time, the drug may have had time to accumulate in the tumor via the "enhanced permeability and retention" effect, which is thought to enable liposomes or other large particles to accumulate in a tumor due to permeable blood vessels and restricted clearance (Matsumura and Maeda 1986). K_{trans} measurements were obtained at an earlier time point, so this effect (if it were present for Gd-DTPA) would not have time to occur.

Such issues may also explain why we did not see a good correlation between the doxorubicin delivery and the K_{trans} measurements for Gd-DTPA. Such a correlation would have been useful since it could enable one to noninvasively estimate drug concentrations. Several studies have found a good correlation between signal intensity changes in contrast enhanced MRI and that of a drug or tracer in the normal brain or in tumor models (Treat et al 2007, Kinoshita et al 2006, Yang et al 2010). Most relevant to this study, Treat et al. compared MRI signal enhancement after GD-DTPA injection and doxorubicin concentration in the normal rat brain after FUS-BBBD (Treat et al 2007). Unlike in this work those experiments, which also used liposomal doxorubicin, a good correlation was observed between these two measurements. These results highlight differences between normal brain and tumor blood vessels. In contrast to the normal brain, where FUS-induced permeability changes to the BBB might be expected to be relatively reproducible, the vascularity, permeability, and other factors in a tumor can vary widely and can change over time, and it is likely that predicting the sonication-induced changes to the BTB is not straightforward.

Future work investigating agents with a range of different sizes would be necessary to fully understand these findings. In particular it would be useful to repeat these experiments using a liposomal MRI contrast agent. Experiments that obtained doxorubicin concentrations closer in time to the DCE-MRI acquisition and that obtained K_{trans} measurements at standard times after sonication could also be illuminating. Investigating K_{trans} changes as a function of the exposure level or other factor could help understand whether a permeabilization plateau exists for the BTB similar to what has been observed in the BBB (Vlachos et al 2010). Finally, the 9L gliosarcoma is a highly aggressive tumor model; it would be interesting to investigate less aggressive models to further understand how tumor development influences the ability to enhance delivery of drugs of different sizes.

The fact that FUS appeared to be more effective in delivering a drug encapsulated in a large carrier regardless of the tumor stage is encouraging, as it suggests that even late-stage tumors can benefit from FUS-induced drug enhancement. Many promising drug carriers such as liposomes and various other nano-carriers are being developed that may be useful for brain tumors if the challenges created by the BTB and BBB can be overcome. These agents can be more targeted and have fewer side effects compared to the broad-spectrum chemotherapy agent doxorubicin. However, more work is needed to confirm that drug penetration away from the blood vessels, which can be relatively small for large particles, is sufficient to achieve a therapeutic response. Prior work with liposomal doxorubicin and brain tumor models (Treat et al 2012, Aryal et al 2013), and the development of "brain-penetrating" nano-carriers (Nance et al 2014) are encouraging in this regard.

Conclusions

Vascular permeability can increase over time in rapidly growing tumors. These results suggest that significant FUS-induced enhancements in tumor drug delivery with a large, encapsulated chemotherapy agent are relatively consistent over this time, at least in this tumor model. These results are encouraging, as they suggest that even late-stage tumors can benefit from FUS-induced drug enhancement. Corresponding enhancements in K_{trans} to the MRI contrast agent Gd-DTPA were variable after the first week and did not correlate with doxorubicin delivery, perhaps reflecting not only with the size mismatch between it and the liposomal agent but also with the vascularity, permeability, and other factors in a tumor which can vary widely and can change over time. Overall, this work demonstrates that drug delivery to tumors and its enhancement via FUS-induced BBB disruption is complex and needs to take into account a number of factors including drug size, time after administration, and the stage of tumor growth.

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Figure 1. Schematic of the MRI-guided focused ultrasound system used in this work. The function generator, amplifier, and power meter were located outside the MRI room.

Figure 2.

MRI appearance of bilateral 9L gliosarcoma, their growth curves, and doxorubicin concentrations measured approximately 2 hours after the ultrasound experiments as a function of tumor volume. (A) Axial views of MRI at day 9, 14, and 17 after implantation. The rapid growth of the tumor was evident in T2-weighted MRI (T2WI), changes of BBB/BTB permeabilization were observed in contrast-enhanced T1-weighted images (CE-T1WI) after gadolinium injection, and FUS-induced tissue damage was evaluated in T2*-weighted images (T2*WI) which would appear as hypointense spots in images. (B) The growth curves shows no significant difference between the volumes of the sonicated and non-sonicated tumors ($P=0.9$). (C) Doxorubicin concentrations measured in the tumors post mortem in tumor tissue were correlated with tumor volume ($R^2=0.58$, 0.51 for the sonicated

and non-sonicated tumors, respectively). The doxorubicin concentration in the sonicated tumor was generally larger than the non-sonicated tumor.

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Figure 3.

Example results from DCE-MRI at different stages of tumor growth before and after FUS. (Left) Plots of changes in signal intensity vs. time in three tumors measured before and after FUS at different times after implantation. The signal intensity increase after FUS was significantly ($P < 0.05$) higher than the pre-sonication values at day 9, but not at day 14 or 17 compared. (Right) Maps of K_{trans} for Gd-DTPA obtained after two separate injections (before and after FUS) on three different days (9, 14 and 17 days after implantation). The red curves represent the ROI to extract K_{trans} values in tumors before and after FUS. ROI is enlarge in sonicated tumor indicated by arrow with FUS. Before FUS, the K_{trans} values for the two tumors appeared similar on days 9, 14 and 17. They increased, particularly at day 9, for the sonicated tumors (arrow). K_{trans} values are indicated in the color bar.

Figure 4.

Mean K_{trans} estimates ($\pm$ S.D.) before and after FUS for the sonicated and control (non-sonicated) tumors at different stages of tumor growth. (A) The K_{trans} measurements in the non-sonicated tumors were not significantly enhanced between the two DCE-MRI acquisitions (before and after FUS) at any day. (B) For the sonicated tumors, the mean K_{trans} was significantly increased ($P < 0.05$) at day 9 but not at day 14 or 17. (* $P < 0.05$)

Figure 5.

Mean K_{trans} estimates and doxorubicin concentrations ($\pm$ S.D.) at different stages of tumor growth for the sonicated and control (non-sonicated) tumors. (A) The K_{trans} measurements in the sonicated tumors were significantly enhanced compared those in the controls at day 9 but not at day 14 or 17. Note that these data include the three animals that did not have K_{trans} measurements before sonication. (B) Doxorubicin concentrations were significantly enhanced at all days. (* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$)

Figure 6.

Appearance of two 9L gliosarcoma at different days after implantation in histology. (A–B) Appearance of the tumors in an H&E stained section at day 9 (5×) and 14 (1.25×). The tumors appeared as solid masses that replaced large volumes of brain tissue. The bulk of tumors consisted of rapidly dividing spindle-shaped cells with some small necrotic zones evident on day 14 (yellow square in B). (C–D) Magnified views (20×) of the red boxes. Most of the sonicated region appeared unaffected, but in a few areas tiny clusters of extravasated erythrocytes (petechiae) (inset in C, magnified view of yellow square in C) were scattered throughout the sonicated tumor regions.