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Ultrasound increases nanoparticle delivery by reducing intratumoral pressure and increasing transport in epithelial and epithelial-mesenchymal transition tumors

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Abstract

Acquisition of the epithelial-mesenchymal transition (EMT) tumor phenotype is associated with impaired chemotherapeutic delivery and a poor prognosis. In this study, we investigated the application of therapeutic ultrasound methods available in the clinic to increase nanotherapeutic particle accumulation in epithelial and EMT tumors by labeling particles with a positron emission tomography tracer. Epithelial tumors were highly vascularized with tight cell-cell junctions, compared to EMT tumors where cells displayed an irregular, elongated shape with loosened cell-cell adhesions and a reduction in E-cadherin and cytokeratins 8/18 and 19. Without ultrasound, the accumulation of liposomal nanoparticles administered to tumors in vivo was ~1.5 times greater in epithelial tumors than EMT tumors. When ultrasound was applied, both nanoaccumulation and apparent tumor permeability were increased in both settings. Notably, ultrasound effects differed with thermal and mechanical indices, such that increasing the thermal ultrasound dose increased nanoaccumulation in EMT tumors. Taken together, our results illustrate how ultrasound can be used to enhance nanoparticle accumulation in tumors by reducing their intratumoral pressure and increasing their vascular permeability.

Keywords

ultrasound; EMT; drug delivery; hyperthermia; pharmacokinetic modeling

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INTRODUCTION

The delivery of drugs to solid tumors remains a significant challenge, particularly for stromal tumors characterized by an epithelial-mesenchymal transition phenotype (EMT). EMT is a developmental process linked to increased cell proliferation and motility that is important for morphogenic processes that require migratory mesenchymal cells to be recruited at distant sites for epithelial tissue differentiation (1). Tumors have co-opted this transdifferentiation program for enhanced cell migration. While epithelial tumors are highly vascularized with tight cell-cell junctions, EMT tumor cells adopt an irregular, elongated shape with weak cell-cell adhesion and a reduction in E-cadherin, cytokeratin 8/18 and cytokeratin 19 (2). In this study, by controlling the confluency and passage number, epithelial and EMT phenotypes were both produced from identical transgenic Met-1 cells (3). Given the low accumulation and poor penetration of nanotherapeutics in EMT tumors, we set out to test whether nanodelivery in epithelial and EMT tumor phenotypes improves with low levels of therapeutic ultrasound exposure. Using positron emission tomography (PET), we have created imaging-based methods to optimize and quantify drug delivery vehicle distribution (4). Classical optical techniques and analyses were used to validate imaging-based results (5).

Nanoparticle-encapsulated chemotherapeutics exploit the enhanced vascular permeability and retention effect of tumors. Liposomes, with a 100 nm diameter, increase the therapeutic index of chemotherapeutic drugs by reducing systemic toxicities and enhancing the drug concentration in target tissues (6). Albumin, with a smaller diameter than liposomes (~66 kDa, dimensions of 4×4×14 nm), has been used in a similar fashion to carry therapeutics and study permeability (4, 5). However, as compared with small molecule therapeutics, delivery using liposomal or albumin-based carriers is limited by high interstitial pressure and heterogeneous vascular permeability (7, 8).

Both hyperthermia and local ablation have been associated with enhanced nanodelivery to the treated region. The concentration of doxorubicin liposomes has been increased several fold to tissues surrounding tumors treated with radiofrequency ablation (9-11). Further, mild hyperthermia has been shown to increase the effective pore size and enhance liposome extravasation (12). Here, we develop analytic techniques based on the Kedem-Katchalsky equation for the net flux of solute across a blood vessel wall to directly assess nanoparticle transport to the endothelium using non-invasive imaging methods (4, 5, 13, 14).

Ultrasound induced hyperthermia is non-invasive and easily directed to the region of interest. In addition, ultrasound has both mechanical and thermal effects (15, 16), and has been associated with enhancing drug delivery (17), increasing blood supply, activating immune cell trafficking (18-20), inducing mast cell degranulation (21) and prompting macrophage release of fibroblast mitogenic factor for fibroblast proliferation (22). Combining hyperthermia with chemotherapeutic drugs enhances the efficacy of macromolecular and liposomal formulations (23, 24) by increasing tumor blood flow (25) and sensitizing tumor cells to radiation (26). However, there are contradictory reports of the efficacy of ultrasonic enhancement of nanoparticle extravasation (27-29).

Insonation-induced tissue temperature increases depend on the dynamic balance of ultrasound absorption and heat conduction and diffusion in the tissue (30). The resulting thermal effect on tissue increases with increasing temperature and exposure time. Thermal dose is often estimated in terms of the cumulative equivalent minutes at 43°C (CEM43), where CEM43 is defined as $tR^{(43-T)}$, with t being the time of treatment, T the average temperature during treatment, and R a constant that equals 0.25 for temperatures between 37 and 43°C (31, 32) and 0.5 above 43°C. Heat damage to tumor tissue and vasculature varies

amongst species with human tumors being the most heat resistant. In general, treatments resulting in a CEM43 below 0.5 are considered to be reversible and non-lethal (33).

A primary goal of this work is to determine the mechanism and optimal treatment protocol for ultrasound-enhanced nanodelivery to epithelial and EMT tumors, including peak negative pressure (PNP) and thermal dose. In addition, we seek to establish the use of imaging-based pharmacokinetic techniques to estimate vascular permeability and nanoparticle accumulation. We have developed chemistries that can be applied to radiolabel lipid, polymer, and albumin-based particles for pre-clinical and clinical studies (34, 35). Such techniques show promise in facilitating head-to-head comparisons of nanodelivery vehicles and to evaluate techniques (such as ultrasound) to enhance delivery.

MATERIALS AND METHODS

Cell culture and serial transplants

All protocols were approved by the University of California, Davis Animal Care and Use Committee. Met-1^{fvb-2} cells were cultured as previously described (3). Low passage (P9), highly confluent (120-150%) cells produced epithelial tumors. High passage (P16-25), 80-85% confluent cells generated EMT tumors. 1×10^5 cells in 0.02mL were injected into the 4th mammary inguinal fat pads of donor animals. Fresh tumor samples were transplanted into the inguinal mammary fat pad from cell injected donor animals as previously described (3).

Liposome preparation, MicroPET imaging and biodistribution

Liposomes were composed of a total of 10 or 15 mg HSPC:cholesterol:DSPE-PEG2K-OMe:BAT-PEG-lipid = 55.5:39:5.0:0.5, mol/mol/mol/mol. Preparation and radiolabeling followed previously described protocols (34). The radiochemical purity of liposomes on TLC was greater than 95%. Isoflurane anesthetized mice were injected with 1mg/150uL of ⁶⁴Cu-liposomes. PET acquisitions were obtained as previously described (4, 23, 34) using a dedicated small-animal PET scanner (Focus120, Siemens Medical Solutions, USA, Inc.). *In vivo* PET scans were obtained for 30 minutes at 0, 3, 6, 18, 28, and 48 hours post injection. The biodistribution of radioactivity in collected organs was measured in a gamma counter (Perkin-Elmer Life Sciences, MA).

Ultrasound

One tumor per animal was insonified pre-liposome injection. Tumor temperature was either maintained at $37 \pm 0.5^\circ\text{C}$ (no thermal influence) or increased to 42°C for 2, 7, or 18 minutes using feedback-controlled ultrasound as described in (36). The ultrasound pulses consisted of 100-cycle bursts at 1.5 MHz center frequency and 1.1 or 2.4 MPa PNP, with variable pulse-repetition frequency (PRF) ranging from 100 Hz up to 5 kHz. Studies were designed based on CEM43 and PNP goals.

Histology

Excised tumors were fixed overnight in 10% Formalin and dehydrated in 70% ethanol. Four-micron slices were stained with hematoxylin and eosin or deparaffinized for immunohistochemistry with the following antibodies: CD31 (Santa Cruz, sc-1506, 1:800), E-cadherin (Abcam, ab53033, 1:1200), N-cadherin (Epitomics, 2019-1, 1:800), CK8/18 (Fitzgerald/RDI, 20R-CP004, 1:8000), CK19 (Epitomics, 3863-1, 1:1000), and vimentin (Epitomics, 2707-1, 1:1000).

Tumor interstitial pressure

Pressure was measured with the wick-in needle method using a 25G needle with a side-hole as previously described (37).

Transcapillary transport of albumin

The 30 minute clearance of fluorescently labeled albumin was measured as described in detail elsewhere (5, 38). The tissue was dried over three days in an oven to obtain a dry weight measurement.

Statistics

A total of 123 animals with bilateral tumors was studied. Enhanced accumulation was determined by the equation:

$$\text{Accumulation Enhancement} = \frac{\text{Insonified \%ID/g} - \text{Control \%ID/g}}{\text{Control \%ID/g}} * 100$$

Statistical analysis was performed in collaboration with the Division of Biostatistics, UC Davis School of Medicine. The maximum accumulation over time and time-activity data acquired over multiple time points was analyzed using a linear mixed model including repeated measures. When analyzing the effect of ultrasound on epithelial and EMT tumors, multiple comparisons were performed using Turkey's Honest Significant Difference (HSD) test with a repeated measures mixed model. Least squares means (LSM) were calculated to test for differences between paired, treated and control animals after a global F test. A minimum of 7 animals/group were studied with some groups including more than 7 since tumor characterization was not performed until histology was evaluated.

RESULTS

Characterizing tumor phenotypes

In order to differentiate tumor phenotypes, we first characterized epithelial and EMT tumors based on H&E staining for gross anatomy and five immunohistochemical (IHC) markers (E-cadherin, N-cadherin, CK8/18, CK19, vimentin). Epithelial tumors demonstrated a well-defined margin (Fig. 1A) that displaced the host tissue during proliferation and contained large diameter vascular sinuses and vessels (Fig. 1B). EMT Met-1 tumors were highly invasive (Fig. 1C) with a reduced vascular volume ($43.7 \pm 15.0 \mu\text{L}/\text{gram tissue}$), as compared with epithelial tumors ($107.4 \pm 111.6 \mu\text{L}/\text{gram tissue}$, $p < 0.02$) and the quadriceps muscle ($24.5 \pm 9.2 \mu\text{L/g dry tissue}$, $p < 5.0 \times 10^{-5}$) (Figs. 1D, E). The positivity (N positive/N total) of the IHC stains was evaluated to determine protein prevalence (Figs. 2A-M). Epithelial tumors (Figs. 2A-F) were characterized by the presence of high levels of E-cadherin, CK8/18, and CK19 expression (0.63 ± 0.01 , 0.65 ± 0.06 , and 0.32 ± 0.09 , respectively) with low vimentin expression levels (0.28 ± 0.07). N-cadherin was highly expressed in both tumor phenotypes (0.68 ± 0.02 and 0.64 ± 0.02), but was bound to the plasma membrane in epithelial tumors versus cytoplasmic in EMT tumors. Vimentin was upregulated in EMT tumors (0.58 ± 0.09), while E-cadherin, CK8/18, and CK19 levels fell below the 20% detection threshold (Figs. 2G-L).

The accumulation of liposomal particles differed between epithelial (N=39) and EMT (N=58) phenotypes. In the absence of ultrasound, liposomal accumulation was greater in epithelial ($8.2 \pm 3.6 \% \text{ID/g}$) than EMT tumors ($5.5 \pm 2.0 \% \text{ID/g}$, $p < 3.0 \times 10^{-6}$) (Fig. 2N). The time required for maximum accumulation was longer for epithelial tumors (30.3 ± 9.0 hours) compared with EMT tumors (23.5 ± 9.0 hours, $p < 0.0005$) (Fig. 2N).

MicroPET imaging of ^{64}Cu labeled liposomal particles after tumor insonation

Baseline levels of liposomal accumulation were first assessed in both epithelial and EMT tumor phenotypes. Ultrasound was then applied to induce mild hyperthermia and mechanical stress, with a goal of increasing the transport through the endothelium and thus increasing accumulation (Figs. 3A-B). Over the first 18 hours after intravenous injection, radiolabeled liposomes were highly concentrated within the blood pool, evident in the images within the heart chambers and major blood vessels (Fig. 3C). Within 18 hours, liposome accumulation in tumors was observed and accumulation was greatest for the insonified tumor (red arrow) (Fig. 3C). Through region of interest analysis and biodistribution data (Fig. 3D), the increased accumulation of particles within the insonified tumor was quantified (Fig. 3D), validating the enhanced accumulation in the insonified tumors. Across EMT and epithelial tumors (N=97), therapeutic ultrasound treatment resulted in a 1.5 fold increase in liposomal particle accumulation over control tumors (Figs. 3C and D) ($p < 0.001$). For these long circulating particles, tumor accumulation peaked between 18 and 28 hours (Figs. 3D) with particle clearance occurring through liver metabolism and urinary and intestinal excretion. Both the maximum accumulation over time and the accumulation at the final 48-hour time point increased in insonified (as compared with control) tumors. Accumulation was greatest when the particles were injected within one hour of ultrasound treatment ($8.5 \pm 2.8\% \text{ID/g}$) as compared with injection times exceeding 1 hour after insonation ($6.9 \pm 3.1\% \text{ID/g}$, $p < 0.01$, N=11 for both <1 and >1 hour) (Fig. 3E).

The effect of ultrasound differed with both thermal and mechanical ultrasound dose. Increasing the thermal ultrasound dose (CEM43) from <1 to 4.5 enhanced the accumulation of particles in EMT tumors (5.3 ± 1.8 , 5.3 ± 2.6 , 6.1 ± 1.8 , 8.0 ± 4.1 , $9.2 \pm 3.0\% \text{ID/g-tumor}$ for a CEM43 of 0, <1 , 1.4, 1.6 and 4.5, respectively). Accumulation in epithelial tumors peaked at $\sim 15\% \text{ID/cc}$ with CEM43 of 1.4 and a PNP of 1.1 MPa (Figs. 4A, 4B, 5A). When ultrasound with a PNP of 2.4 MPa was applied with the tumor temperature remaining within $\pm 0.5^\circ\text{C}$ of 37°C , accumulation was not increased in the group tested (EMT tumors) (5.6 ± 1.7 versus $5.3 \pm 2.6\% \text{ID/g}$) (Fig. 5B).

The rate of particle extravasation was then evaluated using an image-driven pharmacokinetic model with transport between the vasculature and tumor estimated over the first 28 hours after insonation and injection. Apparent permeability (P_{AP}) is a measure of particle transport, combining convective and diffusive transport through modified intercellular junctions and specialized transendothelial pathways such as transendothelial gaps and vesiculo-vacuolar organelles (13). Averaged across all insonation parameters, for both epithelial and EMT tumors, transport was increased by approximately 73% with insonation. For epithelial tumors, P_{AP} increased from $4.1 \times 10^{-8} \pm 2.8 \times 10^{-8} \text{ cm/sec}$ to $6.7 \times 10^{-8} \pm 4.6 \times 10^{-8} \text{ cm/sec}$ (Fig. 5C, $p < 0.001$). P_{AP} in EMT tumors was significantly lower than in epithelial tumors prior to insonation ($2.7 \times 10^{-8} \pm 1.4 \times 10^{-8} \text{ cm/sec}$, $p < 0.005$); however, insonation increased P_{AP} to $5.1 \times 10^{-8} \pm 3.6 \times 10^{-8} \text{ cm/sec}$ ($p < 1.0 \times 10^{-5}$). A general trend of increasing apparent permeability with increasing CEM43 for EMT tumors was observed with $R^2 = 0.93$ (Fig. 5D).

During insonation, intratumoral pressure decreases, apparent permeability increases, and the extravasation of particles was enhanced

The mechanisms for the effect of ultrasound on particle accumulation were also investigated. Ultrasound reduced the intratumoral pressure and increased P_{AP} , thereby enhancing the vascular clearance of nanoparticles into the tumor interstitium (Figs. 6A-C). Intratumoral pressure in control tumors averaged $13.6 \pm 5.4 \text{ mmHg}$. As the tumor was heated from body temperature (37°C) to 42°C , intratumoral pressure was unchanged (control pressure normalized to 1 versus 0.95 ± 0.19). When tumor temperature was maintained at

42°C and insonation continued for five minutes (CEM43 ~1.4, PNP 2.4 MPa) in a live animal, intratumoral pressure declined (1 versus 0.82 ± 0.24 , $p < 0.02$, $N = 15$). As expected, intratumoral pressure fell after euthanasia (from 1 to 0.84 ± 0.084 , $p < 0.005$, Fig. 6B). Tumor insonation after euthanasia also resulted in an intratumoral pressure reduction; a significant change was not observed during the 37-42°C incline, but intratumoral pressure dropped during a 5 minute insonation at 42°C (from 0.84 ± 0.084 to 0.76 ± 0.11 $p < 0.05$, $N = 7$).

For comparison with classical permeability studies, the clearance of fluorescently-labeled albumin was evaluated before and after insonation (Fig. 6C). The 30 minute clearance of albumin from the tumor vasculature into the tumor interstitium was faster in insonified (85.5 ± 40.6 uL/g dry tissue) than control tumors (64.9 ± 33.8 uL/g dry tissue, $p < 0.001$, $N = 18$), and greater than quadriceps muscle clearance (8.6 ± 3.1 uL/g dry tissue). Intratumoral water content did not change after ultrasound (Fig. 6D, $N = 19$), but exceeded that obtained from the quadriceps muscle (4.2 ± 0.4 mL/g), collected as an internal control.

DISCUSSION

The use of ultrasound to enhance the extravasation of drugs and nanoparticles has been widely reported; however, reports of significant enhancements have been accompanied by reports of failed protocols. Alternatively, reports of enhanced accumulation by radiofrequency ablation or by mild hyperthermia have led to consistent, well-characterized protocols. Here, by labeling liposomes and albumin with a positron emission tomography or fluorescent tracer, individualized protocols for ultrasound treatment of epithelial and EMT tumors have been developed.

Without ultrasound, we found that the accumulation of liposomes was ~1.5 times greater in epithelial, as compared with EMT, tumors. When ultrasound was applied within 1 hour of particle injection, accumulation was enhanced in the insonified, as compared with the contralateral, tumor with a concomitant increase in both the rate and volume of accumulation. Across both epithelial and EMT tumors, maximum and mean accumulation in the tumor interstitium were increased in the insonified tumors. The effect of ultrasound differed with both thermal and mechanical ultrasound indices. Increasing the CEM43 from 1.6 to 4.5 increased the accumulation of particles in EMT tumors to a maximum of $9.2 \pm 3.0\%$ ID/g for a CEM43 of 4.5, while accumulation peaked in epithelial tumors at $14.7\% \pm 3.0\%$ ID/g. Given the narrow therapeutic window for current chemotherapeutics, this increase in tumor accumulation could significantly improve efficacy.

Enhanced delivery to the EMT phenotype is of particular interest as such tumors are aggressive and frequently difficult to treat (39). Here, a significant enhancement of accumulation was observed in both the EMT and epithelial models, yet the parameters required to maximize the effect differed with the tumor biology. Accumulation increased in both EMT and epithelial tumors with a PNP of 2.4 MPa; however, a greater thermal dose decreased accumulation in epithelial tumors. The mean vascular volume in epithelial tumors was more than double that in the EMT phenotype. We hypothesize that the decreased accumulation in epithelial tumors with a higher thermal dose results from vascular stasis in the large vessels that are prevalent in the epithelial (but not EMT) phenotype (12). On the contrary, accumulation in the EMT phenotype was greatest at the highest thermal dose evaluated here, where small regions of heat-mediated necrosis were evident. For EMT tumors, the proportional increase in transport with increasing CEM43 likely results from decreased interstitial pressure, increased apparent permeability and cell damage (40).

Mechanical effects of ultrasound stem from multiple sources, including cavitation, radiation pressure, and expansion and contraction of space between lipid membrane bilayers (41).

Potential benefits of leaflet stretching include stimulation of mechano-sensitive membrane proteins, rearrangement of cytoskeletal elements, membrane perforation increasing drug uptake, and increased tissue permeability (42). As demonstrated in figure 5A, accumulation was enhanced in epithelial tumors at a PNP of 1.1 and 2.4 MPa; however, accumulation was not maximized in EMT tumors until a PNP of 2.4 MPa was applied. Following the transformation of Met-1 cells from an epithelial to an EMT phenotype, we observed downregulation of E-cadherin, cytokeratin 8/18 (CK8/18), and CK19 expression, but upregulation of vimentin. E-cadherin expression is important for maintaining cell-cell contacts and tissue organization (43, 44). Vimentin has a number of critical functions within the cell that involve attachment, migration, and cell signaling (45). In addition, vimentin modulates the negative effects of mechanical and thermal stresses (46, 47). Our results suggest that imposing a mechanical stress by ultrasound (in addition to heat) enhances accumulation in the EMT phenotype, presumably due to weakened cell-cell adhesion and increased vimentin expression.

The mechanisms for the effect of ultrasound on apparent permeability and particle accumulation were also investigated. First, techniques for the estimation of vascular volume, clearance rate and water content were validated by measurements for the quadriceps muscle (internal control), where values were similar to those previously reported for C57BL/6 mice (5, 38). Ultrasound reduced the intratumoral pressure and increased P_{ap} in our tumor model without significant alterations to vascular volume and tissue water content. The measured changes in these two parameters likely contributed to the enhanced accumulation of particles within the tumor. Interestingly, ultrasound reduced intratumoral pressure even after euthanasia; therefore, the effect cannot be solely attributed to changes in blood flow, protein expression or macrophage recruitment. We hypothesize that this reduction is likely due to the effect of insonation and hyperthermia on the extracellular matrix and will evaluate this effect further in future studies. Thermal absorption and mechanical agitation can influence the extracellular matrix, particularly collagen integrity (48). Also, collagen content is known to influence extracellular matrix hydraulic conductivity (49).

PET provided a convenient method to assess the accumulation and apparent vascular permeability over time and across the tumor volume; such measurements can be directly compared with classical estimates of vascular clearance. PET-based pharmacokinetic modeling has previously been developed for small molecule therapeutics, but nuclear medicine-driven techniques to evaluate the pharmacokinetics of nanoparticles are currently unavailable. While small molecules distribute in seconds to minutes, nanoparticles extravasate from the blood pool in hours to tens of hours, and therefore nanoparticle distribution at the time of injection provides a direct measurement of organ and tumor vascular volume. With PET, the circulating radioactivity can be measured based on cardiac chamber activity. With estimates of circulating particle concentration and tumor vascular volume, PET can be applied to estimate the rate of nanoparticle extravasation for each acquisition.

In our supplemental information, we detail the relationship between the P_{AP} parameter derived through the image-guided model with the classical parameters of solute transport coefficient, clearance, and 30 minute albumin clearance (Cl_{30}). P_{AP} and Cl_{30} are related as described by SI Eq6,

$$P_{AP} = \frac{Cl_{30}}{1800} \frac{r}{2\eta V_T}$$

where V_T is the tumor volume, r is the mean radius of the blood vessels within the tumor, and η is the plasma volume fraction in the tumor. In our study, both the apparent permeability (as determined by PET) and the clearance (as determined by fluorescent albumin tracers) were increased as a result of ultrasound. Not surprisingly, due to the difference in diameter, the ultrasound-induced increase in apparent permeability for liposomes was greater than the induced increase in clearance for albumin (~30% increase in Cl_{30} for albumin and ~70% increase in P_{AP} for liposomes as assessed by PET, averaged across epithelial and EMT tumors). One limitation of these studies is that we were not able to determine whether the net flux of solute into the tumor interstitium was due to increased extravasation or a decrease in the net clearance from the tumor. Regardless of the mechanism, both albumin clearance and PET imaging of liposomes assess net flux of solute between the tumor vasculature and interstitium, and demonstrated the effectiveness of ultrasound to enhance accumulation. A further limitation is that we selected one peak treatment temperature to study (42°C) and the optimal protocol is expected to vary with changes in this treatment temperature.

In summary, we applied ultrasound to increase nanoparticle-based drug extravasation and accumulation in both epithelial and EMT tumors. Using both the thermal and mechanical properties of ultrasound, effective treatment times were shorter than times typically used in mild hyperthermia treatments (5-18 minutes compared to durations of 1 or more hours). In addition, we observed a decrease in intratumoral pressure after only 5 minutes as compared to the reported 6 hours required for IFP reduction with whole body hyperthermia (50). Our study clearly demonstrates that differences in tumor phenotype must be recognized in treatment planning and suggests that imaging techniques, such as those applied here, may provide the information required to optimize treatment for individual characteristics. Such individual differences can be viewed as an opportunity or obstacle for such treatments. For our ultrasound treatment parameters and a target temperature of 42°C, thermal dose must be limited when treating highly vascular epithelial tumors; whereas delivery can be increased in the poorly vascularized EMT phenotype with a higher mechanical and thermal dose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol.* 2003; 15:740–6. [PubMed: 14644200]
2. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol.* 2008; 9:628–38. [PubMed: 18628784]

3. Borowsky AD, Namba R, Young LJ, Hunter KW, Hodgson JG, Tepper CG, et al. Syngeneic mouse mammary carcinoma cell lines: two closely related cell lines with divergent metastatic behavior. *Clin Exp Metastasis*. 2005; 22:47–59. [PubMed: 16132578]
4. Rygh CB, Qin S, Seo JW, Mahakian LM, Zhang H, Adamson R, et al. Longitudinal Investigation of Permeability and Distribution of Macromolecules in Mouse Malignant Transformation Using PET. *Clin Cancer Res*. 2011; 17:550–9. [PubMed: 21106723]
5. Curry FR, Rygh CB, Karlsen T, Wiig H, Adamson RH, Clark JF, et al. Atrial natriuretic peptide modulation of albumin clearance and contrast agent permeability in mouse skeletal muscle and skin: role in regulation of plasma volume. *J Physiol*. 2010; 588:325–39. [PubMed: 19948658]
6. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv Enzyme Regul*. 2001; 41:189–207. [PubMed: 11384745]
7. Sharma G, Anabousi S, Ehrhardt C, Ravi Kumar MN. Liposomes as targeted drug delivery systems in the treatment of breast cancer. *J Drug Target*. 2006; 14:301–10. [PubMed: 16882550]
8. Krol A, Maresca J, Dewhirst MW, Yuan F. Available volume fraction of macromolecules in the extravascular space of a fibrosarcoma: implications for drug delivery. *Cancer Res*. 1999; 59:4136–41. [PubMed: 10463619]
9. Ahmed M, Monsky WE, Girnun G, Lukyanov A, D'Ippolito G, Kruskal JB, et al. Radiofrequency thermal ablation sharply increases intratumoral liposomal doxorubicin accumulation and tumor coagulation. *Cancer Research*. 2003; 63:6327–33. [PubMed: 14559820]
10. Goldberg SN, Kamel IR, Kruskal JB, Reynolds K, Monsky WL, Stuart KE, et al. Radiofrequency ablation of hepatic tumors: Increased tumor destruction with adjuvant liposomal doxorubicin therapy. *American Journal of Roentgenology*. 2002; 179:93–101. [PubMed: 12076912]
11. Monsky WL, Kruskal JB, Lukyanov AN, Girnun GD, Ahmed M, Gazelle GS, et al. Radio-frequency ablation increases intratumoral liposomal doxorubicin accumulation in a rat breast tumor model. *Radiology*. 2002; 224:823–9. [PubMed: 12202721]
12. Kong G, Braun RD, Dewhirst MW. Characterization of the effect of hyperthermia on nanoparticle extravasation from tumor vasculature. *Cancer Res*. 2001; 61:3027–32. [PubMed: 11306483]
13. Qin S, Seo JW, Zhang H, Qi J, Curry F-RE, Ferrara KW. An Imaging-Driven Model for Liposomal Stability and Circulation. *Molecular Pharmaceutics*. 2009; 7:12–21. [PubMed: 19621944]
14. Dreher MR, Liu W, Michelich CR, Dewhirst MW, Yuan F, Chilkoti A. Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers. *J Natl Cancer Inst*. 2006; 98:335–44. [PubMed: 16507830]
15. Miller MW, Miller DL, Brayman AA. A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. *Ultrasound Med Biol*. 1996; 22:1131–54. [PubMed: 9123638]
16. Caskey CF, Stieger SM, Qin S, Dayton PA, Ferrara KW. Direct observations of ultrasound microbubble contrast agent interaction with the microvessel wall. *J Acoust Soc Am*. 2007; 122:1191–200. [PubMed: 17672665]
17. Baker KG, Robertson VJ, Duck FA. A review of therapeutic ultrasound: biophysical effects. *Phys Ther*. 2001; 81:1351–8. [PubMed: 11444998]
18. Hu ZL, Yang XY, Liu YB, Morse MA, Lysterly HK, Clay TM, et al. Release of endogenous danger signals from HIFU-treated tumor cells and their stimulatory effects on APCs. *Biochemical and Biophysical Research Communications*. 2005; 335:124–31. [PubMed: 16055092]
19. Wu F, Wang ZB, Lu P, Xu ZL, Chen WZ, Zhu H, et al. Activated anti-tumor immunity in cancer patients after high intensity focused ultrasound ablation. *Ultrasound in Medicine and Biology*. 2004; 30:1217–22. [PubMed: 15550325]
20. Zhou Q, Zhu XQ, Zhang J, Xu ZL, Lu P, Wu F. Changes in circulating immunosuppressive cytokine levels of cancer patients after high intensity focused ultrasound treatment. *Ultrasound in Medicine and Biology*. 2008; 34:81–7. [PubMed: 17854983]
21. Fyfe MC, Chahl LA. Mast cell degranulation and increased vascular permeability induced by 'therapeutic' ultrasound in the rat ankle joint. *Br J Exp Pathol*. 1984; 65:671–6. [PubMed: 6498084]

22. Young SR, Dyson M. Macrophage responsiveness to therapeutic ultrasound. *Ultrasound in Medicine & Biology*. 1990; 16:809–16. [PubMed: 2095011]
23. Kheirloomoom A, Mahakian LM, Lai CY, Lindfors HA, Seo JW, Paoli EE, et al. Copper-Doxorubicin as a nanoparticle cargo retains efficacy with minimal toxicity. *Mol Pharm*. 2010; 7:1948–58. [PubMed: 20925429]
24. Lewis G, Wang P, Olbricht W. Therapeutic Ultrasound Enhancement of Drug Delivery to Soft Tissues. *AIP Conference Proceedings*. 2009; 1113:403–7.
25. Song CW, Park HJ, Lee CK, Griffin R. Implications of increased tumor blood flow and oxygenation caused by mild temperature hyperthermia in tumor treatment. *Int J Hyperthermia*. 2005; 21:761–7. [PubMed: 16338859]
26. Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, et al. Hyperthermia in combined treatment of cancer. *Lancet Oncol*. 2002; 3:487–97. [PubMed: 12147435]
27. O'Neill BE, Vo H, Angstadt M, Li KP, Quinn T, Frenkel V. Pulsed high intensity focused ultrasound mediated nanoparticle delivery: mechanisms and efficacy in murine muscle. *Ultrasound Med Biol*. 2009; 35:416–24. [PubMed: 19081668]
28. Locke J, Zeug A, Thompson D Jr, Allan J, Mazzarella K, Novak P, et al. Localized versus regional hyperthermia: comparison of xenotransplants treated with a small animal ultrasound system and waterbath limb immersion. *Int J Hyperthermia*. 2005; 21:271–81. [PubMed: 16019853]
29. Frenkel V, Etherington A, Greene M, Quijano J, Xie J, Hunter F, et al. Delivery of Liposomal Doxorubicin (Doxil) in a Breast Cancer Tumor Model: Investigation of Potential Enhancement by Pulsed-High Intensity Focused Ultrasound Exposure. *Academic Radiology*. 2006; 13:469–79. [PubMed: 16554227]
30. Barnett SB, Rott HD, terHaar GR, Ziskin MC, Maeda K. The sensitivity of biological tissue to ultrasound. *Ultrasound in Medicine and Biology*. 1997; 23:805–12. [PubMed: 9300983]
31. Dewhirst MW, Vujaskovic Z, Jones E, Thrall D. Re-setting the biologic rationale for thermal therapy. *International Journal of Hyperthermia*. 2005; 21:779–90. [PubMed: 16338861]
32. Tang D, Khaleque MA, Jones EL, Theriault JR, Li C, Wong WH, et al. Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. *Cell Stress Chaperones*. 2005; 10:46–58. [PubMed: 15832947]
33. Yarmolenko PS, Moon EJ, Landon C, Manzoor A, Hochman DW, Viglianti BL, et al. Thresholds for thermal damage to normal tissues: an update. *Int J Hyperthermia*. 2011; 27:320–43. [PubMed: 21591897]
34. Seo JW, Mahakian LM, Kheirloomoom A, Zhang H, Meares CF, Ferdani R, et al. Liposomal Cu-64 labeling method using bifunctional chelators: poly(ethylene glycol) spacer and chelator effects. *Bioconjug Chem*. 2010; 21:1206–15. [PubMed: 20568726]
35. Seo JW, Zhang H, Kukis D, Meares C, Ferrara K. A Novel Method to Label Preformed Liposomes with Cu-64 for Positron Emission Tomography (PET) Imaging. *Bioconjugate Chemistry*. 2008; 19:2577–84. [PubMed: 18991368]
36. Kruse DE, Lai CY, Stephens DN, Sutcliffe P, Paoli EE, Barnes SH, et al. Spatial and temporal-controlled tissue heating on a modified clinical ultrasound scanner for generating mild hyperthermia in tumors. *IEEE Trans Biomed Eng*. 2010; 57:155–66. [PubMed: 20064754]
37. Oldberg A, Kalamajski S, Salnikov AV, Stuhr L, Morgelin M, Reed RK, et al. Collagen-binding proteoglycan fibromodulin can determine stroma matrix structure and fluid balance in experimental carcinoma. *Proc Natl Acad Sci U S A*. 2007; 104:13966–71. [PubMed: 17715296]
38. Lin YC, Samardzic H, Adamson RH, Renkin EM, Clark JF, Reed RK, et al. Phosphodiesterase 4 inhibition attenuates atrial natriuretic peptide-induced vascular hyperpermeability and loss of plasma volume. *J Physiol*. 2011; 589:341–53. [PubMed: 21098005]
39. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci U S A*. 2009; 106:13820–5. [PubMed: 19666588]
40. Krol A, Dewhirst MW, Yuan F. Effects of cell damage and glycosaminoglycan degradation on available extravascular space of different dextrans in a rat fibrosarcoma. *Int J Hyperthermia*. 2003; 19:154–64. [PubMed: 12623638]

41. Krasovitski B, Frenkel V, Shoham S, Kimmel E. Intramembrane cavitation as a unifying mechanism for ultrasound-induced bioeffects. *Proc Natl Acad Sci U S A*. 2011; 108:3258–63. [PubMed: 21300891]
42. Gruden G, Thomas S, Burt D, Lane S, Chusney G, Sacks S, et al. Mechanical stretch induces vascular permeability factor in human mesangial cells: mechanisms of signal transduction. *Proc Natl Acad Sci U S A*. 1997; 94:12112–6. [PubMed: 9342371]
43. Chanson L, Brownfield D, Garbe JC, Kuhn I, Stampfer MR, Bissell MJ, et al. Self-organization is a dynamic and lineage-intrinsic property of mammary epithelial cells. *Proc Natl Acad Sci U S A*. 2011; 108:3264–9. [PubMed: 21300877]
44. Douezan S, Guevorkian K, Naouar R, Dufour S, Cuvelier D, Brochard-Wyart F. Spreading dynamics and wetting transition of cellular aggregates. *Proc Natl Acad Sci U S A*. 2011; 108:7315–20. [PubMed: 21504944]
45. Ivaska J, Pallari HM, Nevo J, Eriksson JE. Novel functions of vimentin in cell adhesion, migration, and signaling. *Exp Cell Res*. 2007; 313:2050–62. [PubMed: 17512929]
46. Toivola DM, Tao GZ, Habtezion A, Liao J, Omary MB. Cellular integrity plus: organelle-related and protein-targeting functions of intermediate filaments. *Trends Cell Biol*. 2005; 15:608–17. [PubMed: 16202602]
47. Coulombe PA, Wong P. Cytoplasmic intermediate filaments revealed as dynamic and multipurpose scaffolds. *Nat Cell Biol*. 2004; 6:699–706. [PubMed: 15303099]
48. Goss SA, Dunn F. Ultrasonic propagation properties of collagen. *Phys Med Biol*. 1980; 25:827–37. [PubMed: 7454768]
49. Netti P, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK. Role of Extracellular Matrix Assembly in Interstitial Transport in Solid Tumors. *Cancer Res*. 2000; 60:2497–503. [PubMed: 10811131]
50. Sen A, Capitano ML, Sperryak JA, Schueckler JT, Thomas S, Singh AK, et al. Mild elevation of body temperature reduces tumor interstitial fluid pressure and hypoxia and enhances efficacy of radiotherapy in murine tumor models. *Cancer Res*. 2011; 71:3872–80. [PubMed: 21512134]

Fig. 1. Gross anatomical differences in epithelial versus EMT tumors. Four micrometer (A, C) H&E and (B, D) CD31 slices of (A, B) epithelial and (C, D) EMT tumors with vessels indicated by arrows in (B, D). Epithelial tumors have a well-defined margin, surrounded by spindle cells, that gradually displaces the host mammary fat pad. (E) Vessel diameter and volume were greater for epithelial tumors in CD31 images (B, D) as measured by vascular volume estimates with Alexa 555-labeled albumin (* $P < 0.02$; ** $P < 0.005$; *** $P < 5.0 \times 10^{-5}$).

Fig. 2. Defining characteristics of (A-F) epithelial and (G-L) epithelial-mesenchymal transition (EMT) tumors. (A) Large blood vessels and solid nests of tumor cells separated by connective tissue are observed in the microscopic H&E sections. (G) The EMT tumor architecture is characterized by a heterogeneous population of cells, including loosely packed, elongated (spindle) tumor cells (B-F, H-L, M). During EMT there is a significant reduction in E-cadherin, CK8/18 and CK19 expression, with a concomitant upregulation of vimentin. (N) Without ultrasound, the EMT phenotype is also associated with lower liposomal accumulation than epithelial tumors, although the rate of accumulation was higher for EMT. (* $P < 0.005$; ** $P < 0.0005$; *** $P < 0.0001$; **** $P < 3.0 \times 10^{-6}$).

Fig. 3.

Effect of ultrasound treatment on liposomal accumulation. (A, B) Therapeutic ultrasound treatment enhanced liposomal extravasation into the tumor interstitium. (C) Decay-corrected PET images confirmed enhanced particle accumulation after ultrasound treatment in epithelial and EMT tumors. Arrow indicates insonified tumor, contralateral tumor was control. Radioactive particles are evident in the blood pool at early time points. (D) Time-activity curves and tumor accumulation measurements were averaged across all insonified tumors and compared with contralateral control tumors and blood pool. (E) Accumulation was greatest when the time between ultrasound treatment and particle injection was less than 1 hour. (* $P < 0.01$; ** $P < 0.001$) Abbreviation: PEG indicates PEGylated.

Figure 4. Time activity curves for (A) epithelial and (B) EMT tumors as a function of cumulative equivalent minutes at 43°C (CEM43). There were no statistical differences among groups for epithelial tumors. However there was significant difference between <1 and 4.5 CEM43 for EMT tumors (* $P < 0.02$).

Fig. 5.

Ultrasonic enhancement of accumulation in epithelial and EMT tumors. (A) Epithelial accumulation was greatest with a CEM43 of 1.4, although accumulation also increased (as compared with control) with a CEM43 of 1.6 and less than 1. Enhanced accumulation was achieved for EMT tumors at a CEM43 of 1.6 and 4.5. A PNP of 1.1 MPa did not increase accumulation in EMT tumors for a CEM43 below 1.4. Statistical significance was tested against no ultrasound, contralateral control tumors (not shown). Table shown below plot (A) summarizes percentage of increase in accumulation relative to control. (B) In EMT tumors, insonation without a concomitant increase in temperature did not significantly increase particle accumulation as compared with a no ultrasound control. In the legend, “mechanical” indicates insonation with a PNP of 2.4 MPa for 35 minutes, achieving a temperature increase below 0.5°C. (C) Without insonation (shown here as control), PAP was significantly higher for epithelial, as compared with EMT, tumors. Insonation significantly increased PAP in both epithelial and EMT tumors. (D) EMT tumors show a linear increase in PAP with increasing CEM43, $R^2 < 0.93$. (* $P < 0.05$; ** $P < 0.02$; *** $P < 0.005$; + $P < 0.001$; and ++ $P < 1.0 \times 10^{-5}$).

Fig. 6.

Tumors responded to the combined thermal and mechanical effects of ultrasound by decreasing intratumoral pressure both (A) before and (B) after euthanasia. (C) The 30 minute clearance of albumin was also increased ($p < 0.001$) in the insonified tumors, (D) without a significant increase in edema. (* $p < 0.01$, ** $p < 0.02$, *** $p < 0.005$, + $p < 0.001$, ++ $p < 1.0 \times 10^{-5}$)