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Adjuvant liposomal doxorubicin markedly affects radiofrequency (RF) ablation-induced effects on periablational microvasculature

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Abstract

Purpose—To evaluate the effects of radiofrequency (RF) ablation without and with adjuvant IV liposomal doxorubicin (Doxil®) on microvessel morphology and patency and intratumoral drug delivery and retention.

Materials and Methods—A total of 133 tumors/animals were used. First, single subcutaneous tumors (R3230 in Fischer rats, and 786-0 in nude mice) were randomized to receive RF alone or no treatment, and sacrificed 0-72hr post-treatment. Next, combined RF/liposomal doxorubicin (1mg given 15min post-RF) was studied in R3230 tumors at 0-72hr post-treatment. Histopathologic assessment including immunohistochemical staining for ced caspase-3), heat shock protein 70 and CD34 were performed to assess morphologic vessel appearance, vessel diameter, and microvascular density. Subsequently, animals were randomized to receive RF alone, RF/liposomal doxorubicin, or control tumors, followed by intravenous fluorescent-labeled liposomes (a surrogate marker) given 0-24hr post-RF to permit qualitative assessment.

Results—RF ablation alone results in enlarged and dysmorphic vessels from 0-4hr, peaking at 12-24hr post-RF, occurring preferentially closer to the electrode. The addition of doxorubicin resulted in earlier vessel contraction (mean vessel area $47539 \pm 9544 \mu\text{m}^2$ vs. $1854 \pm 458 \mu\text{m}^2$ for RF alone at 15min, $p < 0.05$). Combined RF/liposomal doxorubicin produced similar fluorescence 1hr post-treatment ($40.88 \pm 33.53 \text{ AU}/\mu\text{m}^2$ vs. $22.1 \pm 13.19 \text{ AU}/\mu\text{m}^2$, $p = 0.14$), but significantly less fluorescence at 4hr ($24.3 \pm 3.65 \text{ AU}/\mu\text{m}^2$ vs. $2.8 \pm 3.14 \text{ AU}/\mu\text{m}^2$, $p < 0.002$) compared to RF alone denoting earlier reduction in microvascular patency.

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Conclusion—RF ablation induces morphologic changes to vessels within the ablation zone lasting up to 12-24hr post-treatment. The addition liposomal doxorubicin causes early vessel contraction and a reduction in periablational microvascular patency. Such changes will likely need to be considered when determining optimal drug administration and imaging paradigms.

Keywords

Radiofrequency ablation; thermal; microvasculature; liposomal doxorubicin; animal tumor model

Introduction

Radiofrequency (RF) ablation of solid tumors has been progressively widely adopted into clinical practice for small <3 cm tumors because of its high treatment efficacy and low risk of complications(1). However, the incomplete treatment of larger tumors, which often leads to local tumor progression, has led many to explore strategies to improve overall ablation efficacy (2-5) including the addition of adjuvant treatments such as chemotherapy (6, 7). Several studies have demonstrated complementary interactions when combining RF ablation with the concurrent administration of the chemotherapeutic agent doxorubicin, particularly in the form of liposomal preparations which have longer circulation times, and greater periablational drug delivery compared to conventional doxorubicin) (8-15). Indeed, prior studies demonstrate up to 6-fold increases in liposomal drug deposition in periablational tumor (11, 12) with concomitant gains in animal end-point survival and increased tumor coagulation in clinical studies (16-18). Subsequently, additional nanoparticle-based agents (such as paclitaxel or quercetin, or combined with radionuclides such as ¹⁸⁶Rhenium) have also been incorporated into this paradigm with similar improvements on RF ablation-induced tumor destruction(14, 19-21).

Several potential mechanisms underlie the observed increases in tumor destruction and drug delivery with combination therapy, including upregulation of hyperthermia-induced cellular stress and apoptosis in the rim of tissue surrounding the ablation zone (13). Additionally, some studies suggest that RF ablation-induced sublethal (42-50°C) hyperthermia also effects intratumoral and periablational microvasculature. For example, Kruskal et al have previously shown that RF-induced sub-lethal hyperthermia results in microvascular stasis and increased endothelial leakiness, likely contributing to the observed increases in liposomal drug delivery (22). However, the effects of RF ablation on microvascular morphology, and additional potential effects of adjuvant liposomal doxorubicin, remain poorly characterized. As newer adjuvant nanoparticle agents are developed, an understanding of microvascular changes will be critical to developing appropriate adjuvant drug dosing regimens to maximize drug delivery within the tumor and immediately surrounding the zone of ablation.

Accordingly, our purpose was to investigate the effects of RF ablation on vessel morphology in and around the ablation zone alone and in combination with adjuvant liposomal doxorubicin (Doxil®). Additionally, we sought to characterize the effects of RF with and without liposomal doxorubicin on vessel patency and intratumoral drug delivery and retention.

Materials and Methods

Experimental Overview

Approval of the Institutional Animal Care and Use Committee was obtained prior to the start of this study. The study was performed in three phases to systematically investigate the potential effects of RF ablation without and with adjuvant liposomal doxorubicin on

intratumoral microvasculature [Fig 1]. For this study, references to “doxorubicin” imply liposomal encapsulation as this was the sole method of drug administration throughout the study. Furthermore, references to ‘periphery of the ablation zone’ refer to areas of coagulation within the zone of ablation, while ‘periablational rim’ refers to areas outside the traditional margin of RF-induced coagulation in the traditional “red zone” surrounding white coagulation.

Phase I. Effect of RF tumor ablation on vessel morphology—For this phase, studies were performed in two animal models with varying degrees of hypervascularity (to determine if RF ablation-induced changes varied from model to model. First, single subcutaneous R3230 breast adenocarcinoma tumors (implanted in 30 Fischer 344 female rats) were randomized to receive RF alone (n=25) or no treatment (control tumors; n=5). Second, single subcutaneous 786-O human renal cell carcinoma (RCC) tumors were implanted in 30 nude mice and were similarly randomized to RF ablation alone or no treatment. For tumors treated with RF ablation, animals were sacrificed and tumors harvested 0-72hr post-treatment (5 time points; n=5 for each group). Histopathologic assessment was performed, including immunochemical staining both individually and with double-staining for apoptosis (cleaved caspase 3, CASP3), thermal injury (heat shock protein 70, [HSP70]) and CD34, an endothelial cell marker (13, 23). Outcome measures included morphologic vessel appearance, vessel diameter, and microvascular density.

Phase II. Effect of RF ablation combined with adjuvant intravenous (IV) liposomal doxorubicin on vessel morphology—Next, single subcutaneous R3230 breast adenocarcinoma tumors (implanted in Fischer 344 female rats) were treated with standardized RF ablation combined with IV liposomal doxorubicin (1mg, 0.5ml IV injection administered 15min post-ablation). Animals were sacrificed and tumors harvested 0-72hr post-treatment (5 time points post-RF ablation: 15 min, 4 hr, 12 hr, 24 hr, and 72 hr; $\times$ n=5 per group = 25 animals). Outcome measures similar to those described for Phase I were used.

Phase III. Characterization of microvascular patency post-RF ablation alone or with adjuvant liposomal doxorubicin—For part A of this phase, a total of 48 animals were used. First, 32 animals/tumors (R3230 breast tumors in Fischer 344 rats) were randomized to: RF alone or RF/liposomal doxorubicin administered 15min post-RF (n=16 each group). Animals were sacrificed at 4 time points (1, 4, 8, and 24hr) after the last treatment (n=4 animals/time point $\times$ 4 groups = 16 animals for either RF alone or RF/liposomal doxorubicin = 32 animals in total). In each treatment group, liposomes containing only fluorescent rhodamine-labeled were administered IV 1 hr prior to animal sacrifice (n=3 per group $\times$ 4 time points $\times$ 2 treatment arms=24 animals) to determine microvascular patency at the time of sacrifice. At each data point, an additional animal was sacrificed without injection of the fluorescent-labeled liposome to serve as a comparison control including identification of any background fluorescence post-treatment (n=1 $\times$ 4 time points $\times$ 2 treatment arms=8 animals). Outcome measures included qualitative assessment with fluorescent microscopy and fluorescent pixel quantification.

In Part B of this phase, 8 animals/tumors were randomized to receive RF alone or RF/liposomal doxorubicin. To assess for residual FITC in the treated tumor as a surrogate marker for drug/liposome retention, 3 out of 4 animals from both groups received IV FITC-labeled empty liposomes (0.5 ml, no doxorubicin) 15 min after ablation. Here too, one animal from each group served as a control. Animals were sacrificed 24 hr post-treatment (n=4 each group $\times$ 1 time points $\times$ 2 treatment arms= 8 animals). Outcome measures include qualitative assessment with fluorescent microscopy and fluorescent pixel quantification (pixel number per area and pixel fluorescence intensity).

Animal Models

For all experiments and procedures, anesthesia was induced with intraperitoneal (IP) injection of a mixture of ketamine (50 mg/kg, Ketaject; Phoenix Pharmaceutical, St. Joseph, MO) and xylazine (5 mg/kg, Bayer, Shawnee Mission, KS). Animals were sacrificed with an overdose (0.2 mL/kg) of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL).

Experiments were performed using two animal models. The first is a well-characterized established R3230 mammary adenocarcinoma cell line implanted in female Fisher 344 rats (150±20g; 14–16 weeks old, Charles River, Wilmington, MA) (11, 12). Tumor implantation, evaluation, and preparation techniques were performed as previously described (11). Briefly, one tumor was implanted into each animal by slowly injecting 0.3–0.4 mL of tumor suspension into the mammary fat pad of each animal via an 18-gauge needle. 1.3–1.5 cm solid non-necrotic tumors were used (18–21 days after implantation), randomized to different treatment arms.

The second model is a well-characterized 786-O human renal cell carcinoma tumor implanted in nude NIH III mice (23). Tumor line propagation was similar to that described above. Tumor implantation was performed in the right flank of all animals. Animals were monitored every 2–3 days until tumors reached a size of 1.3–1.5 cm diameter, at which point solid, non-necrotic tumors were randomly assigned to treatment arms.

RF Application

Conventional monopolar RFA was applied by using a 500-kHz RFA generator (model 3E; Radionics, Burlington, Mass), as has been previously described (13, 23). Briefly, the 1-cm tip of a 21-gauge electrically insulated electrode (SMK electrode; Radionics) was placed at the center of the tumor using palpatory and ultrasound guidance. RF was applied for 5 min with generator output titrated to maintain a designated tip temperature (70±2°C, mean 90.1±21.6 mA, range 48–156 mA, continuous monitoring via a thermocouple in the electrode tip). This standardized method of RF application has been demonstrated previously to provide reproducible coagulation volumes with use of this conventional RFA system (11, 12). To complete the RF circuit, the animal was placed on a standardized metallic grounding pad (Radionics).

Preparation and administration of adjuvant intravenous liposomal agents

For liposomal doxorubicin, a commercially-available preparation (Doxil; ALZA Pharmaceuticals, Palo Alto, CA) was used. Fluorescent labeled liposomes were prepared using the following protocol; the lipid film was formed in a round bottom flask by solvent removal on a rotary evaporator of chloroform solutions of hydrogenated soy phosphatidylcholine, cholesterol and polyethyleneglycolphosphatidylethanolamine (PEG2000-PE) (57.25:37.57:5.18 mol % respectively, total lipid 13.4mg/mL). The lipid film was then rehydrated with 1 mL of phosphate buffered saline, pH 7.4 and the preparation was probe-sonicated with a Sonic Dismembrator (Model 100, Fisher Scientific, Pittsburgh, PA) at a power output of 7 watts for 30 min. To remove any titanium particles which may have been shed from the tip of the probe during sonication, the sample was centrifuged for 10 min at 2000 rpm. For fluorescent labeling the liposomes in addition contained 1 mol% of FITC-PE or rhodamine PE added during liposome preparation.

The liposomal size was 110 ± 21nm and 118± 33nm for liposomes labeled with FITC-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein)) and rhodamine-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- (lissaminerhodamine B

sulfonyl) (ammonium salt)), respectively. The liposome composition was similar to that of the commercially-available Doxil formulation.

Liposomal doxorubicin (1 mg, 0.5 ml) was slowly administered via tail vein injection in specified animals using a 27-gauge needle 15 min post-RF ablation. This timing of IV liposomal doxorubicin administration was selected based upon prior studies demonstrating maximum tumor coagulation and intratumoral drug accumulation with this paradigm (11, 12). Fluorescent liposomes in Phases II and III were also injected in a similar manner. When two agents (i.e., liposomal doxorubicin and fluorescent liposomes) were administered at the same time point, both agents were loaded in the same syringe to ensure simultaneous administration thereby minimizing any potential variability from varying temporal administration. In all instances, agents were stored in at 4°C in light-proof containers prior to use and loaded exactly at time of administration to avoid bleaching of fluorescent agents and/or precipitation.

Tumor harvesting

In Phase I and II, animals were sacrificed and tumors harvested and sectioned perpendicularly to the direction of electrode insertion and placed in cassettes containing the central section of tumor. All tissues were fixed in 10% formalin overnight at 4°C, embedded in paraffin, and sectioned at a thickness of 5 µm. Tissues were stained with H&E for gross pathology.

In Phase III, harvested tumors were also sectioned perpendicularly to the direction of electrode insertion but flash frozen in optimal cutting temperature (OCT) media, to allow fluorescent analysis. Tissues were sectioned at thickness of 5 µm. For each tumor one slide was stained for H&E for gross pathology and slides prepared for confocal fluorescent microscopy were counterstained with DAPI nuclear staining.

Immunohistochemical staining

For Phase I and II, at least 3 samples from each treatment group underwent IHC staining using previously described techniques(13, 19, 20). Antibodies included CASP3, (Cell Signaling Technology, Danvers, MA), and HSP70, (Stressgen, Chicago, MI) (24), to detect evidence of apoptosis and HSP production, respectively; and CD34 antibodies (R&D systems, Minneapolis, MN) were used to detect vascular endothelial cell lining (23). The staining protocol used has been previously described for CASP3 and HSP70, modified to use a secondary anti-goat antibody (Dako, Carpinteria, CA) incubated for 30 min, then washed with TBST, followed by staining with a tertiary anti-mouse antibody (Dako) for 30 min.

Specimen slides were imaged and analyzed using a Micromaster I microscope (Fisher Scientific, Pittsburgh, PA) and Micron Imaging Software (Westover Scientific, Inc., Mill Greek, WA). Temporal evolution of cellular morphology and the spatial distribution of intratumoral microvessels were determined in relation to the central zone of RF coagulation and to one another. Vessel morphology was evaluated on CD34 stained slides that facilitate their identification. Additionally, comparison of regions of CASP3, HSP70 and CD34 staining was performed by double immunochemical staining when required to visualize spatial differences in distribution in the periablational rim.

Quantitative analysis was performed using two metrics: the average microvessel diameter (as measured in three separate sites) and the % cells that stain per high-powered field (hpf, 40×) within the selected areas in the rim of tumor/tissue surrounding the central ablation zone where it has been previously shown that the maximum effects of apoptosis and HSP70 expression occur (13, 19, 20). Microvessel quantification was performed at the center of the

ablation zone (designated Zone I), and at the periphery of the ablation zone (Zone II). To facilitate comparison, quantification of this 'center' (Zone I) was always performed 1-2 high 10× fields away from electrode insertion (equivalent to 1-2 mm away from the electrode insertion). The peripheral area (Zone II) was quantified 3-4 10× fields away (in a direct line from the site of insertion to the edge of the ablation zone) from the electrode insertion point. Peripheral fields were confirmed to be within the ablation zone based upon visualization of expected post-ablation architecture and confirmation on some sections with co-staining with HSP70 (which has previously been shown outlines the edge of the ablation zone (13)). Five random high power fields were analyzed for a minimum of 3 specimens for each parameter and scored in a blinded fashion to remove observer bias. As an additional control to insure uniformity of staining, whenever direct comparisons were made, IHC was repeated with all relevant comparison slides stained at the same time. Accuracy of the final data was verified by the senior author (MA), who was blinded to treatment group.

Confocal microscopy and fluorescent quantification

A Zeiss LSM 510 Inverted Live-Cell Confocal System (Carl Zeiss Microscopy, Thornwood, NY) was used for image acquisition and tiling. In brief, slides were counterstained with Gold anti-fade reagent with DAPI (Life Technologies, Grand Islands, NY) and left overnight in a flat position, in a light proof container. The next morning image acquisition was performed. For each sample, at 10× magnification, a minimum of 250 fields were imaged and automatically tiled by the microscope software, Ziess LSM image examiner (Carl Zeiss Microscopy). Tiled images allowed subjective assessment and quantification of the entire tumor section. Images were then quantified for fluorescence using Volocity 6.0 software (PerkinElmer, Waltham, MA). For each tumor section, the values, means and sums of fluorescent pixel count and fluorescent intensity were quantified.

Statistical Analysis

The SPSS 13.0 software package (SPSS Inc., Chicago, IL) was used for statistical analysis. All data were provided as mean plus or minus SD. Immunohistochemistry results and fluorescence quantification were compared using analysis of variance (ANOVA). Additional post-hoc analysis was performed with paired, two-tailed Student's T-test, if and only if, the analysis of variance achieved statistical significance. A *P* value of less than 0.05 was considered significant.

Results

Phase I. RF alters vessel morphology after tumor ablation

In R3230 tumors treated with RF alone, vessels within the ablation zone had enlarged and dysmorphic vacuolar spaces on hematoxylin-eosin and CD34 staining at early (0-4 hr) time points after treatment (Fig 2). Two dominant zones of altered microvasculature were identified. Within the central portion (Zone I) of the ablation zone, dysmorphic vessels progressively enlarged at 12 and 24hr post-treatment before contracting to a smaller size at 72hr. In contrast, vessels in Zone II (closer to the margin of the ablative zone) contracted early, and were of smaller caliber throughout the time points observed (15min - 72hr). Quantification of vessel diameter demonstrated significant increases for vessels in tumors treated with RF alone ($p < 0.01$, Table 1, Fig 3). Similar morphologic changes were also observed in Zone I for 786-O RCC tumors, though the maximum effect was observed at a later time point, 24hr post-RF ablation (Fig 4). A much smaller and more inconsistent Zone II (composed of smaller vessels) was observed in this model and more difficult to reliably quantify. IHC co-localization staining with HSP70 and CASP3 demonstrated that the observed morphologic changes in the microvasculature occurred inside the edge of the ablation (as determined by HSP70).

Phase II. Liposomal doxorubicin reverses RF-induced morphologic changes to vasculature

Hematoxylin-eosin and CD34 staining for R3230 tumors treated with RF/liposomal doxorubicin demonstrated early contraction of vessels (0-15 min), which persisted throughout the observed period (24-72hr) (Fig 5). Similar to RF ablation alone, co-localization staining with HSP70 and CASP3 demonstrated that the observed morphologic changes in the microvasculature occurred inside the edge of the ablation zone (Fig 6). Quantification of vessel diameter demonstrated a significant decrease for vessels with RF/liposomal doxorubicin, greatest at 12hr after treatment ($p < 0.05$, Table 2, Fig 7). Unlike RF alone in the same model, no differences in diameter or time course were noted regardless of proximity (Zone I or II) to the RF electrode.

Phase III. Liposomal doxorubicin shortens the window of microvascular patency within the RF-induced coagulation zone

Fluorescent liposome accumulation was observed in the periablational rim for tumors treated with RF alone at both 1 and 4 hr after treatment ($40.88 \pm 33.53/\text{AU}$ and $24.36 \pm 3.65/\text{AU}$) (Fig 8A, C, Table 3). Tumors treated with RF/liposomal doxorubicin demonstrated a similar ($22.15 \pm 13.19/\text{AU}$) amount of increased fluorescence at 1 hr post-treatment when compared to tumors treated with RF alone ($p = 0.49$, Fig 8B). However, RF/liposomal doxorubicin demonstrated significantly less fluorescence at 4 hr compared to RF alone consistent with earlier reduction in microvascular patency ($2.84 \pm 3.14/\text{AU}$, $p < 0.002$) (Fig 8D). For both RF alone and RF/liposomal doxorubicin treated tumors, diminished fluorescence was observed 8-24 hr post-ablation (Fig 8E, F).

When FITC-labeled liposomes were administered immediately following RF ablation with or without IV liposomal doxorubicin and animals were sacrificed later, combined RF/liposomal doxorubicin resulted in significantly less fluorescence in the periablational zone compared to RF alone at 24 hr post-treatment (mean fluorescence retention per high power field $9.62 \pm 5.44 \mu\text{m}^2$ vs. $46.0 \pm 20.68 \mu\text{m}^2$, $p < 0.04$). Tumors treated with IV liposomal doxorubicin alone or control untreated tumors demonstrated minimal to no uptake of FITC-labeled liposomes at 24 hr post-injection throughout the tumor.

Discussion

RF tumor ablation generates focal high temperatures within the target tumor in a dose-dependent heterogeneous and geographic distribution (1). To date, this variability and the resultant effects of such gradient tissue heating on the tumor microenvironment are only partly understood. Here, we have studied the effect of RF ablation on the tumor microvascular morphology and demonstrate that RF ablation results in progressively enlarging and dysmorphic vascular spaces within the ablation zone that ultimately evolve to take on a more shrunken and contracted appearance. Traditional understanding of RF heating-tissue interactions in the ablative zone suggest that coagulative tissue death that results from heating to temperatures above $50-55^\circ\text{C}$ results from immediate heat fixation and is static for all tissue constituents. Nevertheless, our findings run contrary to this conventional wisdom as we demonstrate that changes in vessel caliber, in particular in the central portion of the ablation (Zone 1) are fluid, and evolve over time.

The findings of the current study also highlight the spatial variability in tissue heating that occurs in the ablation zone, as tissue heat transmission which radiates from the RF electrode results in a thermal gradient, and therefore over a range of temperature exposures in the treated area. The differing responses in vessel caliber and appearance based upon location (i.e., central vs. peripheral) in the ablation zone underscore the large gradient in tissue

thermal dosimetry, and subsequent physiologic responses, that occur even in ablated regions previously thought to undergo immediate cell death. These results further show that changes in endothelial appearance over time do correlate to observations in the evolution of apoptosis that occurs in various regions of the ablative zone from 0-24 hr (13). Yet, the temporal pattern (i.e., time of maximum effect) varied between two tumor models, with a longer effect occurring in the 786-O RCC tumor. This highlights the variability (and potentially resistance) in tumor cell response to tissue heating between different cell lines, an effect that has been reported in low-temperature hyperthermic treatments (25-27), but not assessed in the setting of high temperature tumor ablation.

This study further demonstrates that the post-ablation vasculature changes seen can be modulated using combination therapies. Indeed, RF ablation combined with near simultaneous administration of adjuvant IV liposomal doxorubicin results in much more rapid contraction of these affected vessels in the central portion of the ablation zone. This suggests that some liposomal doxorubicin drug delivery not only occurs immediately but also permeates into the coagulated region, and that this translates into an early and relatively quick effect on endothelial cells, potentially representing another mechanism for the efficacy of combined treatment. This adds to prior studies by Chen et al, which demonstrated that intravascular release of doxorubicin from a different nano-carrier -- thermosensitive liposomes (in the setting of low-level 39-42°C hyperthermia) -- can have direct cytotoxic effects on the vascular endothelium, resulting in microvascular occlusion (which would inhibit further drug delivery) (28). Most recently, Manzoor et al have demonstrated that 'fast-release' thermosensitive liposomal preparations result in early intravascular release and exposure of endothelial cells to doxorubicin. These preparations are being studied with thermal ablation, and may contribute to even greater vascular injury (18, 29).

While we did not observe significant penetration of fluorescent-labeled liposomes in the central ablative zone, we know from microvascular changes in this study, and prior studies demonstrating increased free radical generation and apoptosis with combination therapy (13), that liposomal doxorubicin does penetrate into the central ablation zone. One explanation may be that the levels of fluorescent agent that penetrate (and therefore, the amount of drug required to see an effect) are very low and are below the level of detectability of our imager. Regardless, as the dysmorphic enlarged vessels in tumors treated with RF alone ultimately evolve to a more contracted appearance, we believe that, similar to the increased apoptosis that has been previously observed, adjuvant liposomal doxorubicin reaches these central areas and likely accelerates this process. It is, of course, possible that both treatment paradigms reach the same endpoint at different rates, though prior studies (including a clinical study) suggests that increased drug penetration does target residual viable tumor cells even in areas thought to have been encompassed by RF ablation (13, 17).

More importantly, in addition to morphologic/anatomic changes to tumor microvasculature, adjuvant liposomal doxorubicin also causes earlier reduction in microvascular patency and perfusion (from as early as 2-4 hr after treatment) in the periablational rim of tumors compared to RF alone. The noted increases in microvascular perfusion from RF alone in the periablational rim are likely reflective of sub-lethal hyperthermia induced reversible effects on the microvasculature (including increased permeability and endothelial gap junction leakiness) as was shown by Kruskal et al. and others in non-tumor tissues (22). For RF combined with liposomal doxorubicin, vessels remain patent very early after treatment, but lose patency quickly compared to RF ablation alone. Thus, early increased intratumoral drug delivery likely leads to early vascular injury and reduced perfusion. Resulting hypoxic conditions likely potentiates the effects of doxorubicin locally and contributes to the increased apoptosis that has been observed with combination RF/liposomal doxorubicin (13).

Several prior studies in the same model using combination RF/liposomal doxorubicin have demonstrated increases in overall (including ablated and non-ablated tumor) intratumoral drug deposition over time (0-72 hr), peaking at 24-48 hr (11). Here though, we note microvessel patency in the immediate periablational rim (surrounding the ablation zone) decreases early with combination therapy suggesting only a finite window for drug uptake in this geographic area. We reconcile our current findings with the known increase in liposomal doxorubicin deposition within the post-ablated tumor, as this likely indicates preferential continued deposition of the liposomal agent over time to areas peripheral to the periablational zone that remain viable within the tumor and therefore benefit most from the increased drug exposure. Thus, our findings underscore the importance of both spatial and temporal distribution of drug delivery within tumors. Ultimately, the pattern of microvascular changes observed here speaks to the clinical relevancy of combination therapy that targets separate areas of the treatment zone with early central drug penetration (based upon morphologic changes), reductions in periablational vessel patency (and therefore, likely viability) with overall persistent increases in intratumoral drug accumulation in a more delayed fashion.

A reduction in periablational perfusion and fluorescent liposome accumulation was observed with combined RF/liposomal doxorubicin earlier than with RF alone – an effect which persisted regardless of when the fluorescent agent was injected (either immediately after RF ablation or at later time points). This suggests that little additional liposomal accumulation/penetration in the ablation zone or its immediate periablational rim occurs after the single dose of adjuvant liposomal doxorubicin is given. Many of the preliminary experimental and clinical studies combining RF ablation with liposomal chemotherapy, including an active randomized controlled trial studying RF and thermosensitive liposomal doxorubicin, use a single IV dose administered either 24 hr before or 15-30 min before or after RF ablation (11, 17, 18). While, this approach takes advantage of the long circulating half-life of nanoparticle carriers (compared to free drug) and the ‘ease of use’ inherent to single dose regimens, these results highlight a potentially short window for optimal administration of additional drugs/doses, in particular if attempts are made to combine adjuvant therapies in a manner that requires a staggered administration paradigm. For example, RF ablation combined with adjuvant IV liposomal paclitaxel and IV liposomal doxorubicin requires temporally separate administrations (19). Particularly, as newer nanoparticle agents are developed (that have varying encapsulation and drug payload characteristics) and combined with ablative treatments, administration algorithms will need to take into account periablational microvascular patency. Furthermore, as ablative strategies are expanded and modified to improve drug delivery (rather than using adjuvant nanoparticles to improve ablation), additional characterization of these effects will be critical to maximizing interstitial drug delivery.

One clinically relevant insight is that RF induced effects to vessels may also contribute the slight contrast enhancement seen commonly after RF ablation within the ablation zone. Thus, in combination therapies, and indeed, potentially for RF alone, the use of intravascular contrast agents to assess for perfusion/patency of vessels as a very early (within 15-30 min after ablation) surrogate marker for adequacy of treatment or residual tumor viability (as is the current standard practice) may not always be as accurate or reliable as is currently accepted. Additionally, our findings likely explain some of the discrepancy noted in the post-procedure imaging during a very early clinical pilot study using RF/liposomal doxorubicin for primary and secondary hepatic malignancies, and subsequently also observed in a recent Phase I study combining RF ablation with thermosensitive liposomal doxorubicin (17, 18). Specifically, in many of the tumors treated with combination therapy, peripheral areas of enhancement in the treated tumor, initially interpreted as ‘viable’ on CT immediately after, subsequently underwent necrosis or loss of enhancement on follow-up

studies. While one interpretation of these findings speaks to an evolution of nitrative/oxidative cellular stress leading to apoptosis over time, our findings suggest that an additional contributing factor might be the early microvascular patency that is present and then subsequently suppressed several hours later.

There are several limitations of this study worth mentioning. Although the well-characterized R3230 and 786-O tumor models allow comparison to other studies of RF ablation and liposomal chemotherapy performed in these models, careful interpretation and application of the results is required. For example, the specific effects of liposomal doxorubicin identified in this model may vary in other models based upon tumor-drug susceptibility. Similarly, application of these findings, in particular the effects on microvasculature, to different nanoparticle-drug combinations must be done with caution. For example, use of smaller nanoparticles, such as micelles, may result in greater interstitial drug penetration and therefore, have fewer effects on the microvasculature itself. Similarly, use of thermosensitive preparations may result in very early release of all their contents into the vascular space, and therefore may have even more prominent cytotoxic effects on the vasculature. Regardless, as multidrug and multi-dosing paradigms may ultimately contribute to maximizing tumor ablation efficacy or intratumoral drug delivery, continued investigation is required.

In summary, RF ablation alone results in enlarging morphologically distorted blood vessels within the ablation zone that are markedly suppressed/diminished with adjuvant liposomal doxorubicin. Furthermore, RF/liposomal doxorubicin results in earlier periablational microvascular occlusion and loss of patency compared to RF alone. These findings suggest that incorporation of multiple drug and dosing regimens into treatment paradigms will require additional investigation.

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References

1. Ahmed M, Brace CL, Lee FT Jr, Goldberg SN. Principles of and advances in percutaneous ablation. *Radiology*. 2011; 258:351–369. [PubMed: 21273519]
2. Goldberg SN, Hahn PF, Tanabe KK, et al. Percutaneous radiofrequency tissue ablation: does perfusion-mediated tissue cooling limit coagulation necrosis? *J Vasc Interv Radiol*. 1998; 9:101–111. [PubMed: 9468403]
3. Livraghi T, Goldberg SN, Lazzaroni S, et al. Hepatocellular carcinoma: radio-frequency ablation of medium and large lesions. *Radiology*. 2000; 214:761–768. [PubMed: 10715043]
4. Rossi S, Garbagnati F, Lencioni R, et al. Percutaneous radio-frequency thermal ablation of nonresectable hepatocellular carcinoma after occlusion of tumor blood supply. *Radiology*. 2000; 217:119–126. [PubMed: 11012432]
5. Yang W, Chen MH, Yin SS, et al. Radiofrequency ablation of recurrent hepatocellular carcinoma after hepatectomy: therapeutic efficacy on early- and late-phase recurrence. *AJR Am J Roentgenol*. 2006; 186:S275–283. [PubMed: 16632688]
6. Ahmed M, Moussa M, Goldberg SN. Synergy in cancer treatment between liposomal chemotherapeutics and thermal ablation. *Chem Phys Lipids*. 2012
7. Poon RT, Borys N. Lyso-thermosensitive liposomal doxorubicin: an adjuvant to increase the cure rate of radiofrequency ablation in liver cancer. *Future Oncol*. 2011; 7:937–945. [PubMed: 21823888]

8. Ahmed M, Goldberg SN. Combination radiofrequency thermal ablation and adjuvant IV liposomal doxorubicin increases tissue coagulation and intratumoural drug accumulation. *Int J Hyperthermia*. 2004; 20:781–802. [PubMed: 15675672]
9. Ahmed M, Liu Z, Lukyanov AN, et al. Combination radiofrequency ablation with intratumoral liposomal doxorubicin: effect on drug accumulation and coagulation in multiple tissues and tumor types in animals. *Radiology*. 2005; 235:469–477. [PubMed: 15858089]
10. Ahmed M, Lukyanov AN, Torchilin V, Tournier H, Schneider AN, Goldberg SN. Combined radiofrequency ablation and adjuvant liposomal chemotherapy: effect of chemotherapeutic agent, nanoparticle size, and circulation time. *J Vasc Interv Radiol*. 2005; 16:1365–1371. [PubMed: 16221908]
11. Ahmed M, Monsky WE, Girnun G, et al. Radiofrequency thermal ablation sharply increases intratumoral liposomal doxorubicin accumulation and tumor coagulation. *Cancer Res*. 2003; 63:6327–6333. [PubMed: 14559820]
12. Monsky WL, Kruskal JB, Lukyanov AN, et al. Radio-frequency ablation increases intratumoral liposomal doxorubicin accumulation in a rat breast tumor model. *Radiology*. 2002; 224:823–829. [PubMed: 12202721]
13. Solazzo S, Ahmed M, Schor-Bardach R, et al. Liposomal doxorubicin increases radiofrequency ablation-induced tumor destruction by increasing cellular oxidative and nitrate stress and accelerating apoptotic pathways. *Radiology*. 2010; 255:62–74. [PubMed: 20160000]
14. Soundararajan A, Dodd GD 3rd, Bao A, et al. Chemoradiation therapy with ¹⁸⁶Re-labeled liposomal doxorubicin in combination with radiofrequency ablation for effective treatment of head and neck cancer in a nude rat tumor xenograft model. *Radiology*. 2012; 261:813–823. [PubMed: 22025735]
15. Staruch R, Chopra R, Hynynen K. Localised drug release using MRI-controlled focused ultrasound hyperthermia. *Int J Hyperthermia*. 2011; 27:156–171. [PubMed: 21158487]
16. D'Ippolito G, Ahmed M, Girnun GD, et al. Percutaneous tumor ablation: reduced tumor growth with combined radio-frequency ablation and liposomal doxorubicin in a rat breast tumor model. *Radiology*. 2003; 228:112–118. [PubMed: 12808127]
17. Goldberg SN, Kamel IR, Kruskal JB, et al. Radiofrequency ablation of hepatic tumors: increased tumor destruction with adjuvant liposomal doxorubicin therapy. *AJR Am J Roentgenol*. 2002; 179:93–101. [PubMed: 12076912]
18. Wood BJ, Poon RT, Locklin JK, et al. Phase I study of heat-deployed liposomal doxorubicin during radiofrequency ablation for hepatic malignancies. *J Vasc Interv Radiol*. 2012; 23:248–255. e247. [PubMed: 22178041]
19. Yang W, Ahmed M, Elian M, et al. Do Liposomal Apoptotic Enhancers Increase Tumor Coagulation and End-Point Survival in Percutaneous Radiofrequency Ablation of Tumors in a Rat Tumor Model? *Radiology*. 2010; 257:685–696. [PubMed: 20858851]
20. Yang W, Ahmed M, Tasawwar B, et al. Radiofrequency ablation combined with liposomal quercetin to increase tumor destruction by modulation of heat shock protein production in a small animal model. *Int J Hyperthermia*. 2011; 27:527–538. [PubMed: 21846189]
21. Yang W, Ahmed M, Tasawwar B, et al. Combination radiofrequency (RF) ablation and IV liposomal heat shock protein suppression: Reduced tumor growth and increased animal endpoint survival in a small animal tumor model. *J Control Release*. 2012
22. Kruskal JB, Oliver B, Huertas JC, Goldberg SN. Dynamic intrahepatic flow and cellular alterations during radiofrequency ablation of liver tissue in mice. *J Vasc Interv Radiol*. 2001; 12:1193–1201. [PubMed: 11585886]
23. Hakime A, Hines-Peralta A, Peddi H, et al. Combination of radiofrequency ablation with antiangiogenic therapy for tumor ablation efficacy: study in mice. *Radiology*. 2007; 244:464–470. [PubMed: 17641366]
24. Theriault JR, Adachi H, Calderwood SK. Role of scavenger receptors in the binding and internalization of heat shock protein 70. *J Immunol*. 2006; 177:8604–8611. [PubMed: 17142759]
25. Basile A, Biziato D, Sherbet GV, Comi P, Cajone F. Hyperthermia inhibits cell proliferation and induces apoptosis: relative signaling status of P53, S100A4, and Notch in heat sensitive and resistant cell lines. *J Cell Biochem*. 2008; 103:212–220. [PubMed: 17497677]

26. Jordan A, Scholz R. Selection and characterization of heat-resistant variants of multidrug-resistant human gastric carcinoma cell lines. *Exp Oncol.* 2005; 27:18–23. [PubMed: 15812352]
27. Tokalov SV, Pieck S, Gutzeit HO. Varying responses of human cells with discrepant p53 activity to ionizing radiation and heat shock exposure. *Cell Prolif.* 2007; 40:24–37. [PubMed: 17227293]
28. Chen Q, Tong S, Dewhirst MW, Yuan F. Targeting tumor microvessels using doxorubicin encapsulated in a novel thermosensitive liposome. *Mol Cancer Ther.* 2004; 3:1311–1317. [PubMed: 15486198]
29. Manzoor AA, Lindner LH, Landon CD, et al. Overcoming Limitations in Nanoparticle Drug Delivery: Triggered, Intravascular Release to Improve Drug Penetration into Tumors. *Cancer Res.* 2012; 72:5566–5575. [PubMed: 22952218]

Figure 1. Overview of experimental design

Figure 2. Microvessel characterization in R3230 tumors treated with RF alone

Immunohistochemistry with CD34 staining demonstrates dysmorphic enlargement of vessels (red arrows) within the ablation zone that starts at 0-4 hr (B, C) and peaks at 12-24 hr (D, E) compared to control, untreated tumor (A). By 72 hr after RF ablation, vessels within the ablation zone demonstrate a small, contracted appearance (F).

Figure 3. Quantification of regional differences in mean microvessel diameter after RF ablation R3230 tumors were treated with RF alone, followed by CD34 staining and quantification of microvessel surface area in inner (RF ZI) and outer (RF ZII) areas within the RF ablation zone. Within the inner portion (Zone I) of the ablation zone, dysmorphic vessels progressively enlarged at 12 and 24 hr post-treatment before contracting to a smaller size at 72 hr. In contrast, vessels in Zone II (closer to the margin of the ablative zone) contracted early, and were of smaller caliber throughout the time points observed (15 min - 72 hr).

Mean microvessel surface area change after RF ablation in 786-0 RCC tumors

Figure 4. Quantification of microvessel surface area with and without RF ablation in the 786-0 renal cell carcinoma model

RF ablation results in dysmorphic enlargement of microvessels within the ablation zone compared to control tumors. This effect peaks at 24 hr post-RF ablation.

Figure 5. Microvessel characterization in R3230 tumors treated with RF/liposomal doxorubicin
Immunohistochemistry for microvessel (red arrows) with CD34 staining for R3230 tumors treated with RF/liposomal doxorubicin demonstrated early contraction of vessels (0-15 min), which persisted throughout the observed period (24-72hr).

Microvessel surface area changes after RF with and without liposomal doxorubicin

Figure 6. Comparison of regional differences in microvessel size for R3230 tumors treated with RF ablation without and with adjuvant liposomal doxorubicin

RF ablation alone results in enlargement of microvascular surface area, predominantly in the central ablation zone (RF ZI) which peaks at 12-24 hr. Combining RF ablation with liposomal doxorubicin markedly suppresses this effect – as microvessel size is much smaller in RF ZI at 12-24 hrs ($p < 0.05$, all comparisons).

Figure 7. Co-localization staining for HSP70 and CD34 in a tumor treated with RF/liposomal doxorubicin

This merged tile image demonstrates increased HSP70 expression (blue stain, blue arrows) at the edge of the ablation zone extending outwards. Morphologic changes in the appearance of microvessels (in this case contracted) are outlined by CD34 staining (brown, red arrows), both centrally (ZI) and closer to the edge of the ablation zone (ZII).

Figure 8. Rhodamine-labeled liposome accumulation after RF ablation without or with liposomal doxorubicin

Fluorescent liposome accumulation was observed in the periablational rim for tumors treated with RF alone at both 1 and 4 hr after treatment. Tumors treated with RF/liposomal doxorubicin demonstrated significantly less fluorescence at 4 hr compared to RF alone consistent with earlier reduction in microvascular patency ($p < 0.002$). For both RF alone and RF/liposomal doxorubicin treated tumors, diminished fluorescence was observed 8-24hr post-ablation.

Quantification of microvessel diameter after RF ablation. Quantification of vessel diameter demonstrated significant increases for microvessel surface area in tumors treated with RF alone, with greatest increase at 12 and 24 hr after RF ablation when compared with control tumors ($p<0.02$).

Table 1

Microvessel Surface area/ μm^2 at various times after treatment					
	0 hr	4 hr	12 hr	24 hr	72 hr
RF ZI	18154 $\pm$ 4908	12121 $\pm$ 7555	47539 $\pm$ 9544	57198 $\pm$ 14336	12778 $\pm$ 3268
RF ZII	30716 $\pm$ 9433	9775 $\pm$ 8601	7270 $\pm$ 3192	22355 $\pm$ 17467	14217.06 $\pm$ 6096.39
Control	1575 $\pm$ 1500	623 $\pm$ 152	1273 $\pm$ 376	1298 $\pm$ 247	585 $\pm$ 148

Table 2
Comparison of microvessel diameter between RF without and with adjuvant liposomal doxorubicin

Adjuvant liposomal doxorubicin resulted in earlier contraction of RF induced microvessel increase in diameter when combined with RF ablation. Greatest reduction was observed between 12 and 24 hr after RF/liposomal doxorubicin ($p<0.05$).

Microvessel Surface area/ μm^2 at various times after treatment					
	0 hr	4 hr	12 hr	24 hr	72 hr
RF/ZI	18154 $\pm$ 4908	12121 $\pm$ 7555	47539 $\pm$ 9544	57198 $\pm$ 14336	12778 $\pm$ 3268
RF/ZII	30716 $\pm$ 9433	9775 $\pm$ 8601	7270 $\pm$ 3192	22355 $\pm$ 17467	14217 $\pm$ 6096
RF+Dox ZI	12617 $\pm$ 7505	10564 $\pm$ 4661	1854 $\pm$ 458	8801 $\pm$ 2216	4312 $\pm$ 3826
RF+Dox ZII	6688 $\pm$ 3260	14205 $\pm$ 15482	3517 $\pm$ 1382	19200 $\pm$ 19443	7562 $\pm$ 819
Control	1575 $\pm$ 1500	623 $\pm$ 152	1273 $\pm$ 376	1298 $\pm$ 247	585 $\pm$ 148

Table 3
Quantification of fluorescent liposome accumulation for RF ablation without and with liposomal doxorubicin

No significant difference was observed in mean periablational rim fluorescence when comparing RF ablation to RF/liposomal doxorubicin at 1 hr after RF ablation ($p=0.49$). By contrast, animals sacrificed 4 hr after RF ablation showed greater rim mean fluorescence intensity for RF/liposomal doxorubicin compared to RF alone ($p<0.002$). At 8 and 24 hr minimal to no fluorescence could be detected in both groups.

Mean fluorescence intensity per $\mu\text{m}^2/\text{AU}$ at various times after treatment				
	1 hr post-RF	4 hr post-RF	8 hr post-RF	24 hr post-RF
Mean intensity per μm^2 - RF	40.88 ± 33.53	24.36 ± 3.65	0.05 ± 0.04	0 ± 0
Mean intensity per μm^2 - RF+Dox	22.15 ± 13.19	2.84 ± 3.14	0.07 ± 0.06	0 ± 0