Radiofrequency Thermal Ablation Sharply Increases Intratumoral Liposomal Doxorubicin Accumulation and Tumor Coagulation


ABSTRACT

Combining radiofrequency (RF) ablation with i.v. liposomal doxorubicin (Doxil) increases intratumoral doxorubicin accumulation and tumor destruction. The purpose of this study was to characterize and better define the specific parameters of such treatment in an animal tumor model. Four hundred R3230 mammary adenocarcinoma nodules were implanted in 250 Fischer rats. First, paired tumors received combined standardized RF (70°C ± 2°C, 5 min) followed 30 min later with i.v. Doxil (1 mg) or Doxil alone. Intratumoral doxorubicin uptake was evaluated using fluorospectrophotometry 2–120 h after therapy (n = 110). The effects of varying i.v. Doxil doses (0.0625–7.0 mg; n = 100) and the RF tip temperatures (45°C–90°C; n = 190) on subsequent intratumoral doxorubicin uptake and induced tumor necrosis were evaluated. Intratumoral doxorubicin accumulation increased to a maximum at 72 h with greater uptake in the RF-ablated tumors compared with controls (P < 0.01). Greater dose-dependent intratumoral doxorubicin increases (to 37.3 ± 7.7 μg/g) were seen with combined RF/Doxil therapy (P < 0.01). RF ablation reduced the i.v. Doxil dose needed to achieve intratumoral doxorubicin uptake of 13 μg/g from 7 to 2 mg. Increasing tip temperatures from 50°C to 90°C increased the ratio of doxorubicin in RF to nonablated tumors from 1.2 ± 0.4 to 5.9 ± 3.8 (P < 0.01). At all temperatures, greater tumor necrosis was identified for RF/Doxil-treated tumors compared with tumors treated with RF alone (P < 0.05). The threshold for inducing necrosis was 5°C lower for tumors receiving combined therapy (P < 0.01). RF tumor ablation sharply increases intratumoral Doxil accumulation over i.v. Doxil alone, enabling a reduction of systemic dose while obtaining higher intratumoral concentrations than otherwise achievable. Combined therapy also increases tumor destruction over either therapy alone.

INTRODUCTION

Whereas surgical resection has traditionally been the standard of care for the treatment of focal malignancies, recent improvements in imaging technologies have enabled the development of minimally invasive high-temperature thermal tumor ablation, a technique that uses imaging guidance for the accurate percutaneous placement of needle-like applicators (1). The primary mechanism of tumor destruction for these methods is based upon subjecting the entire tumor volume to cytotoxic temperatures (>50°C) for short durations (4–12 min) to induce tumor coagulation and necrosis from energy sources such as RF (2, 3), microwave (4, 5), ultrasound (6, 7), and laser (8–11). Thermal ablation strategies for focal tumor destruction have been gaining increasing clinical attention and rapid adoption as minimally invasive alternatives to surgical resection in the treatment of localized malignancies in a wide range of sites, including liver (12, 13), breast (14), kidney (15–17), lung (18), and bone (11, 19). Clinical studies using RF ablation for liver tumors report that local tumor control can be achieved in 80–90% of cases where the tumors measure <2.5 cm in diameter, with less satisfactory results (50–75% success) for the treatment of larger (>3.5 cm) tumors (8). Additionally, with further long-term follow-up of ablation therapy, there has been an increased incidence in detection of progressive local tumor growth for all tumor types and sizes, despite initial indications of adequate therapy (20–22). This suggests that there are residual patches of viable tumor cells in a substantial but unknown number of cases, a result that falls far short of a goal of completely eradicating all tumor treated by RF ablation. Therefore, strategies that can increase the uniformity and completeness of tumor destruction are needed.

Increasing tumor destruction by combining chemotherapy with high-temperature thermal ablation can potentially overcome many of the current limitations posed when performing RF ablation alone. Based upon well-documented antitumor effects of chemotherapy combined with lower-temperature hyperthermia (41.5°C–45°C), several recent studies have begun to explore the potential of effects that can be achieved with a combination of chemotherapy and RF ablation (23–26). For example, in a rat breast adenocarcinoma model, Goldberg et al. (23) reported significant increases in coagulation necrosis over RF alone or doxorubicin alone when RF was combined with the intratumoral injection of free doxorubicin and noted even greater increases in tumor destruction using a liposomal doxorubicin preparation (Doxil) administered i.v. (24, 26). Subsequently, Monsky et al. (25) observed a 5-fold increase in intratumoral uptake of liposomal doxorubicin in tumors treated with RF compared with tumors that received the drug alone, with preferential liposome accumulation in the hyperemic zone surrounding the ablated area. These advances have already gained substantial clinical interest because preliminary results from a randomized study using combined RF/ liposomal doxorubicin therapy in patients with primary and secondary liver tumors demonstrated significant increases in tumor necrosis compared with RF ablation alone (26).

Whereas earlier studies have demonstrated the complementary role of i.v. liposomal doxorubicin therapy administered in conjunction with RF ablation both in terms of increased intratumoral drug uptake and tumor coagulation, further work needs to be done to better define the parameters for this treatment paradigm. In this study, we have demonstrated an increased efficacy of the combined application of RF ablation and liposomal doxorubicin in a rat breast adenocarcinoma model and investigated some specific parameters of such treatment. Special attention was paid to elucidate the pharmacokinetic characteristics of intratumoral liposomal doxorubicin uptake over time after RF treatment and characterize the effect of increased focal heating and i.v. liposomal doxorubicin dose on both induced necrosis and intratumoral doxorubicin uptake within treated tumors.
MATERIALS AND METHODS

Animal Model

Approval of the Institutional Animal Care and Use Committee was obtained before the initiation of these studies. For all experiments and procedures, anesthesia was induced using i.p. injection of a mixture of ketamine (50 mg/kg; Ketaject; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and xylazine (5 mg/kg; Bayer, Shawnee Mission, KS). When necessary, booster anesthesia injections at one-tenth the dose were administered every 30–60 min i.p.

Experiments were performed using a well-characterized (24, 27) R3230 mammary adenocarcinoma cell line obtained from the laboratory of Dr. Ralph Weissleder (Center for Molecular Imaging, Massachusetts General Hospital, Boston, MA). Fresh tumor (measuring approximately 1 cm in diameter) was initially harvested from a live carrier. Within 30 min of this tumor dissection and removal, the tumor was homogenized with a tissue grinder (Model 23, Kontes Glass Co., Vineland, NJ) using aseptic technique and suspended in 7 ml of RPMI 1640 (INC Biomedicals, Aurora, IL). Prior control experiments have documented that this produces a concentration of approximately 1 x 10^6 cells/ml, with >95% cellular viability. Under direct visualization, 0.2–0.3 ml of the tumor suspension was injected slowly via an 18-gauge needle into the mammary fat pad of 250 female Fischer 344 rats, the strain of animals from which this tumor was initially derived. In an attempt to maximize the usable tumor yield of this model, two injections of tumor cells were administered within the mammary fat pad, one on each side of the abdomen in each animal, for a total of 500 tumor inoculations. Animals were monitored every 3–4 days to measure tumor growth. Solid, nonneurotic tumors (as determined by ultrasound) measuring 14–16 mm in diameter were used for ablation studies. Typically, tumors grew for 14–24 days until the desired tumor size was achieved. Although each animal had two tumors implanted, variation in tumor size growth and the development of cystic cavitation in larger tumors permitted the use of only one tumor in some animals, and these animals were used for the studies measuring coagulation necrosis, where only one tumor was required.

Overall Experimental Design

A total of 400 tumors, implanted in 250 animals, were used for this study. Two types of studies were performed, those quantifying the amount of intra-tumoral doxorubicin uptake, and those assessing the amount of coagulation necrosis that was induced by different RF and/or liposomal doxorubicin treatment regimens. All quantitative doxorubicin uptake studies were performed in rats with two paired tumors of approximately equal size (±2 mm in diameter), implanted on the ventral aspect of each animal near the left fore leg and right hind leg, respectively, approximately 4 cm apart. One tumor received a specified RF application. This was followed by administration of liposomal doxorubicin i.v. 30 min after RF ablation to ensure that tumors returned to baseline temperatures. Thus, there was exposure of both tumors to the chemotherapy, such that the other nonablated tumor received i.v. liposomal doxorubicin alone, functioning as an internal, non-RF control. Qualitative studies measuring gross tumor destruction were performed in animals with a single usable tumor.

The study was performed as four separate experiments. In the first experiment, the intratumoral uptake of liposomal doxorubicin over time after administration was characterized for both combination RF with liposomal doxorubicin therapy and i.v. liposomal doxorubicin alone. In the second experiment, the differences in intratumoral doxorubicin concentration were compared for escalating doses of i.v. liposomal doxorubicin for tumors receiving either RF with i.v. liposomal doxorubicin or liposomal doxorubicin alone. The last two experiments compared both qualitative differences in induced coagulation necrosis (gross tumor destruction) and quantitative differences in intratumoral doxorubicin uptake between RF with liposomal doxorubicin groups and relevant control groups (either RF alone or i.v. liposomal doxorubicin alone) for escalating RF tip temperatures.

Characterization and Quantitation of Intratumoral Liposomal Doxorubicin Accumulation over Time After Administration. Fifty-five animals with paired tumors were used in this experiment (total n = 110). Each animal received a standardized dose of i.v. liposomal doxorubicin (1 mg of doxorubicin equivalent), and one tumor of each pair underwent RF ablation (70°C for 5 min) as described below. Initially, animals were randomly assigned to eight groups (n = 5 animals/group) that were sacrificed at specified time intervals (2–120 h posttreatment). Tumor and liver tissue samples were removed and analyzed for doxorubicin content using acid alcohol extraction techniques as described below (28). As an additional control, 15 animals received similar RF and liposomal doxorubicin treatments followed by sacrifice at 24, 48, and 72 h (n = 5 animals/group). Tumor and liver tissue samples were removed and analyzed for doxorubicin content using both silver nitrate and acid alcohol extraction techniques as described below (28, 29).

Quantitative Assessment of Escalating i.v. Liposomal Doxorubicin Dose. A range of liposomal doxorubicin doses (0.0625–7.0 mg, 10 discrete doses) were administered i.v. to 50 animals with paired tumors (total n = 100). As for the previous experiment, one tumor of each pair received a standardized RF application (70°C for 5 min). Animals were sacrificed 24 h after treatment, and tumor and liver tissues were removed and processed, and doxorubicin was quantified.

Quantitative Assessment of Escalating RF Tip Temperature. A total of 45 animals with two tumors each (n = 90) were used. Initially, animals were randomized into six different groups of five paired tumors each (n = 60) and subjected to RF with probe temperatures ranging from 40°C to 90°C (10°C increments). A nonlinear optimization technique was used to further characterize areas of the curve showing the greatest change (30). For this, an additional three temperature points (45°C, 52°C, and 55°C) were also included (n = 30). RF was administered according to a standardized protocol described below. All animals then received a single dose of i.v. liposomal doxorubicin as described below.

Effect of Escalating RF Tip Temperature on Tumor Destruction. The experiment comprised a total of 100 tumors. Initially, 10 tumors each were randomized into six different groups (n = 60) and subjected to RF with probe tip temperatures ranging from 40°C to 90°C (10°C increments). Each group of tumors was randomly subdivided into groups receiving either RF with i.v. liposomal doxorubicin or RF alone in the standardized manner as described below. A nonlinear optimization technique was used to further characterize areas of the curve showing the greatest change. For this, an additional four temperature points (35°C, 45°C, 55°C, and 65°C) were also included (n = 40). Animals were sacrificed 48 h after treatment, and histological studies were performed to evaluate the extent of tumor destruction (i.e., coagulation necrosis), as described below.

RF Application

Conventional, monopolar RF was applied using a 500 kHz RF generator (CC-1; Radionics, Burlington, MA). To complete the RF circuit, the animal was placed on a standardized metallic grounding pad (Radionics). Contact was ensured by shaving the animal’s back and by the liberal use of electrolytic contact gel. The 1-cm tip of a 21-gauge electrically insulated electrode was placed at the center of the tumor using ultrasonographic guidance. The distal 1-cm tip of this needle was not insulated to permit RF deposition. RF was applied for 5 min with the generator output titrated to maintain a designated tip temperature (±2°C; 90.4 ± 25.8 mA; 48–160 mA range). These parameters were chosen because they have previously been shown to ablate a zone of tumor measuring 6–7 mm (half of the tumor diameter in this model), thereby allowing us to measure the effect of combined therapy (23, 27). A thermometer at the tip of the RF electrode constantly measured the local ablation temperature, thereby enabling proper generator adjustment. Parameters of the RF ablation procedure including tip temperature, tissue impedance, and applied current were recorded at baseline and thereafter at 60-s intervals for the duration of RF application.

i.v. Liposomal Doxorubicin Administration

Unless otherwise specified (as for drug dosing studies where concentration varied as specified), 1 mg in 500 μl (approximately 8 mg/kg body weight) i.v. polyethylene glycol-stabilized, long-circulating, liposomal doxorubicin (Doxil; ALZA Pharmaceuticals, Palo Alto, CA) was administered via a direct femoral injection. This dose was selected because it has previously been shown to increase coagulation from 6.7 mm with RF ablation alone to 13.5 mm with combined RF/liposomal doxorubicin therapy over 48 h following RF ablation using the above parameters (23). To avoid any artificial increase in observed intratumoral doxorubicin concentration resulting from the release of doxorubicin from the liposomes present within the tumor during the application of focal hyperthermia, liposomal doxorubicin was injected 20–30 min after the...
RF procedure. The timing of liposomal doxorubicin application was selected to avoid the release of doxorubicin from the liposomes based upon hyperthermic environments (31, 32). Indeed, temperature measurements in the first 10 animals confirmed results from our previous work, which documented the return of tissue temperatures to normal levels (33–34°C) within this time interval (24, 25). Furthermore, (24) previously published time course studies performed by Goldberg et al. demonstrated maximum effect with this protocol. Access to the femoral vein was obtained through a femoral cut-down. Before injection, the right inguinal crease was incised, and the femoral vein was exposed via blunt dissection. The agent was injected under direct visualization followed by applied pressure to the vein for 1 min to prevent excessive bleeding from the injection site. Incision margins were then sutured together using 2-0 silk suture (Ethicon, Inc., Somerville, NJ).

Quantification of Doxorubicin in Tissue Samples

The fluorescent properties of doxorubicin were used to quantify doxorubicin in tumor and liver samples. Twenty-four h after treatment, animals were sacrificed with pentobarbital overdose (Nembutal; 0.2 ml/kg; Abbots Laboratories, North Chicago, IL). This time point was selected because prior studies have demonstrated maximum intrahepatic doxorubicin uptake 24 h after administration (25). Two extraction techniques were performed within the framework of this study. Doxorubicin was extracted using a well-documented acid alcohol (28) or silver nitrate technique (29). For our primary method, tumors were harvested, weighed, and homogenized in acid alcohol (0.3N HCL, 70% EtOH), and doxorubicin was extracted for 24 h at 5°C. As a control, the left lobe of the liver was also harvested from each rat and subjected to similar doxorubicin extraction. For samples undergoing further extraction with silver nitrate, half of the homogenate underwent incubation in acid alcohol for 2 h before the addition of 0.5 ml of 33% silver nitrate (Sigma, St. Louis, MO). The other half of the homogenate was placed in acid alcohol alone. Doxorubicin was then extracted for 24 h at 5°C. Doxorubicin extracted from all tumor or liver homogenate supernatant samples was quantified by fluorimetry using an excitation wavelength of 470 nm and measuring the intensity of emission at 590 nm (25) and plotted on a standard curve of liposomal doxorubicin serially diluted in acid alcohol.

Quantification of Tumor Coagulation Necrosis and Histological Studies

Induced coagulation necrosis (tumor destruction) was measured 48 h after treatment, based upon results from prior studies (24). Animals were sacrificed with pentobarbital overdose, and tumors were excised and sectioned. In addition to H&E staining, histopathological studies included staining for mitochondrial enzyme activity by incubating representative tissue sections for 30 min in 2% 2,3,5-triphenyl tetrazolium chloride at room temperature. This latter test was used to identify irreversible nonspecific cellular injury (33) during the early stages of RF-induced tissue necrosis (23, 27) and has previously been shown to correlate with histopathological findings of RF-induced tumor coagulation (24, 27). Gross measurements of tumor destruction were performed on both 2,3,5-triphenyl tetrazolium chloride-stained and unstained sections, and the extent of visible coagulation was measured with calipers. Coagulation diameter [longest measurement perpendicular to the inserted electrode (23)] was determined by consensus of two observers who jointly measured and verified the findings (M. A. and S. N. G.). Previous studies have documented close correlation between gross pathological and histopathological findings for RF-induced coagulative necrosis (34).

Statistical Analysis

For all experiments, coagulation diameter was measured, and results were compared using routine statistical analysis. All data are provided as mean ± SE. Pairwise t-tests (α = 0.05, two-tailed test) based upon the least square means were subsequently performed if and only if the overall P was significant. The extent of coagulation or intratumoral doxorubicin uptake was correlated with pharmacokinetic data and thermal and drug dosing data using linear and higher-order regression analyses to determine the best-fit, least squares functions (35). The goodness of fit was assessed using either χ² or modified χ² statistics.

RESULTS

Characterization and Quantitation of Intratumoral Liposomal Doxorubicin Accumulation over Time after Administration. Intratumoral accumulation of doxorubicin for both i.v. liposomal doxorubicin alone and combination RF followed by i.v. liposomal doxorubicin groups increased over time after administration to a maximum at 72 h before decreasing at 120 h (Fig. 1). RF ablation combined with liposomal doxorubicin resulted in significant increases in intratumoral uptake of liposomal doxorubicin over tumors receiving i.v. liposomal doxorubicin alone at 6–72 h after treatment was administered (P < 0.05). The maximum intratumoral liposomal doxorubicin uptake was observed at 72 h after combination RF/liposomal doxorubicin treatment (8.8 ± 1.9 μg/g) compared with 3.5 ± 0.1 μg/g in tumors receiving i.v. liposomal doxorubicin alone. No difference was observed in intratumoral doxorubicin extraction between acid alcohol (6.0 ± 0.6, 6.9 ± 0.4, and 8.8 ± 1.9 μg/g) and silver nitrate (6.0 ± 0.1, 6.8 ± 1.4, and 8.4 ± 0.6 μg/g) techniques for any of the time points tested (24, 48, and 72 h, respectively; P = nonsignificant).

Quantitative Assessment of Escalating i.v. Liposomal Doxorubicin Dose. A linear increase in intratumoral liposomal doxorubicin uptake was observed in tumors receiving either combination RF/liposomal doxorubicin treatment (R² = 0.92) or i.v. liposomal doxorubicin alone (R² = 0.94) or in normal liver (R² = 0.96) over a range of i.v. liposomal doxorubicin doses of 0.0625–7.0 mg (Fig. 2). A significant increase in intratumoral doxorubicin was observed at all points for tumors treated with combination therapy compared with liposomal doxorubicin alone (P < 0.01, all points). Additionally, RF enabled even greater uptake in tumor compared with liver at all doses higher than 1 mg (P < 0.01). The highest accumulation of 37.6 ± 3.1 μg/g was achieved in tumors treated with RF/liposomal doxorubicin at a 7.0-mg dose compared with only 13.0 ± 0.3 μg/g in tumors receiving i.v. liposomal doxorubicin alone (P < 0.01). Significantly higher doses of i.v. liposomal doxorubicin were required to achieve equivalent amounts of intratumoral doxorubicin uptake in tumors that did not receive RF. For example, the maximum intratumoral accumulation in tumors treated with i.v. liposomal doxorubicin and no RF was 13.0 ± 0.3 μg/g at the 7.0-mg dose of i.v. liposomal doxorubicin. However, only 2.0 mg of i.v. liposomal doxorubicin were required in
IMPROVED DOXIL ACCUMULATION WITH RF TUMOR ABLATION

Quantitative Assessment of Escalating RF Tip Temperature. Increases in intratumoral doxorubicin concentration to a maximum of $8.1 \pm 2.9 \mu g/g$ at $90^\circ C$ were achieved when a standard 1-mg dose of liposomal doxorubicin was administered i.v. with increasing tip temperatures (Table 1). However, for all groups, equivalent intratumoral doxorubicin was found in the paired control tumors receiving i.v. liposomal doxorubicin alone ($P = \text{nonsignificant}$). In this experiment, a threshold effect was observed. The intratumoral doxorubicin uptake was equivalent for both RF/liposomal doxorubicin and i.v. liposomal doxorubicin alone tumor groups to a tip temperature of $50^\circ C$, above which the intratumoral doxorubicin uptake increased significantly for tumors receiving RF/liposomal doxorubicin compared with tumors receiving i.v. liposomal doxorubicin alone (Fig. 3; $P < 0.05$). Beyond the threshold, regression showed a log dose-response to the formula $y = 6.7396 \ln(x) - 24.188$ ($R^2 = 0.88$). All values represent mean ± SE of mean.

Table 1  A comparison of intratumoral doxorubicin uptake in tumors receiving a combination of RF/liposomal doxorubicin versus i.v. liposomal doxorubicin alone for a range of tip temperatures $(45^\circ C$-$90^\circ C$).

<table>
<thead>
<tr>
<th>Temperature $(^\circ C)$</th>
<th>RF/liposomal doxorubicin $(\mu g/g)$</th>
<th>i.v. liposomal doxorubicin alone $(\mu g/g)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.1</td>
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<tr>
<td>50</td>
<td>2.7 ± 0.3</td>
<td>2.5 ± 0.4</td>
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<tr>
<td>52</td>
<td>4.5 ± 0.5</td>
<td>1.7 ± 0.2</td>
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<tr>
<td>55</td>
<td>7.0 ± 0.9</td>
<td>2.3 ± 0.3</td>
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<tr>
<td>60</td>
<td>6.5 ± 0.6</td>
<td>2.9 ± 1.0</td>
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<tr>
<td>70</td>
<td>8.1 ± 1.1</td>
<td>1.8 ± 0.1</td>
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<tr>
<td>80</td>
<td>5.9 ± 1.1</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>90</td>
<td>8.1 ± 2.9</td>
<td>1.7 ± 0.6</td>
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DISCUSSION

Combination RF Ablation with i.v. Liposomal Doxorubicin Therapy. Strategies that combine focal high-temperature thermal ablation with adjuvant chemotherapeutics are being explored as a way to improve image-guided thermal ablation by increasing the uniformity and completeness of RF tumor destruction. In preliminary work by Goldberg et al. (23, 24), a combination of adjuvant liposomal doxorubicin administered systemically 30 min after the administration of RF ablation of rat breast tumors resulted in 13.1 mm of coagulation necrosis, a significant increase upon results of 11 mm for RF com-

tumors that had previously received RF to achieve an equivalent amount of doxorubicin accumulation.

Effect of Escalating RF Tip Temperature on Tumor Destruction. Significant differences in tumor destruction were identified between combined RF/liposomal doxorubicin groups and groups treated with RF alone at all tip temperatures to a maximum of $80^\circ C$ ($P < 0.05$), with a mean increase of $0.25 \pm 0.17$ cm of coagulation for combined RF/liposomal doxorubicin therapy over RF alone. A similar linear increase in coagulation diameter was observed as tip tempera-
tures were increased from $35^\circ C$ to $90^\circ C$ for both RF/liposomal doxorubicin ($R^2 = 0.94$) and RF alone ($R^2 = 0.95$) groups (Fig. 4). Coagulation at tip temperatures of $90^\circ C$ was equal for tumors receiving RF with or without i.v. liposomal doxorubicin ($1.55 \pm 0.03$ cm, $P = \text{nonsignificant}$) because both regimens enabled complete ablation of the maximum tumor size treated in the study. Based upon linear data plots, the x intercept, representing the temperature at which any coagulation occurred, was $35^\circ C$ for tumors receiving RF/liposomal doxorubicin compared with $40^\circ C$ for tumors treated with RF alone ($P < 0.01$).
bined with the intratumoral percutaneous injection of free doxorubicin, 6.7 mm for RF alone, or 3 mm for doxorubicin alone. Previous work by D’Ippolito et al. (36) in a tumor growth study in the same animal model, demonstrated significant increases in animal end point survival (with the end point defined as tumor diameter > 3 cm) when tip temperature was increased from 70°C to 90°C for groups receiving RF alone, and even greater increases were seen in end point survival when a similar temperature increase was applied in groups receiving combination RF/liposomal doxorubicin therapy. More importantly, in a randomized pilot clinical study in 10 patients receiving either RF alone or RF combined with liposomal doxorubicin, Goldberg et al. (26) treated several different liver malignancies, including 4 patients with hepatocellular carcinoma, and were able to attain 25–30% increases in coagulation volume by administering liposomal doxorubicin 24 h before RF application. Follow-up imaging studies demonstrated that this particular form of adjuvant therapy resulted in more complete tumor destruction because coagulation progressed over time to include residual tumor foci and patent intratumoral blood vessels.

**Increased Intratumoral Accumulation of Liposomal Doxorubicin with Combination Therapy.** Our results demonstrated an increased intratumoral accumulation of doxorubicin to a peak at 72 h after combination therapy was administered, followed by a decrease in levels at 5 days posttreatment. This conforms to previous data demonstrating corresponding blood circulating times (i.e., 48–72 h) for polyethylene glycol-stabilized liposome preparations (37, 38) and is important in that it shows that there are nonlinear, time-dependent differences in the pharmacokinetics of intratumoral doxorubicin accumulation after the administration of liposomal doxorubicin in the presence of RF. The decrease in intratumoral levels at 5 days most likely represents metabolic conversion of doxorubicin to nonfluorescing by-products, or, alternatively, the doxorubicin fluorescence is quenched by binding to intracellular species including forming cross-links with DNA (39). Interestingly, our findings of identical intratumoral liposomal doxorubicin accumulation with two separate doxorubicin extraction techniques highlight an equivalency between these methods in extracting the combination of bound and unbound intratumoral doxorubicin. Overall, these results have significant implications for the clinical application of this RF/liposomal doxorubicin combination therapy because they provide insight into the appropriate timing of follow-up imaging studies. Indeed, this also helps to explain why changes in patterns of tumor destruction were observed by Goldberg et al. (26) in their clinical pilot study, where significant increases in tumor destruction were observed over time versus immediately after thermal ablation. Whereas follow-up imaging in those cases was performed 2 weeks after treatment, our results suggest that imaging at as early as 3–7 days after treatment may also document similar increases.

**Increased Intratumoral Accumulation of Doxorubicin with Increased RF Probe Temperature.** Prior investigations have demonstrated enhanced antitumoral activity of liposomal doxorubicin in tumors infiltrating the liver and spleen (40), where it has been hypothesized that liposomes that are taken up by the reticuloendothelial system with free doxorubicin are subsequently released to the liver metastases (41). However, the activity against extrahepatic (s.c.) tumors has been reported to be inferior to that of free doxorubicin at milligram equivalent doses (28, 42). Thus, although liposomal vectors are gaining acceptance as a means for increased drug delivery, methods for improved intratumoral drug accumulation are still needed. Our results also provide evidence that when using focal high-temperature hyperthermia, significant increase in intratumoral drug concentrations can be achieved intratumorally by increasing the drug dose to levels that could not be achieved with higher drug doses without RF ablation, or in organs such as the liver that normally have the greatest liposomal uptake. Thus, the linear and diverging increase in intratumoral accumulation of liposomal doxorubicin with increasing drug dose in tumors receiving a standard thermal dose compared with control, non-RF-treated tumors delineates the potential of this combination therapy to overcome a main barrier to i.v. drug delivery to tumors, including high interstitial pressures resulting in reduced intratumoral uptake (43, 44). The results of our study also further confirm the premise of thermal dependency because linear increases in coagulation necrosis were observed for both combination RF/liposomal doxorubicin therapy and RF alone at all temperature points.

**Understanding RF Thermal Ablation.** Classically, the parameter governing tissue destruction for RF ablation has been tissue heating, which induces cellular death via thermal coagulation necrosis (34, 45–47). The volume of RF ablation is therefore governed by the temperature distribution within the tissue. Unlike classic hyperthermia, which is typified by long courses of heating [on the order of hours (48–50)], application of RF energy for ablation tends to be of short duration (1, 21, 51, 52). This is due to the fact that RF ablation strategies have traditionally only taken advantage of the coagulative effects of high-temperature heating. Currently, thermal ablation strategies only take advantage of temperatures that are sufficient by themselves to induce coagulation necrosis (>50°C). However, based upon the exponential decrease in RF tissue heating, there is a steep and changing thermal gradient in tissues surrounding a RF electrode, and as such, a large zone of tissue is heated to temperatures that result in reversible cellular injury. Hence, lowering the temperature threshold at which cell death occurs by taking advantage of ultra-short courses of high-temperature hyperthermia would increase the volume of tumor destruction. Our results confirm this because the x intercept at which the threshold for observing coagulation for tumors treated with combination therapy was 35°C, which is 5°C lower than results for RF ablation alone. This suggests that by combining RF ablation with adjuvant chemotherapy, we are able to increase tumoricidal effects in the sizable zone of elevated but sublethal temperatures adjacent to regions of heat-induced coagulation, including the periphery of the treatment zone. The constant increase in the diameter of coagulation necrosis observed over a wide range of tip temperatures in our experiments nevertheless represents an increase in volumetric tumor destruction, and this may account for the enhanced intratumoral doxorubicin uptake that is observed with increasing temperature.

**Time Factor in Post-RF Ablation Liposomal Doxorubicin Accumulation.** Much work has been done examining the complementary nature of low-temperature hyperthermic environments (42°C–45°C) and i.v. liposomal drugs (53). Kong et al. (31) documented a 2-fold increase in tissue uptake of liposomal doxorubicin in conventional low-temperature hyperthermia monitored for a 12-h period after treatment. However, very little data have been published using higher temperatures (>50°C) with ultra-short heating times (~5 min) in conjunction with liposomal vectors. The preliminary results of this study suggest that the use of short courses of focal high-temperature hyperthermia may indeed be extremely beneficial in improving intratumoral drug uptake and should therefore receive increased attention. Indeed, in this study, we were able to achieve up to 5-fold increases in intratumoral liposome deposition, which is at least equivalent to results reported by Kong et al. (31) and Matteucci et al. (54) for lower temperatures. Our results also suggest that whereas posttreatment monitoring of liposome accumulation for conventional hyperthermia occurs for up to 12 h after therapy, monitoring for longer periods may document even greater accumulation.

**Physiological Mechanisms of Intratumoral Drug Accumulation and Distribution.** The characterization of intratumoral liposomal doxorubicin uptake over a range of tip temperatures suggests that preferential drug accumulation in tissue surrounding the RF ablative
zone occurs in a temperature-dependent fashion with an apparent threshold at 50°C. This conforms to several postulated mechanisms of the increased uptake that is observed when liposomal chemotherapeutics are administered in a hyperthermic environment. Hyperthermia (and hence thermal ablation) has been proposed to increase vascular permeability, likely as a result of endothelial injury (44, 55, 56), which results in improved intratumoral delivery of cytotoxic agents. Under normal conditions, the intratumoral delivery of systemic chemotherapeutics is limited (44, 57). Although tumor microvasculature is known to be “hyperpermeable,” most tumors have regions of hypopervascular microvessels, where the transvascular channels are limited in number and or size, limiting the extravasation of larger (>100 nm) antineoplastic agents such as liposome vectors (57, 58). This in turn results in less than optimum therapeutic efficacy. However, Kong et al. (31) have demonstrated that low-temperature hyperthermia increases the vascular endothelial pore size, which remains patent for several hours after tissue heating, and hence allows for greater deposition of liposomes containing doxorubicin. Hence, noncoagulative hyperthermia in regions adjacent to the thermally mediated RF-induced coagulation may behave as other permeabilizing agents, opening interendothelial gaps, thus augmenting microvascular permeability (and transvascular transport) to drug. Alternatively, low-temperature hyperthermia (42°C) doubles maximum blood flow, which could increase drug delivery, although higher temperatures (>45°C) cause temporary vascular stasis, as shown by Duda and Jain (55). Kruskal et al. (59) have recently demonstrated the reversibility of this process with increased blood flow during reperfusion in this zone. This correlates with results published by Monsky et al. (25), where, using autoradiography to visualize intratumoral accumulation of radiolabeled liposomes administered after RF ablation, the authors observed significant liposome deposition immediately adjacent to the central zone of RF-induced coagulation in tissue exposed to sublethal temperatures.

Mechanisms of Increased Tumor Destruction with Combination Therapy. Several investigators have also examined the role of hyperthermia in augmenting intratumoral liposomal accumulation and facilitating liposomal content release in both thermally stable and labile liposomes (32, 53). Specifically, whereas several studies have documented increased intratumoral drug accumulation for both types of liposomes, greater liposomal drug release and intracellular uptake have been documented with the use of thermosensitive liposomes in low-temperature hyperthermic environments (32, 53, 60). Our study was designed to isolate the effect of focal high-temperature hyperthermia on intratumoral liposomal drug accumulation by using a thermally stable liposomal preparation, administered in a manner that minimized temperature dependent-drug release. As such, our results confirm that a similar phenomenon of temperature-dependent increased accumulation also occurs at higher temperature ranges than have been previously studied and, more importantly, that this accumulation occurs from thermal effects on tissue that persist after tumors have returned to normal temperatures. However, whereas this study characterizes increased intratumoral liposomal doxorubicin accumulation at high temperatures, further work is still required to identify the mechanisms underlying liposomal drug release in this setting. Indeed, earlier research has documented that intratumoral doxorubicin release from liposomes occurs over an extended period of time (up to weeks) in the absence of hyperthermic treatments (61). Given that increases in tumor necrosis have been observed when liposomal doxorubicin was administered after tumor temperatures returned to normal and that this is likely, at least in part, an effect of released doxorubicin, which has well-documented tissue necrotic action (62, 63), further study is warranted into the potential mechanisms behind accelerated liposomal drug release after focal high-temperature treatment.

The synergy that has been observed between RF ablation and adjuvant administration of i.v. liposomal doxorubicin is likely to be a multifactorial phenomenon. Three specific factors, namely, RF ablation, doxorubicin, and the liposome vector, have already identified contributory roles in the enhanced tumor destruction that is observed (24). Indeed, several studies have previously documented the synergistic potential of lipid agents with both low-temperature (64) and high-temperature (24) hyperthermic situations. Given the multifactorial nature of this tumor destruction, the actual mechanism of cellular death, whether through necrosis or apoptosis, is likely determined by which component is predominant, based upon location within the tumor (i.e., the effect of focal high-temperature hyperthermia predominates in the central zone of the tumor, whereas lipid and doxorubicin effects predominate at the margins of tissue heating). Further study of this relationship is still required to characterize the specific mechanisms that underlie this synergy. Additionally, given the small size of our tumor model, a further characterization of the range of RF-induced tissue temperatures at which increased liposomal accumulation occurs in a larger animal tumor model would both be welcome and serve as a confirmation of our current and independently valid results.

The results of this study clearly confirm beneficial effects of combining RF thermal ablation with i.v. liposomal doxorubicin application in improving coagulation necrosis and enhanced intratumoral liposomal doxorubicin accumulation. Tumor necrosis induced by a combination of focal RF thermal ablation and adjuvant systemically administered liposomal doxorubicin occurs in a temperature-dependent manner. Furthermore, intratumoral liposomal doxorubicin accumulation can be significantly increased in tumors treated with focal high-temperature heating, and this increase is influenced by both thermal and drug dose in a linear fashion. These insights will yield significant gains toward the characterization of this strategy for eventual wider clinical implementation, both in terms of minimally invasive image-guided thermal therapy and methods for improved targeting of drug delivery.

Still, whereas the results of this study provide insight into the factors that can potentially improve the outcomes of combination therapy, further validation in tumor models of larger sizes and different types will be a necessary next step to confirming the utility of this combination therapy before its adoption in a wider clinical setting. Further work exploring all of the factors that influence intratumoral liposome delivery, including liposome size, charge, circulation time, and composition, should also be performed. This will enable the production of a liposome vehicle that is tailored for adjuvant use with focal high-temperature thermal ablation, permitting both a maximum volume of coagulation and intratumoral drug uptake. All these are subjects of our current research.

REFERENCES


Radiofrequency Thermal Ablation Sharply Increases Intratumoral Liposomal Doxorubicin Accumulation and Tumor Coagulation
