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Radiofrequency Ablation–Induced Upregulation of Hypoxia-Inducible Factor-1 α Can Be Suppressed with Adjuvant Bortezomib or Liposomal Chemotherapy

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Abstract

Purpose—To characterize upregulation of hypoxia-inducible factor (HIF)-1 α after radiofrequency (RF) ablation and the influence of an adjuvant HIF-1 α inhibitor (bortezomib) and nanodrugs on modulating RF ablation–upregulated hypoxic pathways.

Materials and Methods—Fisher 344 rats ($n = 68$) were used. First, RF ablation–induced periablation HIF-1 α expression was evaluated in normal liver or subcutaneous R3230 tumors (14–16 mm). Next, the effect of varying RF ablation thermal dose (varying tip temperature 50°C–90°C for 2–20 minutes) on HIF-1 α expression was studied in R3230 tumors. Third, RF ablation was performed in R3230 tumors without or with an adjuvant HIF-1 α inhibitor, bortezomib (single intraperitoneal dose 0.1 mg/kg). Finally, the combination RF ablation and intravenous liposomal chemotherapeutics with known increases in periablation cellular cytotoxicity (doxorubicin, paclitaxel, and quercetin) was assessed for effect on periablation HIF-1 α . Outcome measures included immunohistochemistry of HIF-1 α and heat shock protein 70 (marker of nonlethal thermal injury).

Results—RF ablation increased periablation HIF-1 α in both normal liver and R3230 tumor, peaking at 24–72 hours. Tumor RF ablation had similar HIF-1 α rim thickness but significantly greater percent cell positivity compared with hepatic RF ablation ($P < .001$). HIF-1 α after ablation was the same regardless of thermal dose. Bortezomib suppressed HIF-1 α (rim thickness, $68.7 \mu\text{m} \pm 21.5$ vs $210.3 \mu\text{m} \pm 85.1$ for RF ablation alone; $P < .02$) and increased ablation size ($11.0 \text{ mm} \pm 1.5$ vs $7.7 \text{ mm} \pm 0.6$ for RF ablation alone; $P < .002$). Finally, all three nanodrugs suppressed RF ablation–induced HIF-1 α (ie, rim thickness and cell positivity; $P < .02$ for all comparisons), with liposomal doxorubicin suppressing HIF-1 α the most ($P < .03$).

Conclusions—RF ablation upregulates HIF-1 α in normal liver and tumor in a temperature-independent manner. This progrowth, hypoxia pathway can be successfully suppressed with an adjuvant HIF-1 α -specific inhibitor, bortezomib, or non-HIF-1 α -specific liposomal chemotherapy.

Although radiofrequency (RF) ablation of solid tumors has been progressively widely adopted into clinical practice, a key limitation of RF ablation has been the difficulty in achieving a complete ablative margin for larger tumors where residual untreated tumor leads to local tumor progression (1). Strategies that can increase the uniformity and completeness of RF tumor destruction are required. One such strategy has been to study ablation-tissue interactions that take place in and around the ablation zone to identify and characterize cellular responses that occur in tissues exposed to sub-lethal thermal injury (40°C–45°C) (2). Prior studies have identified several responses in incompletely injured tumor tissue, including partial apoptosis, increased heat shock protein expression, and increased proangiogenic growth factor expression (3–6). Such mechanisms can be successfully targeted (both pharmacologically and geographically) to affected areas with the administration of specific adjuvant chemotherapy agents often encapsulated in nanoparticle delivery vehicles, such as liposomes, that can promote drug deposition within the periablational rim (7,8).

Increased hypoxia-inducible factor-1 α (HIF-1 α) has been identified as a key driver of tumor growth and angiogenesis and is increasingly cited for pharmacologic targeting with agents such as bortezomib, camptothecin, and mitomycin C (9,10). Studies have demonstrated that RF ablation can also increase HIF-1 α expression in the rim of viable cells immediately adjacent to the ablation zone (11). RF-induced increased HIF-1 α activity appears to be a promising target for adjuvant pharmacologic modulation to reduce growth in untreated tumor after thermal ablation. However, on a basic mechanistic level, RF ablation-induced upregulation of HIF-1 α in the periablational rim remains poorly characterized to date. Many clinically relevant questions remain regarding tissue-specific and thermal dose-specific variability and the potential role for combining RF ablation with various adjuvant drugs to modulate HIF-1 α activity either directly with HIF-1 α -specific inhibitors or indirectly by targeting cells producing HIF-1 α in the periablational rim.

The purpose of the present study was to (i) characterize RF ablation-induced effects on HIF-1 α expression in a rat breast tumor model and normal rat liver (representing a tumor and the necessary-to-treat normal periablational tissue); (ii) study the effects of varied thermal dose (a key influence on periablational tissue responses such as heat shock proteins) (5) on RF-induced increased HIF-1 α expression; (iii) combine RF ablation of breast tumors with adjuvant administration of bortezomib, a known inhibitor of HIF-1 α protein synthesis to determine whether this pathway can be modulated in the setting of ablation; and (iv) determine whether other adjuvant liposomal chemotherapy agents (eg, doxorubicin, paclitaxel, and quercetin) that increase local cellular cytotoxicity when combined with RF ablation are active partly because of suppression of RF-induced HIF-1 α expression.

MATERIALS AND METHODS

Experimental Overview

Approval of the Institutional Animal Care and Use Committee was obtained. The study was performed in four phases to investigate systematically RF ablation–induced upregulation of HIF-1 α in and around the ablation zone without and with adjuvant pharmacologic modulation (Fig 1). Tissue was obtained from 68 new animals for phases 1–3, and tissue for phase 4 was obtained from 15 samples from a histology bank.

Phase 1: Characterization of RF Ablation–Induced Upregulation of HIF-1 in and around Ablation Zone—The purpose of phase 1 was to determine and compare the pattern of increased periablational HIF-1 α expression in clinically relevant tissue types (tumor and normal liver). First, single subcutaneous R3230 breast adenocarcinoma tumors (implanted in 18 Fisher 344 female rats) were randomly assigned into an RF ablation group, treated with standardized RF ablation (21-gauge electrode, 1-cm active tip, tip temperature 70°C $\times$ 5 min) with animals randomly assigned to be sacrificed at five time points (0 h, 4 h, 12 h, 24 h, and 72 h after RF ablation; $n = 3/\text{group}$; total $n = 15$ animals) or into the control untreated tumor group ($n = 3$). This wide range of time points was initially selected to determine the temporal kinetics of periablational HIF-1 α expression. Concomitant heat shock protein 70 (HSP70) expression was also evaluated in this phase because it is a known surrogate marker for the edge of the ablation zone (5,7). Costaining for both HSP70 and HIF-1 α expression permitted geographic localization of where RF ablation–induced increases in HIF-1 α occur. Next, 15 female Fisher rats were randomly assigned to (a) RF ablation (standardized RF ablation of the liver, sacrificed at 4 h and 24 h after RF ablation (5 rats per group $\times$ 2 time points, total $n = 10$) or (b) control untreated liver ($n = 5$). These time points were selected to enable optimal colocalization of HIF-1 α and HSP70 expression (previously shown to peak at 24 h after ablation) and comparison with existing literature where 24 hours is a commonly evaluated time point after RF ablation (5,7,12). Outcome measures included gross pathologic evaluation, histopathologic evaluation (hematoxylineosin stain), and immunohistochemical staining for HIF-1 α and HSP70 (including quantification of rim thickness and percent cell positivity).

Phase 2: Effect of Thermal Dose on RF Ablation–Induced Periablational HIF-1 Expression—To determine whether varying thermal dose affects periablational HIF-1 α expression, single subcutaneous R3230 breast adenocarcinoma tumors (implanted in 25 Fisher 344 female rats) were treated with five different thermal doses by modifying tip temperature and ablation time: 50°C $\times$ 2 minutes, 50°C $\times$ 20 minutes, 70°C $\times$ 5 minutes, 70°C $\times$ 20 minutes, 90°C $\times$ 2 minutes ($n = 5/\text{group} \times 5$ groups, total $n = 25$). These tip temperature and time combinations were selected to provide a range of thermal doses, while ensuring that there was sufficient incompletely treated tumor at the margin to assess the RF ablation–induced HIF-1 α response. All animals were sacrificed at 24 hours after RF ablation. Outcome measures included gross and histopathologic evaluation and immunohistochemistry as described for phase 1.

Phase 3: Effect of Combining RF Ablation with Adjuvant Bortezomib on RF Ablation–Induced Periablational HIF-1 Expression—To determine whether RF ablation combined with a HIF-1 α inhibitor can increase local tumor ablation efficacy, single subcutaneous R3230 tumors implanted in 10 Fisher rats were randomly assigned to receive RF ablation alone or RF ablation combined with intraperitoneal (IP) injection of bortezomib (a drug known to suppress HIF-1 α activity through binding of the C terminus of the HIF-1 α molecule) (13) administered immediately after RF ablation ($n = 5/\text{group} \times 2$ groups, total $n = 10$). All animals were sacrificed at 24 hours after RF ablation. Outcome measures included gross and histopathologic evaluation and immunohistochemistry for HIF-1 α expression.

Phase 4: Effect of Combining RF Ablation with Adjuvant Liposomal Chemotherapy on RF Ablation–Induced Periablational HIF-1 Expression—To determine whether adjuvant liposomal chemotherapy (loaded with doxorubicin, paclitaxel, or quercetin) could reduce periablational HIF-1 α expression after RF ablation, slides for immunohistochemistry evaluation were prepared from sample tumors available in our histopathologic bank of R3230 tumors (previously implanted in Fisher female rats) treated with standardized RF ablation (tip temperature 70°C $\times$ 5 min) combined with intravenous (IV) liposomal doxorubicin, liposomal paclitaxel, or liposomal quercetin and tissues harvested 24 hours after RF ablation ($n = 5/\text{group} \times 3 = 15$ tumor samples). Primary data for these specimens (including coagulation and apoptosis staining) have been previously reported (5,7,14). Outcome measures included gross and histopathologic evaluation and immunohistochemistry for HIF-1 α expression.

Animal Models

For all experiments and procedures, anesthesia was induced with 0.5 mL/kg IP injection of a mixture of ketamine, 50 mg/kg (Ketaject; Phoenix Pharmaceutical, Inc, St. Joseph, Missouri), and xylazine, 5 mg/kg (Bayer Animal Health, Shawnee Mission, Kansas). Animals were sacrificed with an overdose of ketamine and xylazine—1.5 mL/kg. Experiments were performed in R3230 mammary adenocarcinoma tumors implanted in female Fisher 344 rats (150 g $\pm$ 20; 14–16 wk old; Charles River Laboratories, Wilmington, Massachusetts) (12). Tumor implantation, evaluation, and preparation techniques were performed as previously described (12). 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. Tumors used were 1.3–1.5 cm solid nonnecrotic tumors. For experiments performed in normal liver, similarly sized female Fisher rats were used. Briefly, after achieving deep anesthesia, the rat's abdomen was shaved and prepared using povidoneiodine (Betadine; Carefusion, San Diego, California) and alcohol. A minilaparotomy was achieved by a 1.5-cm subcostal incision exposing the right lobe of the liver. After therapy administration, the abdomen was closed in layers using interrupted sutures.

RF Ablation Application

Conventional monopolar RF ablation was applied by using a 500-kHz RF ablation generator (model 3E; Radionics, Inc, Burlington, Massachusetts) (12). Initially, the 1-cm tip of a 21-gauge electrically insulated electrode (SMK electrode; Radionics, Inc) was placed at the

center of the tumor. RF ablation was applied for 5 minutes with generator output titrated to maintain a designated tip temperature (eg, $70^{\circ}\text{C} \pm 2$; mean, $90.1 \text{ mA} \pm 21.6$; range, 48–156 mA). For phase 2, five different combinations of tip temperature and time were compared (described previously). This standardized RF ablation application provides reproducible coagulation volumes with use of this conventional RF ablation system (12,15). To complete the RF ablation circuit, the animal was placed on a standardized metallic grounding pad (Radionics, Inc).

Preparation and Administration of Adjuvant Agents

A commercially available bortezomib powder (Selleckchem, Houston, Texas) was used. Fresh stock was prepared 2 hours before administration and stored at 4°C temperature in a lightproof container. The desired weight of bortezomib was dissolved in dimethyl sulfoxide, after which distilled water was used to dilute the solution to the desired concentration. A $150 \text{ g} \pm 20$ rat was injected with a single IP dose of 0.1 mg/kg (ie, 1.5 mL of bortezomib was administered only once, 15 min after RF ablation). The times and routes of IV administration and techniques of preparation of liposomal doxorubicin, paclitaxel, and quercetin have been previously optimized (5,7,14). Briefly, in samples evaluated in this study, liposomal doxorubicin was administered via IV injection 15 minutes after RF ablation (1 mg, 0.5 mL), liposomal paclitaxel was administered via IV injection 24 hours before RF ablation (0.12 mg in 0.5 mL), and liposomal quercetin was administered via IV injection 24 hours before RF ablation (0.29 mg in 0.5 mL). Each of these administration paradigms have demonstrated maximal effect on tumor coagulation and periablational cell cytotoxicity when the agent was combined with RF ablation (5,7,14).

Tumor Harvesting

Animals were sacrificed and tumors harvested and sectioned perpendicularly to the direction of electrode insertion. Gross measurements of tumor destruction were performed by staining for mitochondrial enzyme activity by incubating representative tissue sections for 30 minutes in 2% 2,3,5-triphenyl-2H-tetrazolium chloride measured by calipers (12). Coagulation diameter (longest measurement perpendicular to the inserted electrode) was determined by blinded consensus of two observers (M.M., 3 y experience with models and techniques; M.A., 15 y experience with models and techniques). Previous studies have documented close correlation between gross pathologic and histopathologic findings for RF-induced coagulative necrosis (12). Next, the remaining half of the tumors was placed in cassettes and fixed in 10% formalin overnight at 4°C , embedded in paraffin, and sectioned at a thickness of $5 \mu\text{m}$. Tissues were stained with hematoxylineosin for histopathology.

Immunohistochemical Staining and Analysis

In all immunohistochemistry assays, at least three samples from each treatment group underwent immunohistochemical staining using previously described techniques (5,7,14). Antibodies to HSP70 (StressGen, Ann Arbor, Michigan) (16) and HIF-1 α (Abcam, Cambridge, Massachusetts) were used. Briefly, $5\text{-}\mu\text{m}$ -thick paraffin sections were freshly cut; slides were baked at 60°C for 1–3 hours to remove wax, after which heat-mediated (120°C for 30 min) antigen retrieval was performed. After hydrogen peroxidase block, primary antibody was applied for 1 hour at room temperature. Afterward a mouse secondary

antibody (Dako, Carpinteria, California) was applied to HSP70 antibodies. A Tyramide Signal Amplification (TSA) system (PerkinElmer, Waltham, Massachusetts) was applied to HIF-1 α slides followed by a secondary antibody (Dako) and streptavidin block. Finally, slides were developed using 3,3'-diaminobenzidine and placed in a coverslip for imaging.

Specimen slides were imaged at the periablational rim at 10 $\times$ and 40 $\times$ magnification and analyzed using a Micromaster I microscope (Fisher Scientific, Pittsburgh, Pennsylvania) and Micron Imaging Software (Westover Scientific, Inc, Mill Creek, Washington) to determine rim thickness and percent cell positivity. Additionally, HIF-1 α index (multiplication of rim thickness and percent cell positivity of each sample) was calculated when statistical significance was not achieved in both parameters. Five random high-power fields were analyzed for a minimum of three specimens for each parameter and scored in a blinded fashion to remove observer bias (7,14). As an additional control to insure uniformity of staining, whenever direct comparisons were made, immunohistochemistry was repeated with all relevant comparison slides stained at the same time.

Statistical Analysis

Microsoft Office 2010 Excel software (Microsoft Corp, Redmond, Washington) was used for statistical analysis. All data were provided as mean $\pm$ SD. Immunohistochemistry results and fluorescence quantification were compared using analysis of variance. Additional post-hoc analysis was performed with paired, two-tailed Student *t* test if the analysis of variance achieved statistical significance. A *P* value $< .05$ was considered significant.

RESULTS

Phase 1: Characterization of RF Ablation–Induced Upregulation of HIF-1 α in and around Ablation Zone

Standardized RF ablation (70°C $\times$ 5 min) of R3230 tumors resulted in central coagulation (7.6 mm $\pm$ 1.1) at 24 hours after treatment. Immunohistochemistry demonstrated increased HIF-1 α expression (percent cell positivity and rim thickness) in rimlike geographic distribution abutting the margin of the ablation zone circumferentially at all time points from 0–72 hours, although this was greatest in the 24- to 72-hour range (Table 1, Fig 2a–e). Colocalization staining of tumors at 24 hours after RF ablation performed with both HIF-1 α and HSP70 demonstrated an inner circumferential rim of increased HIF-1 α expression that partially overlapped with an outer circumferential rim of HSP70 staining (approximately 50%) (Fig 3a, b). A similar pattern of increased HIF-1 α expression was observed in normal liver treated with RF ablation. No difference was observed between HIF-1 α rim thickness between liver and tumor treated with standardized RF ablation at either 4 hours or 24 hours (100.8 μ m $\pm$ 40.0 vs 125.8 μ m $\pm$ 28.2 at 24 hours; *P* = .4). However, significantly greater HIF-1 α percent cell positivity was detected in tumors versus livers at 24 hours after RF ablation (14.8% $\pm$ 4.6 vs 37.9% $\pm$ 4.3; *P* = .001).

Phase 2: Effect of Thermal Dose on RF Ablation–Induced Periablational HIF-1 α Expression

Varying thermal doses did not increase reactive HIF-1 α expression that was observed in the periablational rim for any of the five thermal doses tested in R3230 tumor (*P* = .1, rim

thickness and percent cell positivity) (Table 2). In comparison, greater increases in HSP70 expression by periablational rim thickness only (no difference in percent cell positivity were seen) were observed for increasing thermal dose (Table 2). For a given thermal dose, HSP70 rim thickness was consistently wider than HIF-1 α rim thickness ($P < .01$ for all comparisons).

Phase 3: Effect of Combining RF Ablation with Adjuvant Bortezomib on RF Ablation–Induced Periablational HIF-1 α Expression

RF ablation combined with adjuvant IP bortezomib increased the coagulation zone in R3230 tumors at 24 hours after RF ablation ($11.0 \text{ mm} \pm 1.5$) compared with RF ablation alone ($7.7 \text{ mm} \pm 0.6$; $P < .002$) (Fig 4a, b). Adjuvant bortezomib also suppressed RF ablation–induced upregulation of HIF-1 α expression in the periablational rim (Fig 5a, b). Rim thickness was $68.7 \mu\text{m} \pm 21.5$ for combined therapy compared with $210.3 \mu\text{m} \pm 85.1$ for RF ablation alone ($P < .02$), cell positivity for combined therapy was $21.0\% \pm 11.2$ compared with $37.9\% \pm 13.9$ for RF ablation alone ($P = .08$), and HIF-1 α index for combined therapy was 14.5 ± 12.1 compared with 95.0 ± 41.6 for RF ablation alone ($P < .01$).

Phase 4: Effect of Combining RF Ablation with Adjuvant Liposomal Chemotherapy on RF Ablation–Induced Periablational HIF-1 α Expression

RF ablation combined with all of the liposomal chemotherapies (doxorubicin, paclitaxel, or quercetin) significantly reduced periablational HIF-1 α expression (rim thickness $\times$ percent cell positivity = HIF-1 α index) compared to RF ablation alone (6.5 ± 1.4 , 12.2 ± 8.4 , and 25.3 ± 3.7 at 24 h vs 48.5 ± 15.9 ; $P < .05$ for all comparisons) (Fig 6a–d, Table 3). When combined with RF ablation, liposomal doxorubicin reduced periablational HIF-1 α expression to the greatest degree compared with RF ablation combined with either liposomal paclitaxel or liposomal quercetin for rim thickness (6.5 ± 1.4 vs 12.2 ± 8.4 and 25.3 ± 3.7 at 24 h; $P < .02$ for all comparisons) (Table 3).

DISCUSSION

HIF-1 α has been identified as a key driver of tumor angiogenesis and proliferation and is increasingly studied for pharmacologic targeting (9). Existing literature suggests that variable expression of HIF proteins may be predictive of tumor cell survival and aggressive biology (17,18). Earlier studies demonstrated that RF ablation can increase HIF-1 α expression in the rim of viable cells immediately adjacent to the ablation zone, possibly related to reversible cell injury or regional reduction in tumor perfusion (11,19). This increased HIF-1 α expression has been associated with increased growth rates in residual viable cells exposed to nonlethal hyperthermic temperatures (6,11,20). For example, Nijkamp et al (11) observed increases in HIF-1 α after RF ablation in a small animal model of colorectal liver metastases.

The present study confirms that RF tissue ablation increases HIF-1 α expression at the ablative margin. This study expands on current available literature by demonstrating that variable or increasing thermal dose does not appear to change the degree to which RF ablation induces HIF-1 α expression in the periablational rim (as designated by rim

thickness); this is in contrast to periablational HSP70 expression, which varied widely in concert with varying thermal doses (increasing thermal dose leads to increasing HSP70 rim thickness), as expected given the known spectrum of low temperatures (40°C–47°C) to which tissues around the ablation zone are exposed as tissue heating falls off (21). This finding suggests that RF ablation–induced HIF-1 α upregulation is not thermally based or has a very narrow window of inciting injury (eg, only at temperatures of 45°C–47°C) or may be dependent on secondary effects of hyperthermia (eg, endothelial injury or vascular thrombosis to incite hypoxia). Additionally, a similar amount of HIF-1 α upregulation occurs for different-sized ablation zones, suggesting no greater degree of upregulation when ablating larger or multiple tumors. This finding is particularly clinically relevant in that it runs counter to the current conventional understanding whereby enlarging ablation zones create larger rims of surrounding partial thermal injury (21) (enlarging zones of secondary reactions)—which is not the case for RF ablation–induced HIF-1 α expression.

Differences in the degree of HIF-1 α cell positivity were also observed based on tissue type—with greater cell positivity in the periablational rim observed in R3230 tumors compared with normal liver. The biologic processes that are taking place in the periablational zone are likely complex given that this study shows variable HIF-1 α response between two different tissue types (normal liver vs R3230 tumor), the exact cellular sources of which require further study. In addition, the cellular composition in the periablational rim involves diverse groups of cells (ie, native parenchymal, tumor, or incoming scavenger and immunogenic cells), several types of which are likely producing different proteins in response to sublethal hyperthermic exposure or other inciting processes (endothelial injury or hypoxia). Regardless, further characterization of specific cell types responsible for the various proteomic reactions in the periablational rim may be helpful in refining techniques to suppress RF ablation–induced HIF-1 α expression, given that it is implicated in stimulating distant tumor growth in intrahepatic colorectal metastases in animal studies (11,20,22,23).

This study also demonstrates that RF ablation resulted in periablational HIF-1 α expression in normal liver parenchyma. These findings suggest that the secondary effects of RF ablation–induced HIF-1 α upregulation, such as increased growth rates in residual tumor cells, likely occur in many (if not all) cases of RF ablation. For example, given these findings of increased HIF-1 α after ablation of normal liver, similar increases likely occur in clinical cases where complete ablation (and apparent clinical success) is achieved with the required 5–10 mm ablative margin of normal liver tissue. HIF-1 α has well-established and wide-ranging downstream pro-oncogenic effects, with activation of > 70 hypoxia-related and angiogenesis-related genes associated with tumor growth and invasiveness, including potential interaction and synergy with heat shock proteins such as the HSP70 studied here as a comparative marker (24–28). Nijkamp et al (11) previously demonstrated that RF ablation of colorectal liver metastases in a small animal model can lead to increased growth of separate intrahepatic metastases. Upregulation of HIF-1 α after RF ablation may also contribute to broader secondary systemic effects of RF ablation, including potentially stimulating distant tumor growth, the clinical significance of which is likely underappreciated, is poorly characterized, and requires further study. Increased HIF-1 α expression has also been reported after transarterial chemoembolization of liver tumors, suggesting the

potential need to study and modulate HIF-1 α for a range of interventional oncologic therapies (29).

In the present study, RF ablation was also successfully combined with an anti-HIF-1 α agent such as bortezomib (a proteasome inhibitor that also blocks HIF-1 α protein production) (13) to suppress the secondary increased expression of HIF-1 α . Bortezomib also has some potentially proapoptotic effects, which are likely responsible for increases in gross tumor coagulation observed with combined RF ablation and bortezomib therapy (30). These results support the rationale of prior studies, such as the study by Poff et al (31); these investigators combined bortezomib with pulsed high-intensity focused ultrasound to increase treatment efficacy in murine squamous cell tumors. In the present study, some degree of HIF-1 α suppression in the periablational rim likely arose from additional cytotoxic targeting of cells partially injured from sublethal hyperthermia (ie, a “two-hit effect”) that are responsible for HIF-1 α production. That several liposomal chemotherapy agents with variable or no direct effect on HIF-1 α production had a similar effect suggests that one mechanism of HIF-1 α suppression, at least, might be increasing cell cytotoxicity in the periablational rim. Prior studies have reported that although different types of adjuvant liposomal chemotherapy agents can induce similar amounts of local tumor coagulation, there can be differences in tumor growth rate in the remaining, untreated tumor (14). The variable suppression of a secondary mechanism, such as HIF-1 α , whose effect is not reflected in an outcome measure such as gross tumor coagulation, might be responsible for the observed disconnect between outcomes of local tumor injury and long-term tumor growth. Regardless, incomplete HIF-1 α suppression suggests that continued investigation of specific HIF-1 α inhibitors is likely warranted.

One advantage of the geographic nature of HIF-1 α expression specific to the zone around the RF ablation zone is that it is particularly amenable to targeting with nanoparticle-encapsulated drugs. Many studies have previously shown that liposome-encapsulated chemotherapies achieve 6-fold to 10-fold higher concentrations in the periablational rim secondary to changes in microvascular patency from sublethal hyperthermia (15,32). The present study demonstrates that combining RF ablation with adjuvant liposomal chemotherapies can at least partially target the cells behind the increased HIF-1 α expression. However, variability in the degree of HIF-1 α suppression with different chemotherapies (with liposomal doxorubicin being most effective) suggests that continued study is required to reduce HIF-1 α expression further.

The present study has several limitations. A single tumor model (R3230 breast adenocarcinomas in rats) was used, and so extrapolation of these findings to other models and human tumors must be made with caution. Bortezomib, an agent with known anti-HIF-1 α effects, also likely has additional interactions and mechanisms of action (30). This study characterizes the effects of RF ablation on HIF-1 α expression only (shown to be the most active hypoxia-induced factor after RF ablation) (11), although characterization of additional hypoxia-induced factors and related heat shock proteins (particularly given differing biologic half-lives of the various proteins) (33,34) may warrant additional study. Confirmation of these findings with other, potentially more specific, HIF-1 α inhibitors might yield even greater HIF-1 α suppression. Finally, although studying early time points

after therapy provides insight into immediate changes in the periablational rim without and with additional adjuvant therapy, longer term growth studies (and studies in metastatic tumor models) would be helpful in determining the true effect of RF combined with HIF-1 α suppression on local and distant tumor growth, particularly given variable stimulation or suppression of tumor growth after RF ablation (35).

In conclusion, this study demonstrates that RF ablation induces increases in HIF-1 α (a mediator with known stimulatory effects on tumor angiogenesis) expression in the periablational rim to a variable extent based on the tissue type being treated (tumor or liver) with HIF-1 α expression occurring in a manner that is at least partly independent of thermal dose. This study also demonstrates that HIF-1 α expression after RF ablation can be effectively suppressed either by using an anti-HIF-1 α agent such as bortezomib or with nonspecific reductions in HIF-1 α production by cellular sources through targeted liposome-encapsulated chemotherapy delivery to the periablational rim.

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ABBREVIATIONS

HIF-1α	hypoxia-inducible factor-1 α
HSP70	heat shock protein 70
IP	intraperitoneal
IV	intravenous

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Figure 1.

Overview of experimental study design. Schematic representation of the four phases, models, and outcome measures assessed as part of this study. IHC = immunohistochemistry.

Figure 2.

RF ablation of R3230 tumor induces increased periablation HIF-1α expression that peaks at 24–72 hours after treatment. Sequential images of periablation RF-induced HIF-1α obtained at various times [0 h (a), 4 h (b), 12 h (c), 24 h (d), 72 h (e)] after standardized RF ablation (70°C × 5 min) of R3230 tumor demonstrates increasing HIF-1α expression over time (arrows), peaking at 24–72 hours. Asterisk denotes the ablation zone inside the periablation rim. Within the periablation rim, cells stained red are positive for HIF-1α expression. (Available in color online at www.jvir.org.)

Figure 3.

Colocalization staining of HIF-1 α and HSP70 in the periablational rim. R3230 tumors sampled 24 hours after treatment with standardized RF ablation (70°C $\times$ 5 min) were stained for HIF-1 α (blue) and HSP70 (pink) expression. Images at 10 $\times$ (**a**) and 40 $\times$ (**b**) demonstrate an inner rim of HIF-1 α -positive cells (stained blue and adjacent to arrow, **a**) surrounded by an outer rim of HSP70-positive cells with only a partial (~50%) costaining of cells (purple color, arrow, **b**) in between the two rims. Asterisk denotes the ablation zone inside the periablational rim. (Available in color online at www.jvir.org.)

Figure 4.

Gross pathologic specimens of RF ablation without and with adjuvant IP bortezomib. Tumors treated with RF ablation followed 15 minutes later by IP bortezomib (RF+BTZ) and RF ablation alone (RF alone) harvested at 24 hours after treatment and stained for viability (mitochondrial enzyme activity) are presented. The central white area represents treatment-induced tumor necrosis/coagulation (as noted by arrow), with viable tumor staining red. Greater coagulation was observed with RF+BTZ ($11.0 \text{ mm} \pm 1.5$) compared with RF alone ($7.7 \text{ mm} \pm 0.6$, $P = .002$). (Available in color online at www.jvir.org.)

Figure 5.

RF ablation combined with bortezomib reduces periablational HIF-1 α expression compared with RF ablation alone (40 $\times$). Immunohistochemical staining of R3230 tumors sampled 24 hours after standardized RF ablation without (a) and with adjuvant IP bortezomib (b) demonstrates significantly reduced HIF-1 α expression (cells stained with red; arrows) in the periablational rim (rim thickness, $68.7 \mu\text{m} \pm 21.6$ vs $210.3 \mu\text{m} \pm 85.1$, $P = .02$; percent cell positivity, $21.0\% \pm 11.2$ vs $37.9\% \pm 13.9$, $P = .08$) and reduced HIF-1 α index (14.4 ± 12.1 vs 95 ± 41.6 , $P < .01$) for RF alone. Asterisk denotes the ablation zone inside the periablational rim. (Available in color online at www.jvir.org.)

Figure 6.

Histopathologic comparison of periablational HIF-1 α expression for RF ablation combined with liposomal chemotherapies (10 $\times$). R3230 tumors sampled 24 hours after treatment with (a) standardized RF ablation alone and combined with (b) liposomal doxorubicin, (c) liposomal quercetin, or (d) liposomal paclitaxel were stained for HIF-1 α expression (cells stained with red; arrows). All adjuvant liposomal chemotherapy agents reduce the extent of periablational HIF-1 α expression (HIF-1 α index), as evidenced by the thickness and number of cells stained in the periablational rim, compared with RF alone ($P < .05$, all comparisons). However, RF combined with IV liposomal doxorubicin reduced HIF-1 α expression to the greatest degree. Asterisk denotes the ablation zone inside the periablational rim. (Available in color online at www.jvir.org.)

Table 1

Quantitative HIF-1 α Expression (Rim Thickness, Percent Cell Positivity) after RF Ablation at Studied Time Points

Time Point	Rim Thickness ($\mu\text{m} \pm \text{SD}$)	Percent Cell Positivity ($\% \pm \text{SD}$)
Immediate	9.1 $\pm$ 15.8	3.0 $\pm$ 5.2
4 h	65.7 $\pm$ 59.6	17.6 $\pm$ 20.1
12 h	101.3 $\pm$ 18.0	19.3 $\pm$ 1.7
24 h	125.8 $\pm$ 28.2	37.9 $\pm$ 4.3
72 h	211.7 $\pm$ 53.3	52.0 $\pm$ 17.3

HIF-1 α = hypoxia-inducible factor-1 α .

Table 2

Quantitative (Rim Thickness) HIF-1 α and HSP70 Expression after RF Ablation with Varying Thermal Doses at 24 Hours

Thermal Dose	HIF-1 α Rim Thickness ($\mu\text{m} \pm \text{SD}$)	HSP70 Rim Thickness ($\mu\text{m} \pm \text{SD}$)	<i>P</i> Value *
50°C $\times$ 2 min	64.8 $\pm$ 27.7	592.4 $\pm$ 102.2	.0004
50°C $\times$ 20 min	88.5 $\pm$ 6.3	651.8 $\pm$ 275.2	.01
70°C $\times$ 5 min	125.8 $\pm$ 28.2	687.5 $\pm$ 104.5	.00006
70°C $\times$ 20 min	143.8 $\pm$ 46.3	1,300.4 $\pm$ 123.3	.00001
90°C $\times$ 2 min	112.0 $\pm$ 17.1	1,011.9 $\pm$ 188.5	.0002

HIF-1 α = hypoxia-inducible factor-1 α , HSP70 = heat shock protein 70, RF = radiofrequency.

* Statistical significance when comparing HIF-1 α rim thickness with HSP70 rim thickness achieved at a specific thermal dose.

Table 3

Quantitative Assessment of Periablational HIF-1 α Expression (Rim Thickness, Percent Cell Positivity, and HIF-1 α Index) for RF Ablation Combined with Liposomal Chemotherapies

Treatment	HIF-1 α Rim Thickness (μ m $\pm$ SD)	P Value ^{*,†}	HIF-1 α Percent Cell Positivity (% $\pm$ SD)	P Value ^{*,†}	HIF-1 α Index (Index $\pm$ SD)	P Value ^{*,†}
RF ablation alone	125.8 $\pm$ 28.2		37.9 $\pm$ 4.1		48.5 $\pm$ 15.9	
RF ablation + quercetin	90.6 $\pm$ 16.0	.08, .006	27.3 $\pm$ 5.4	.02, .001	25.3 $\pm$ 3.7	.04, .005
RF ablation + paclitaxel	58.7 $\pm$ 6.0	.01, .1	22.6 $\pm$ 6.5	.002, .059	12.2 $\pm$ 8.4	.002, .02
RF ablation + doxorubicin	48.4 $\pm$ 11.8	.007	13.6 $\pm$ 2.1	.0004	6.5 $\pm$ 1.4	.002

HIF-1 α = hypoxia-inducible factor-1 α , HSP70 = heat shock protein 70, RF = radiofrequency.

* P value when comparing HIF-1 α expression of any treatment group with HIF-1 α expression achieved by RF ablation alone.

† P value when comparing HIF-1 α expression of any RF combination group with HIF-1 α expression achieved by RF ablation combined with doxorubicin.