Aspergillus fumigatus Hyphal Damage Caused by Noninvasive Radiofrequency Field-Induced Hyperthermia

Warna D. Kaluarachchi,a Brandon T. Cisneros,a,c Stuart J. Corr,a,c Nathaniel D. Albert,b Steven A. Curley,a,d Dimitrios P. Kontoyiannisb

Department of Surgical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USAa; Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USAa; Department of Chemistry and The Richard E. Smalley Institute for Nanoscale Science and Technology, Rice University, Houston, Texas, USAa; Department of Mechanical Engineering and Materials Science, Rice University, Houston, Texas, USAa

We studied the effect of noninvasive radiofrequency-induced hyperthermia on the viability of Aspergillus fumigatus hyphae in vitro. Radiofrequency-induced hyperthermia resulted in significant (>70%, P < 0.0001) hyphal damage in a time and thermal dose-dependent fashion as assessed by XTT [(sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-1H-tetrazolium inner salt)], DiBAC [(bis-(1,3-dibutylbarbituric acid) trimethine oxonol] staining, and transmission electron microscopy. For comparison, water bath hyperthermia was used over the range of 45 to 55°C to study hyphal damage. Radiofrequency-induced hyperthermia resulted in severe damage to the outer fibrillar layer of hyphae at a shorter treatment time compared to water bath hyperthermia. Our preliminary data suggest that radiofrequency-induced hyperthermia might be an additional therapeutic approach to use in the management of mold infections.

Aspergillosis is the most common opportunistic invasive mold infection and is a frequent cause of death in patients with hematological malignancies and transplant recipients (1). Despite improvements in diagnosis and antifungal drug development, novel strategies to combat Aspergillus infections are still needed. Such strategies should be specific, potent, and unaffected by resistance to conventional antifungals and should have the potential to provide synergistic activity with other antifungal therapies.

Hyperthermia is a promising therapeutic approach that is being investigated for the treatment of both superficial and deep-seated cancers (2). Specifically, noninvasive radiofrequency hyperthermia (RFHT) has shown promise in the treatment of hepatic and pancreatic carcinomas (3, 4). The radio waves used in RFHT have a frequency of 13.56 MHz, allowing tissue penetration to depths greater than 30 cm. This contrasts with the superficial penetration depths (<1 cm) of hyperthermia delivery systems that use near-infrared and microwave frequencies (5). Also, RF-HT-based therapy for cancer can be enhanced and targeted using antibody-conjugated gold nanoparticles (4, 5).

Because invasive aspergillosis and cancer have similar biological patterns of invasion and mass-like growth (6), we sought to determine the effect of RFHT on the viability of Aspergillus fumigatus hyphae. We hypothesized that RFHT could damage A. fumigatus hyphae. We found that RFHT inhibits A. fumigatus hyphal growth and damages hyphal structures in a dose-dependent fashion. These results indicate that noninvasive RFHT should be validated in vivo as an adjunct modality in treatment of invasive aspergillosis.

MATERIALS AND METHODS

Fungal isolate. The reference A. fumigatus strain Af293 was used in the present study. To obtain conidia, Af293 was plated on yeast agar glucose plates containing 0.5% (wt/vol) yeast extract, 1.0% (wt/vol) dextrose, 0.2% (wt/vol) vitamin mixture, 0.1% (wt/vol) trace elements, 1.5% (wt/vol) agar, and 1.0% (wt/vol) MgSO4 at 37°C. The conidia were harvested 2 days later, counted using a hemocytometer, and suspended in phosphate-buffered saline (PBS). Af293 conidial suspensions (107 conidia/ml) were incubated in RPMI culture medium supplemented with 2% (wt/vol) glucose at 37°C for 16 to 18 h in 12-well plates to generate hyphae.

Water bath hyperthermia. We used water bath hyperthermia (WBHT) to determine the minimum and maximum temperatures (over a constant exposure time) required for maximum destruction of A. fumigatus (Af293) hyphae. Af293 samples were exposed to WBHT for 5 min at a temperature range of 45 to 55°C.

Thermal dose calculations using cumulative equivalent minutes at 43°C. The relationship between thermal exposure (time and temperature) and thermal injury in complex biological systems is multifaceted. This relationship is approximated using cumulative equivalent minutes at 43°C (CEM43) as a unit of the thermal isoeffective dose (7). Converting time and temperature to CEM43 allows the investigator to accommodate the unpredictable temporal variation of temperature as a sample of Af293 hyphae reaches a thermal steady state when determining how much thermal exposure a sample should receive. For Af293 samples exposed to WBHT over the temperature range of 45 to 55°C, we calculated the thermal dose using the following formula:

\[ \text{CEM43} = \sum_{i=0}^{t_{\text{final}}} t_i (43-T_i) \]  

in which \( t_i \) is the exposure time (in minutes), \( T \) is the final temperature (in degrees Celsius), \( R \) = 0.5 for temperatures > 43°C, and \( R \) = 0.25 for temperatures < 43°C.

RFHT. A Kanzius noninvasive external RF generator (Therm Med LLC, Erie, PA) with a fixed frequency of 13.56 MHz was used for the RFHT experiments. The generator power was set to 900 W for all experiments. The experimental setup for the generator is shown in Fig. S1 in the

Received 10 May 2013 Returned for modification 29 May 2013 Accepted 26 June 2013

Published ahead of print 8 July 2013

Address correspondence to Dimitrios P. Kontoyiannis, dkontoyi@mdanderson.org.

B.T.C. and S.J.C. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01017-13.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.
supplemental material. Af293 hyphae culture samples were placed on a polytetrafluoroethylene (Teflon) plate holder in the RF field. The generator was warmed for 10 min before each treatment. Each sample was exposed to the RF electric field (~60 kV/m) for 5 or 10 min. An infrared camera (FLIR Systems Inc., Boston, MA) was used to continuously monitor the temperature of the samples. The starting temperature for the culture medium in all of the experiments was 30°C.

**XTT colorimetric assay.** Immediately after exposure to hyperthermia (WBHT or RFHT), hyphal damage was evaluated using an XTT assay (Sigma-Aldrich) as described previously (8). XTT-treated hyphae were incubated at 37°C for 2 h in the dark. The absorbance of samples was then measured using a microplate spectrophotometer at 492 nm, and the measurements were corrected for background absorbance at 690 nm. The relative hyphal damage was calculated using the change in absorbance (relative to that of an untreated control) according to the equation,

\[
\text{% Relative hyphal damage} = \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}}} \times 100
\]

in which A is the absorbance in arbitrary units. Each experiment was repeated three times with three replicates (n = 9).

**DiBAC staining.** The fluorescent dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC) is capable of penetrating into depolarized cells via damage to the cell walls and binding to intracellular proteins or membranes, resulting in enhanced fluorescence. To assess the A. fumigatus (Af293) hyphal damage induced by RFHT, Af293 conidia were allowed to grow in 12-well plates in RPMI liquid medium with 0.15% (wt/vol) polyacryl acid (Junlon), which promotes dispersed growth of Af293, for 18 h at 37°C to form hyphae. After RFHT-based treatment, hyphal samples were scraped from the plates, placed in 1.5-ml centrifuge tubes, and centrifuged at 8,000 rpm for 10 min at room temperature. The supernatant was removed from each tube, and the hyphae were washed twice with 1× sterile PBS. DiBAC (Molecular Probes, Eugene, OR) staining of hyphal samples was performed as described previously (8). After incubation, hyphae were washed twice, and 10 μl of the hyphal suspension was mounted on a slide to examine the hyphal damage under a Fluoview FV1000 confocal fluorescence microscope (Olympus Imaging America).

**TEM.** Immediately after RFHT exposure, A. fumigatus (Af293) hyphae were prepared for transmission electron microscopy (TEM) analysis to examine the structural changes after the hyperthermia treatment. Hyphae exposed to WBHT at 55°C were used as controls. Briefly, hyphae were fixed with a solution containing 3% (vol/vol) glutaraldehyde and 2% (vol/vol) paraformaldehyde in 0.1 M cacodylate buffer at pH 7.3 for 1 h. After fixation, the hyphae were washed and treated with 0.1% (wt/vol) cacodylate-buffered tannic acid, postfixed with 1% (wt/vol) buffered osmium tetroxide for 30 min, and stained en bloc with 1% (wt/vol) uranyl acetate. The hyphae were dehydrated in ethanol and embedded in LX-112 medium. The hyphae were then polymerized in a 70°C oven for 2 days. Ultrathin hyphal sections were cut using an Ultracut microtome (Leica Microsystems, Deerfield, IL), stained with uranyl acetate and lead citrate in an EM stainer (Leica Microsystems, Deerfield, IL), and examined using a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images of hyphae were obtained using an AMT imaging system (Advanced Microscopy Techniques Corp, Danvers, MA).

**Statistical analysis.** Statistical analysis was conducted using the Prism software program (version 5.0; GraphPad Software). For analysis of each data set, an unpaired t test or one-way analysis of variance with Bonferroni’s multiple comparison tests was used with 95% confidence intervals. All experiments were repeated three times, and P values that were <0.05 were considered significant.

**RESULTS**

We evaluated the A. fumigatus (Af293) hyphal damage caused by WBHT using an XTT assay at a constant exposure period (5 min) and various temperatures. As shown in Fig. 1a, WBHT exposure...
caused significant hyphal damage at temperatures above 51°C with no damage observed at 45°C. In contrast, RFHT exposure resulted in damage of >70% of the hyphae (P < 0.0001) over 5 min (Fig. 1b) at a much lower average final media temperature of 42.13°C. Moreover, a 10-min exposure to RFHT at an average final medium temperature of 48.09°C caused extreme hyphal damage (>90%) (Fig. 1b). We used a mathematical model described previously (7) to calculate the time required in a water bath at 43°C (CEM43) to produce an equivalent hyphal damage with 5 min and 10 min exposure to RFHT at 43°C. The calculated log10 CEM43 values of 0.755 (5.69 CEM43) and 3.54 (3,517.59 CEM43) corresponded to RFHT exposures for 5 and 10 min, respectively (Fig. 1c). We observed comparable hyphal damage with WBHT exposure, with log10 CEM43 values of 4.62 (41,686.94 CEM43) and 5.24 (173,780.08 CEM43) for RFHT exposure periods of 5 and 10 min, respectively. These results indicated that destruction of A. fumigatus (Af293) hyphae by RFHT requires a much shorter exposure time than that by WBHT. Of note is that we calculated the apparent thermal dose using the duration of exposure and final temperature of WBHT-treated hyphae with formula (Materials and Methods), which may have overestimated the delivered thermal doses for RFHT-treated samples of hyphae since the samples’ starting temperature was 30°C.

Furthermore, we assessed the A. fumigatus (Af293) hyphal damage caused by RFHT using DiBAC staining and fluorescence micrographs. We found time-dependent hyphal damage in RFHT-treated samples but not in untreated samples (Fig. 2). Also, we used TEM to evaluate hyphal morphology after RFHT exposure and compare it to the morphology of control hyphae exposed to WBHT at 55°C for 5 min. Untreated A. fumigatus (Af293) hyphae had no structural changes, whereas hyperthermia-treated hyphae (RFHT at 42.13°C, RFHT at 48.09°C, and WBHT at 55°C) exhibited destruction and alteration of extracellular structures (Fig. 3). Cellular stress induced by hyperthermia resulted in the destruction of the outer fibrillar layers of the hyphal cell walls. This damage increased as the hyperthermia exposure period increased.

DISCUSSION

Previous studies within our lab have shown several important clinically relevant results in terms of the translational nature of noninvasive RFHT for cancer therapy. Targeted RFHT can be achieved by three means: (i) the use of a copper tape template which attenuates RF energy but allows propagation of RF waves into the patient in areas where there is no copper (4); (ii) the inherent electrical (permittivity) properties of the tumor itself, which allows for increased absorption of RF energy in the tumor relative to normal, healthy tissues (9); and (iii) the use of functionalized nanoparticles conjugated to bioactive molecules or antibodies that act as nano-heat transducers (10).

In light of the results presented here, we think that these three techniques can also be applied to a systemic fungal disease such as invasive aspergillosis and to more chronic, localized forms of deep-seated pulmonary aspergillosis or cutaneous aspergillosis. Both systemic and localized aspergillosis could be treated using
whole-body RFHT exposure with tight temperature constraints (with or without copper tape to cover RF-sensitive areas on the patient, if applicable). Namely, RFHT should not allow the core body temperature to rise above 41.5°C (4). The bodies ability to self-regulate core temperature when exposed to external environmental heat stimuli by means of sweating, heat shock protein production, etc., means that longer periods of RFHT can be tolerated without being detrimental to the patient. Although our results indicate that RFHT can induce severe hyphal damage for a short duration of RF exposure over a temperature range of ca. 42 to 48°C, this is the temperature of the medium in vitro and does not represent the heating mechanisms and dynamics of in vivo environments.

In many of our previous in vivo studies on mice bearing a range of ectopic and orthotopic cancer models, we have shown that RF exposure times <15 min are completely safe, tolerable, and non-lethal, as well as being of therapeutic relevance. As previously stated, this RF time is tolerable due to the body’s ability to self-regulate core body temperature when stressed by external heat factors. Its therapeutic benefit derives from a variety of factors including the mechanism of RF-induced heat production in tumors, as well as the difference in blood networks between normal and cancerous tissue lesions. The leaky vasculature nature of tumor angiogenesis and poor blood flow dynamics throughout the tumor mean they are less able to effectively dissipate heat into their surrounding environment, away from the heat source. This inevitable means the tumor gets hotter compared to the rest of the body. We would also anticipate this effect in A. fumigatus (Af293) given that it has no blood network connected to the patient and is a foreign artifact.

The mechanism of heat induction of RFHT is also different from other forms of hyperthermia such as WBHT, which is mediated through simple heat conduction mechanisms. A time-varying RF electric field induces heat in a variety of materials due to the electrophoretic motion of ions and interactions with molecular dipoles due to the inherent electrical permittivity of the specific material. Each material (e.g., metals, insulators, tissues, organs, etc.) has a unique, frequency-dependent permittivity that describes how it will store and convert electrical energy into heat. We have recently shown that pancreatic and hepatic tumors have larger permittivity loss-tangents than normal, healthy tissues, meaning they will absorb more RF energy, as well as dissipate more RF energy into heat (9). We also anticipate that A. fumigatus (Af293) has an RF-sensitive permittivity, given that hyphal damage was so severe for such a short space of RF exposure time. This would be another targeting strategy for treating both systemic and localized, deep-seated pulmonary aspergillosis and may allow for even shorter RF exposure times.

Our findings demonstrated that RFHT causes severe damage to the outer fibrillar layer of A. fumigatus polysaccharide cell wall, which is a fungus-specific structure not found in mammalian eukaryotic cells and is considered an important target of antifungal drugs. Nakai et al. (11) and Chiu et al. (12) observed similar cell wall damage when they administered cell wall-active agents to treat A. fumigatus infection. The penetration of fungi by antifungal agents is limited by an intact cell wall. Damaged fungal cell wall may therefore facilitate enhanced antifungal drug delivery and accumulation, which may further inhibit the growth of hyphae.

Finally, the use of antifungal drugs and/or cell-wall active agents conjugated to RF-sensitive nanoparticles, such as gold nanoparticles, or ultrashort carbon nanotubes may allow for enhanced targeting methods similar to previous strategies shown in our lab. For example, we have shown that C225 (Cetuximab)-conjugated gold nanoparticles can target and induce intracellular hyperthermia in pancreatic cancer (10) and have also shown that pluronics-wrapped, cisplatin-loaded ultrashort carbon nanotubes (13) can be remotely “triggered” by RF fields to release their therapeutic payload into cancer cells upon activation of the RF field by the user. Both of these strategies could be effectively used for the treatment of A. fumigatus infections.

Further studies using more Aspergillus strains and, non-Aspergillus molds designed to enhance the therapeutic index of RFHT and validation of its application using in vivo model of subcutaneous invasive mold infections (14) are warranted. Our findings presented here should inspire such further studies exploring the effectiveness of RFHT as an adjunct to the local treatment of opportunistic fungal infections.

ACKNOWLEDGMENTS
This study was funded by an unrestricted research grant from the Kanzius Research Foundation (SAC, Erie, PA). This research is supported in part by M. D. Anderson Cancer Center support grant CA016672. D.P.K. is a recipient of the Frances King Black Memorial Professorship for Cancer Research.

We thank Kenneth Dunner, Jr., at the High Resolution Electron Microscopy Facility, University of Texas M. D. Anderson Cancer Center, for providing invaluable assistance with TEM imaging, Fazal Shirazi for useful discussion, and Kristine Ash for administrative assistance.

REFERENCES

September 2013 Volume 57 Number 9 aac.asm.org 4447

Aspergillus Damage Caused by Hyperthermia

