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High intensity focused ultrasound-induced gene activation in sublethally injured tumor cells *in vitro*

Yunbo Liu,

Department of Mechanical Engineering and Materials Science, Duke University, Durham, North Carolina 27708

Takashi Kon,

Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina 27708

Chuanyuan Li, and

Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina 27708

Pei Zhong^a)

Department of Mechanical Engineering and Materials Science, Duke University, Durham, North Carolina 27708

Abstract

Cultured human cervical cancer (HeLa) and rat mammary carcinoma (R3230Ac) cells were transfected with vectors encoding green fluorescent protein (GFP) under the control of hsp70B promoter. Aliquots of 10- μ l transfected cells (5×10^7 cells/ml) were placed in 0.2-ml thin-wall polymerase chain reaction tubes and exposed to 1.1-MHz high intensity focused ultrasound (HIFU) at a peak negative pressure $P^- = 2.68$ MPa. By adjusting the duty cycle of the HIFU transducer, the cell suspensions were heated to a peak temperature from 50 to 70 °C in 1–10 s. Exposure dependent cell viability and gene activation were evaluated. For a 5-s HIFU exposure, cell viability dropped from 95% at 50 °C to 13% at 70 °C. Concomitantly, gene activation in sublethally injured tumor cells increased from 4% at 50 °C to 41% at 70 °C. A similar trend was observed at 60 °C peak temperature as the exposure time increased from 1 to 5 s. Further increase of exposure duration to 10 s led to significantly reduced cell viability and lower overall gene activation in exposed cells. Altogether, maximum HIFU-induced gene activation was achieved at 60 °C in 5 s. Under these experimental conditions, HIFU-induced gene activation was found to be produced primarily by thermal rather than mechanical stresses.

1. Introduction

In recent years, high intensity focused ultrasound (HIFU) has been used successfully as a noninvasive and nonionizing therapeutic modality for the treatment of a variety of cancers, including liver (Chen *et al.*, 1999; Kennedy *et al.*, 2004), prostate (Chapelon *et al.*, 1999; Chaussy and Thuroff, 2003), ocular (Lizzi *et al.*, 1992), and breast (Hynynen *et al.*, 2001) cancers. Extensive physical and biological studies have been carried out, which demonstrate that HIFU, with a spatial peak temporal average intensity (I_{SPTA}) between 10^3 and 10^4 W/cm², can produce well-defined lesions in deep-seated tissue through coagulative necrosis and cavitation damage (ter Haar, 1995, 2001; Bailey *et al.*, 2003; Wu *et al.*, 2004; Kennedy *et al.*, 2003). In addition to thermal ablation, there is also preliminary yet encouraging evidence to suggest that HIFU may induce a distinct stress response in sublethally injured tumor cells surrounding the lesion. For example, following HIFU treatment in patients

a) Author to whom correspondence should be addressed; electronic mail: pzhong@duke.edu

with benign prostatic hyperplasia, (BPH), significant up-regulation of heat shock proteins (hsps) has been observed at the border of HIFU-induced necrosis region (Kramer *et al.*, 2004). It has been further postulated that the up-regulation of hsps may play a critical role in eliciting an anti-tumor immunity (Kramer *et al.*, 2004; Wu *et al.*, 2004). Although heat shock response induced by conventional hyperthermia (42 °C for 30 min) is well documented (Hildebrandt *et al.*, 2002), the physical conditions for HIFU-induced stress response have not been thoroughly investigated. Furthermore, as we will discuss later, a synergistic combination of HIFU-induced stress response and/or gene therapy with HIFU-produced thermal ablation may help to improve the effectiveness of cancer therapy.

When cells are exposed to stressful conditions, such as hyperthermia, environmental toxicity, and even mechanical stresses, the expression of a specific family of genes that control the production of heat shock (or stress) proteins, also known as molecular chaperones, will be dramatically upregulated (Morimoto, 1993). In cells of diverse origins, heat shock proteins are well conserved throughout evolution. In particular, it has been shown that hsp70 protein family represents the most abundant gene product in response to external stresses (Voellmy, 1996). One member of the hsp70 gene family, hsp70B, is strictly stress inducible and absent in unstressed cells (Hildebrandt *et al.*, 2002). Taking advantage of this unique property, various heat-inducible gene constructs have been developed recently for both cytotoxic and immuno-stimulatory gene therapies, which are usually activated by hyperthermia in a 42 °C water bath for 30 min (Li and Dewhirst, 2002). Compared to other physical means such as radiation for gene activation, heat-regulated gene expression under the control of hsp70B promoter has excellent inducibility with very low background, making it an attractive approach for controlling gene expression both temporally and spatially (Huang *et al.*, 2000; Borrelli *et al.*, 2001).

Recently, ultrasound-induced hyperthermia has also been exploited to regulate transgene expression both *in vitro* and *in vivo* (Vekris *et al.*, 2000; Zhong *et al.*, 2003). Following exposure to 2 W/cm² ultrasound for 20 min, Smith *et al.* (2002) demonstrated a locally induced luciferase or FasL gene expression under the control of hsp70B promoter. Using a MRI-guided ultrasound system, Guilhon *et al.* (2003) reported heat inducible transgene (GFP) expression in a rat tumor model after ultrasound-induced hyperthermia within a thermal dose of 44 °C for 20 min to 48 °C for 3 min. However, the profile of heat shock response of tumor cells exposed to temperatures in the range of 50–70 °C for a few seconds is largely unknown. These thermal exposure conditions are most relevant to those experienced by the sublethally injured tumor cells surrounding HIFU-produced necrosis lesion. Moreover, it should be noted that heat shock response could also be induced by pulsed ultrasound without thermal effects (Barneet *et al.*, 1994; Angles *et al.*, 1990; Locke and Nussbaum, 2001) or even by cyclic mechanical shear stress (Xu *et al.*, 2000), usually for long exposure durations of 30 min to 6 h. However, the relative contribution of thermal versus mechanical stresses to ultrasound induced heat shock response in biological cells/tissue has not been systematically evaluated.

The purpose of this study was to determine the activation of transfected GFP marker gene under the control of hsp70B promoter in tumor cells that were sublethally injured during HIFU exposure *in vitro*. Specifically, the goals of this study are threefold: (1) assessing the inducibility of transfected exogenous heat-sensitive gene in sublethally injured tumor cells following HIFU treatment, (2) evaluating the optimal thermal dosage (temperature and duration) for eliciting strong and consistent gene expression both in surviving and total exposed cell populations, and (3) comparing the contribution of underlying physical mechanisms (thermal stress versus mechanical stress) for HIFU-induced gene activation.

II. Materials and Methods

A. Cell culture and gene transfection

Human cervical cancer (HeLa) cell line was maintained routinely in minimal essential medium (MEM) with 10% heat-inactivated fetal bovine serum (FBS) and 5% antibiotics in a humidified incubator at 37 °C containing 5% CO₂. One day before HIFU treatment, HeLa cells were transfected with Adeno-hsp70B-GFP adenovirus vector at a MOI (multiplicity of infection=ratio of infectious virus particles to cells) of 10. In another set of experiments, rat mammary carcinoma (R3230Ac) cells were transfected with hsp70B-EGFP plasmid using lipofectamine (Gibco-BRL, Bethesda, MD). To select stably transfected R3230Ac cells, G418 (400 µg/ml) was added to the transfected cell culture and hyperthermia was performed in a water bath (42 °C-30 min). Colonies that showed high level of inducible GFP expression and low fluorescence background were selected and subsequently expanded and frozen in aliquots for future use. One week before the experiment, a frozen stock of the constructed R3230Ac cells were recovered at low seeding density and cultured in T-25 flasks with DMEM containing 10% FBS and 1% penicillin-streptomycin. Before HIFU treatment, the transfected HeLa and R3230Ac cells were grown to about 60% confluence, trypsinized, pelleted (80 g, 3 min), and then suspended in culture medium with a cell density of 5×10^7 cells/ml.

B. High intensity focused ultrasound (HIFU) apparatus

A 1.1-MHz HIFU transducer (H-102, Sonic Concepts, Seattle, WA) with a focal length of 62 mm was mounted horizontally inside a heated (37 °C) chamber filled with degassed water. As shown in Fig. 1(a), the HIFU transducer with its 50-Ω matching network was driven by sinusoidal tone-burst signals produced by a function generator (33120A, Agilent, Palo Alto, CA), which was connected in series with a 55-dB power amplifier (A150, Electronic Navigation Industries, Rochester, NY). The operation and exposure parameters of the HIFU system were controlled by LABVIEW programs via a GPIB board installed in a PC. A volume of 10-µl cell suspension was loaded in a 0.2-ml Polymerase Chain Reaction (PCR) thin-wall tube (Product No. 3746, Corning, NY), which was placed vertically with its conical bottom aligned within the -6-dB beam focus of the HIFU transducer. Using a calibrated needle hydrophone (TUN001A, NTR Sys, Seattle, WA), the pressure distribution in the focal plane of the transducer was measured, which revealed a peak positive (P^+)/peak negative pressure (P^-) of 3.05/-2.68 MPa and a -6-dB beam diameter of 1.64 mm when the function generator was operated at 0.1 V_{pp} [Fig. 1(b)]. The corresponding spatial average pulse average intensity (I_{SAPA}) of the acoustic field around the beam focus was calculated to be 278 W/cm², based on an established protocol (Harris, 1985). At this output level, different temperature elevations in the cell suspension could be achieved by adjusting the duty cycle (10%-30%) of the tone burst signal at a fixed pulse repetition frequency of 200 Hz.

C. Temperature measurement

Temperature elevation in the cell suspension during HIFU exposure was monitored using a 0.2-mm bare-wire thermocouple (Customer designed IT-23, Physitemp Inc., Clifton, NJ), which minimizes ultrasound beam distortion and measurement artifacts due to viscous and sheathing heating (Hynynen and Edwards, 1989). The thermocouple output voltage was conditioned by an electronically compensated isothermal terminal block (TC-2190, National Instrument, Austin, TX) and registered at 6-Hz sampling rate by a Data Acquisition Board (NI4351, National Instrument, Austin, TX) controlled by a LABVIEW program [Fig. 1(a)]. Using this system, the nonuniform spatial temperature fluctuation inside the cell suspension (2 mm medium high) was determined to be less than 3.0 °C, which was presumably caused by the inhomogeneous ultrasound absorption at the tube wall.

Figure 2 shows two representative temperature profiles that can be produced in the cell suspension during HIFU exposure—heat plateau and heat shock, both of which reached a peak temperature of 60 °C. For heat plateau scheme, the initial temperature rise was linearly proportional to the absorbed energy density in the first 5 s and subsequently the temperature rise slowed down as the local heat conduction became pronounced. With continued ultrasound exposure, a thermal equilibrium was eventually achieved to maintain a temperature plateau during the 15- to 30-s insonification. Such a heat plateau profile is most important for hyperthermia therapy. In comparison, heat shock profile is more relevant for HIFU therapy, in which the initial (5 s) temperature rise becomes dominant. Because of the high ultrasound intensity and short exposure time, a local thermal equilibrium cannot be reached before the end of HIFU exposure (Lizzi and Ostromogilsky, 1987; Hill and ter Haar, 1995).

D. Experimental design

Three series of experiments were carried out in this study. The first series of experiments were designed to provide a transition from hyperthermia-induced gene activation (heat plateau) to HIFU-elicited gene activation (heat shock). Here the temperature in the cell suspension was gradually escalated from 42 to 70 °C while the treatment time was reduced concomitantly from 30 min to 5 s. Multiple durations at 50, 60, and 70 °C temperature levels were evaluated.

Once the feasibility of HIFU-induced gene activation was demonstrated, a second series of experiments were carried out with HIFU-relevant heat shock exposures in which either the treatment time was fixed at 5 s while the peak temperature was increased from 50 to 70 °C or the peak temperature was maintained at 60 or 70 °C while the heat shock duration was increased progressively from 1 to 10 s. Based on these experiments, the optimal exposure conditions were identified for producing maximum gene activation either in the surviving cells or with respect to the total exposed cell population.

In the third series of experiments, the contribution of thermal stress versus mechanical stress to HIFU-induced gene activation was investigated. To evaluate the contribution of thermal stress transfected HeLa cells cultured on etched grid cover slips (Bellco Biotechnology, Vineland, NJ) were plunged rapidly into PBS solutions of 50, 60, or 70 °C, respectively, for a short immersion period of 2 s. Such exposures yielded similar cell death to the corresponding 5-s HIFU heat shock treatment. In comparison, to assess the effect of mechanical stress transfected HeLa cells were treated under the identical acoustic parameters for the 70 °C-5-s HIFU exposure except that the ambient temperature in the water bath was lowered to 5 °C. Using this approach, the peak temperature in the cell suspension during the 5-s HIFU treatment could be kept below 35 °C, thus eliminating the contribution of the thermal stress.

In addition, cavitation activity in cell suspension during HIFU treatment was measured by passive cavitation detection (PCD) using a 15-MHz transducer (M2062, Panametrics, Waltham, MA) positioned perpendicularly to the HIFU beam and 5 mm away from the cell samples. A total of 100 acoustic emission signals (each collected in a 10- μ s window) emanated from the HIFU-induced cavitation bubbles were amplified by a pulse/receiver (5052PR, Panametrics, Waltham, MA) and recorded in sequence mode on a digital oscilloscope (9310A, LeCroy, Chestnut Ridge, NY) during the 5-s exposure period (Chen *et al.*, 2004). Fast Fourier Transform (FFT) spectrums of these wave forms from ten measurements were averaged and compared for different exposure settings. rms amplitude of the broadband noise signals for each FFT spectrum between 12.5 and 17.5 MHz in frequency domain was further calculated and presented in time sequence. The level of inertial cavitation (IC) activity was quantified by computing the cumulated IC dosage (ICD), defined by the integration of the rms amplitude (12.5–17.5 MHz) over the entire exposure period (Chen *et al.*, 2003).

E. Viability and gene expression assays

Immediately following HIFU exposure, cell samples were plated and cultured on the etched-grid cover slips in a six-well plate for 24 h before assayed for cell viability and gene expression. Cell viability was determined by counting viable cells from 10 randomly selected areas of each sample under a microscope after 5 min *in situ* Trypan Blue Staining. The results from HIFU treated groups were normalized by viability of the corresponding control group.

To assess GFP gene expression, the cultured cells were trypsinized and FACS analysis was performed using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA) operated at an exciting wavelength of 488 nm and emission detection wavelength of 530±15 nm.

F. Statistical analysis

Each experiment data point was averaged from six samples except otherwise indicated. Student's t-test was used to determine the statistical significance and $p < 0.05$ was considered to indicate a statistically significant difference between two experimental configurations. All statistics were computed by using SIGMAPLOT 8.0 (Systat Software, Point Richmond, CA).

III. Results

A. Heat plateau- and heat shock-induced gene activation

Inducible GFP gene activation was observed in both cell lines following a wide range of ultrasound exposure. Figure 3 shows the maximum gene expression in surviving cells 24-h post-treatment at four different peak temperature levels: water-bath hyperthermia (42 °C for 30 min), heat plateau (50 °C for 15 s+2 min; 60 °C for 15 s+15 s) and heat shock (70 °C in 5 s). In comparison, the transfected cells in the control group displayed a very low basal fluorescence activity (<1.5%), confirming the highly inducible nature of the hsp70B promoter. The corresponding cell viability is summarized in Table I, which reveals a graduate decrease from 84% for 42 °C-30-min hyperthermia to 13% for 70 °C-5-s heat shock treatment. In addition, the mean GFP fluorescence intensity in the GFP positive cells was calculated and used as a means to evaluate the strength of the heat shock response in each treatment group.

For gene activation in the transfected HeLa cells, the percentage of GFP positive cells in the surviving population was found to be in the range of 54% to 41% for heat plateau and heat shock treatments, which are lower than 66% induced by hyperthermia treatment [Fig. 3(a)]. This is presumably caused in part by the heterogeneous temperature distribution in cell suspension produced by the ultrasound treatment, compared to the uniform temperature distribution in water-bath hyperthermia. However, the mean GFP intensity in HIFU-treated groups was found to be increased by 31-fold to 42-fold from the control group, although no statistical difference in gene expression was found within the heat plateau treatment groups ($p > 0.14$). In comparison, the mean GFP intensity was increased by 21-fold in the hyperthermia treatment group. This finding suggests that stronger heat shock response can be induced in sublethally injured tumor cells that survive the insult of HIFU exposure compared to hyperthermia treatment. Similar patterns of HIFU-induced gene activation were observed in the transfected R3230Ac cells [Fig. 3(b)].

B. Effect of peak temperature on HIFU-induced gene activation

The effect of peak temperature on HIFU-induced gene activation in transfected HeLa cells for a fixed heat shock exposure time of 5 s is shown by phase contrast and fluorescence images in Fig. 4. Detectable level of GFP expression was observed as early as 4–6 h following the HIFU exposure. The gene expression level reached a maximum about 24 h and remained detectable 72 h post HIFU treatment. This temporal profile of inducible gene activation by HIFU is similar to that produced by ultrasound-induced hyperthermia (Smith *et al.*, 2000). Quantitatively, a

low level of GFP expression (4%) was induced at 50 °C peak temperature and its GFP intensity increased about 3-fold compared to the control group [Fig. 5(a)]. However, as the peak temperature was increased to 60 and 70 °C, much stronger GFP expression of 38% and 41% was induced in the surviving cells with concomitant expression intensity increased by 35-fold and 42-fold, respectively [Fig. 5(a)]. However, no statistical difference in gene expression ($p>0.09$) was found between the 60 and 70 °C treatment groups. The inducible gene expression in the surviving cells appeared to reach a saturation threshold at 60 °C peak temperature. In fact, cell viability was 95% and 71% following HIFU exposure at 50 and 60 °C peak temperatures and the corresponding value dropped significantly to 13% post-70 °C exposure (Table I). This transition in HIFU-induced cell necrosis between 60 and 70 °C is comparable to the temperature threshold for tissue necrosis (>60 °C) observed in several animal studies (Yang *et al.*, 1993; ter Haar, 1995).

For assessing the potential therapeutic effect, the gene expression efficiency with respect to the total exposed cells is most relevant. This value can be calculated as the product of cell viability and gene expression efficiency in the surviving cells, and the results are shown in Fig. 5(b). It was found that with respect to the total exposed cells, the maximum gene expression was induced at 60 °C peak temperature. For example, a 20-fold increase (compared to control) was observed at 60 °C peak temperature, which is much higher than the corresponding results of a 3-fold increase for 50 °C and a 4-fold increase for 70 °C, respectively [Fig. 5(b)]. Altogether, these findings suggest that on the one hand, gene expression cannot be activated if the cells are not stressed sufficiently (e.g., 50 °C for 5 s). On the other hand, if cells are overstressed (e.g., 70 °C for 5 s) excessive cell death will be produced, leading to a low gene expression efficiency with respect to the total exposed cells. An optimal window of thermal dosage (around 60 °C for 5 s) exists for inducing the maximum overall gene expression following a 5-s HIFU exposure in our experimental system. It is worth noting that the maximum gene expression induced by HIFU (60 °C-5 s) is about 52% in percentage and 86% in intensity compared to the gene expression induced by 42 °C-30-min water bath hyperthermia [Fig. 5 (b)]. Similar results were obtained from the R3230Ac cells following HIFU treatment (data not shown).

C. Effect of treatment duration on HIFU-induced gene activation

The effect of treatment duration on HIFU-induced gene activation in transfected HeLa cells at 60 °C peak temperature is shown in Fig. 6. It was found that gene expression in the surviving cells increased initially with the exposure duration and gradually saturated after 5-s exposure [Fig. 6(a)]. No statistically significant difference ($p>0.1$) was observed between the gene activation levels produced by the 5- and 10-s exposures with respect to expression percentage and intensity. However, for the total exposed cells HIFU-induced gene activation showed a similar initial increase from 1 to 5 s, followed by a dramatic decline after 10-s exposure. These results suggest that an optimal dosage for HIFU-induced gene activation is 5 s at 60 °C peak temperature, and further increase in treatment time will dramatically increase cell injury and thus significantly diminish the overall inducible gene activation. A similar pattern of HIFU-induced gene activation was also observed at 70 °C peak temperature with an exposure duration ranging from 1 to 10 s; yet the corresponding gene activation efficiency in total cells was lower than its counterpart at 60 °C (data not shown).

D. Physical mechanisms: Thermal stress versus mechanical stress

The thermal stress produced by HIFU exposure was simulated by immersing transfected HeLa cells cultured on etched cover slips in hot PBS solutions with temperature of 50, 60, and 70 °C, respectively, for 2 s. As shown in Fig. 7(a), gene expression can be clearly observed in the 60 and 70 °C groups but barely in the 50 °C group. Quantitatively, GFP intensity in surviving cells was increased 42-fold and 38-fold in the 60 and 70 °C groups [Fig. 7(b)], which are

comparable to the gene activation (35-fold and 42-fold) produced by the 5-s HIFU treatment [Fig. 5(a)]. Because of the much more uniform thermal exposure in the hot PBS immersion system, the resultant gene expression percentage [52% for 60 °C, Fig. 7(b)] was higher than the corresponding value (38%, Fig. 5(a)) induced by the HIFU exposure. In addition, a dramatic decrease in cell viability was also observed from 60 to 70 °C. Therefore, with respect to the total exposed cells, the maximum gene activation level was again produced at 60 °C, confirming the results from HIFU-induced gene activation [Fig. 5(b)].

To assess the effect of mechanical stress in gene activation, HIFU-induced cavitation activities in the cell suspension were measured by PCD under identical acoustic exposure conditions yet with different ambient water temperature: 70 °C-5 s ($T_{\text{waterbath}}=37$ °C) and 35 °C-5 s ($T_{\text{waterbath}}=5$ °C). Representative frequency spectrums of the emitted PCD signals are shown in Fig. 8(a). A significant increase of the broadband noise associated with inertial cavitation was observed during insonification, which is comparable between the two exposure conditions. In addition, subharmonics and higher harmonics (second, third, fourth, etc.) generation can be clearly identified in the frequency domain, which could be produced by the nonlinear scattering of stable cavitation bubbles in the cell culture medium (Miller and Bao, 1998). Moreover, the averaged rms amplitude of the broadband noise in the frequency range of 12.5–17.5 MHz during the 5-s HIFU exposure is plotted in Fig. 8(b), from which the integrated inertial cavitation dosages (ICD) were calculated to be 69.1 ± 10.9 and 61.3 ± 12.5 dBm*s, respectively. Again, no statistically significant difference was found between the two exposure conditions ($p > 0.1$). It is interesting to note that all these indexes for cavitation activity dropped significantly when the culture medium was replaced by degassed water. Altogether, these results suggest that substantial cavitation activities could be induced in cell suspension during HIFU treatment, and comparable bubble activities were produced using either the 70 °C-5 s (thermal+mechanical stresses) or the 35 °C-5-s (mechanical stresses) exposure condition.

The results for cell viability and gene activation produced by the two exposure conditions are shown in Fig. 8(c). At a medium temperature of 70 °C, cell viability dropped to 13% following HIFU treatment; yet gene activation was induced in 41% of the survival cells with a concomitant 42-fold increase in expression intensity. In comparison, at a medium temperature of 35 °C cell viability was 72% with minimal level of gene activation, which is not statistically significant different ($p > 0.1$) from the value in the control group. Together, these results suggest that the dominant mechanism for HIFU-induced cell necrosis and gene activation is thermal stress. The mechanical stress induced during the HIFU treatment contributes to about 1/3 of the cell damage, but it is not sufficient to elicit a measurable stress response (i.e., activation of the hsp70B promoter). However, the mechanical stress and thermal stress may interact synergistically during HIFU exposure to enhance thermal ablation of the tumor.

IV. Discussion

During HIFU treatment, the peak temperature in tissue at the beam focus could increase rapidly above 80 °C, which leads to effective cell killing and coagulative necrosis even for a few seconds exposure (Kennedy *et al.*, 2003). At the boarder of the lesion, a steep temperature gradient exists and multiple layers of cells that are sublethally injured form a sharp demarcation that separate the necrotic tissue and the surrounding untreated tissue. More important, over expression of heat shock proteins in the boarder zone has been observed recently, which may incite inflammation and even stimulate an anti-tumor immune response (Kramer *et al.*, 2004). However, the thermal exposure condition that leads to the heat shock response in the boarder zone was not determined. In this study, using an *in vitro* cell exposure system we have mimicked the response of tumor cells in different temperature regions within the boarder zone of HIFU-induced lesion. Our results suggest that heat shock response can be produced in sublethally injured tumor cells within the temperature range of 50–70 °C. The maximum heat-

induced gene activation could be induced at a peak temperature about 60 °C with a 5-s exposure time. Furthermore, our results suggest that HIFU-induced gene activation is produced primarily by thermal rather than mechanical stresses because of the extremely short exposure duration in HIFU (a few seconds) compared to the long treatment duration (>30 min) in hyperthermia and physiotherapy. In future work, it would be interesting to evaluate stress response of tumor cells that are exposed to a long duration of ultrasound-induced mechanical stresses that yield a similar level of sublethal cellular damage as produced by HIFU-generated thermal stress.

HIFU-induced cell death rises rapidly as the peak temperature is increased from 60 to 70 °C (see Table I). This observation is consistent with the temperature threshold for tissue necrosis (>60 °C) observed in several animal studies (Yang *et al.*, 1993; ter Haar, 1995). However, even at a peak temperature of 70 °C or above (data not shown) a small number of tumor cells can still survive. Similarly, incomplete tissue necrosis has been observed in tissue specimens collected from prostate cancer patients following HIFU treatment (Van Leenders *et al.*, 2000). These results are presumably caused by the heterogeneity in heat conduction and biological response of the tumor cells/tissue to HIFU therapy. In light of this observation, it is desirable to combine therapeutic modalities that are effective against residual tumor cells, such as immunotherapy, with HIFU so that the overall quality of cancer therapy can be improved.

Within the population of cells that were sublethally injured by HIFU treatment, heat-induced gene activation was found to increase initially with either peak temperature (for a fixed treatment duration) or treatment time (for a fixed peak temperature) before reaching a maximum at 60 °C for 5 s. Further increase in peak temperature or treatment duration led to a rapid reduction of gene activation in the total exposed cells [see Fig. 6(b)]. This dose-dependent gene activation may be caused by the two fundamental yet opposite biological responses (i.e., survival and death) that are activated simultaneously when cells are stressed. At the molecular level, the cellular response to stress is represented by the induction of hsp's that may function either as molecule chaperones or proteases (Jolly and Morimoto, 2000). When the stress is low and duration is short (e.g., <60 °C and 5 s), molecule chaperones may assist in protein folding, translocation, refolding of intermediates, and thus restoring homeostasis. In contrast, when the stress is high and the duration is long (e.g., >70 °C and 5 s), the production of proteases such as the ubiquitin-dependent proteasome may increase significantly to degrade efficiently the damaged and short-lived proteins. Furthermore, at high stress levels the induced heat shock response may also lead to apoptosis or necrosis (Barry *et al.*, 1990). The intricate balance between these two opposite pathways and their interplay will determine whether a cell exposed to heat shock will die or survive (Jolly and Morimoto, 2000).

Taking advantage of the ubiquitous heat shock response, several groups have recently exploited the use of heat-induced gene therapy for cancer treatment (Li and Dewhirst, 2002). The most significant advantage of heat-inducible gene therapy is that site-specific control of gene expression in targeted tissues can be achieved. This is particularly important for reducing the systemic toxicity of most therapeutic genes, which are toxic to both tumor and normal tissues. Moreover, significant tumor regression has been produced by hyperthermia-induced cytotoxic (Blackburn *et al.*, 1998) and immunostimulatory (Huang *et al.*, 2000) gene therapy while no such therapeutic effects are seen with hyperthermia or intratumoral injection of the transgene alone. Potentially, there is also a synergistic interaction between hyperthermia and gene therapy, which may be related to hyperthermia-induced Fas and FasL expression in the heated tumor cells (Li and Dewhirst, 2002). However, two major limitations exist for hyperthermia-regulated cancer gene therapy. First, to induce gene activation by hyperthermia the temperature in the target tissue has to be kept closely at 42 °C for a long period of time (>30 min), a condition that is difficult to maintain in internal organs due to blood perfusion (Diederich and Hynnen, 1999). Second, hyperthermia-regulated gene therapy only retards the growth but cannot eradicate the tumor completely (Blackburn *et al.*, 1998; Huang *et al.*, 2000). To be more

effective, hyperthermia-regulated gene therapy has to be used in conjunction with other existing cancer treatment modalities, such as radiation or chemotherapy (Li and Dewhirst, 2002). In contrast, these limitations may be potentially overcome by HIFU-regulated gene therapy, which can be produced simultaneously with HIFU-induced thermal ablation. For example, HIFU can be used to activate cytotoxic or immuno-regulatory genes in sublethally injured peripheral tumor cells while thermally ablating (or reducing) the primary tumor mass. Because of the short exposure duration and temperature gradient produced by HIFU, no rigorous control of the tissue temperature is needed. Moreover, the heat-induced cytotoxic gene products or immunostimulatory factors may significantly improve the effective killing of residual and distal metastasis tumor cells via bystander effects or a boosted anti-tumor immune response (Li and Dewhirst, 2002). Alternatively, a pre-treatment by HIFU-induced gene activation in the target tumor at low intensity (i.e., without thermal ablation) may be used to prime the host anti-tumor immune response before a regular HIFU therapy for complete eradication of the tumor mass. With real-time monitoring of HIFU-induced thermal dosimetry in tumors by MRI (Hynynen *et al.*, 2001; Guilhon *et al.*, 2003), such new treatment strategies should be feasible and may improve the overall quality of cancer therapy by HIFU.

In conclusion, we have demonstrated the feasibility of HIFU-induced transgene activation in sublethally injured tumor cells *in vitro*. Further animal studies are warranted to identify the optimal HIFU exposure condition for maximum gene activation and to explore treatment strategies that can combine synergistically HIFU-induced gene therapy with HIFU-produced thermal ablation for overall improved quality and effectiveness in cancer therapy.

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FIG 1. (Color online) (a) Schematic diagram of the experimental system. (b) Pressure distribution across the focal plane of the 1.1-MHz HIFU transducer in water. P^+ : peak positive pressure and P^- : peak negative pressure.

FIG 2.
(Color online) Two representative temperature profiles (a) heat plateau and (b) heat shock produced by the 1.1-MHz HIFU transducer in cell suspension.

FIG 3. Gene activation in transfected (a) HeLa and (b) R3230Ac cells following various hyperthermia and HIFU exposures.

FIG 4.
(Color online) Representative phase contrast (left column) and fluorescence (right column) images of transfected HeLa cells after 5-s heat shock exposure at different peak temperatures. These images were taken 24 h after the experiment.

FIG 5. Gene expression of transfected HeLa cells in (a) surviving and (b) total cell populations after 5-s heat shock exposure.

FIG 6.
(Color online) Effect of treatment duration on gene expression of transfected HeLa cells in (a) surviving and (b) total cell populations at a peak temperature of 60 °C.

FIG 7.

(Color online) (a) Representative phase contrast (left column) and fluorescence (right column) images of transfected HeLa cells and (b) corresponding cell viability and gene expression in the surviving population after exposure to heated PBS solutions of various temperatures for 2 s. These images were taken 24 h after the experiment.

FIG 8.

(Color online) (a) Representative frequency spectrum of PCD signals measured during different HIFU exposures and medium conditions, (b) time evolution of averaged broadband noise of the PCD signals during the 5-s HIFU treatment, and (c) comparison of thermal +mechanical vs mechanical stresses on HIFU-induced cell viability and gene activation in the surviving population of transfected HeLa cells.

TABLE I

Cell viability of HeLa cells under HIFU exposures.

	Control	Heat plateau			Heat shock				
		42 °C 30 min	50 °C 120 s	60 °C 15 s	50 °C 5 s	60 °C 1 s	60 °C 2 s	60 °C 5 s	70 °C 5 s
Viability (%)	100	84	64	31	95	81	80	71	47
STD (%)	0	±6.9	±15.1	±9.2	±6.7	±6.7	±5.7	±6.2	±9.1
									13 ±7.8