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The Effect of High Intensity Focused Ultrasound Treatment on Metastases in a Murine Melanoma Model

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Abstract

This study aims to assess the risk of high intensity focused ultrasound (HIFU) therapy on the incidence of distant metastases and to investigate its association with HIFU-elicited anti-tumor immunity in a murine melanoma (B16-F10) model. Tumor-bearing legs were amputated immediately after or 2 days following HIFU treatment to differentiate the contribution of the elicited anti-tumor immunity. In mice undergoing amputation immediately after mechanical, thermal, or no HIFU treatment, metastasis rates were comparable (18.8%, 13.3% and 12.5 %). In contrast, with a 2-day delay in amputation, the corresponding metastasis rates were 6.7%, 11.8% and 40%, respectively. Animal survival rate was higher and CTL activity was enhanced in the HIFU treatment groups. Altogether, our results suggest that HIFU treatment does not increase the risk of distant metastasis. Instead, HIFU treatment can elicit an anti-tumor immune response that may be harnessed to improve the overall effectiveness and quality of cancer therapy.

Keywords

High intensity focused ultrasound; metastasis; anti-tumor immunity; bioluminescent imaging; melanoma

High-intensity focused ultrasound (HIFU) has recently emerged as a promising treatment modality for localized solid malignancies [1]. HIFU therapy has the unique advantage of being noninvasive and easily tolerable by the patient, thus allowing for repeated treatments. Despite this, there has been a long-standing concern regarding whether HIFU-induced mechanical damage may lead to the dissemination of cancer cells into the blood circulation, and thus promoting distant metastasis. Several groups have investigated the potential risk of metastasis induced by HIFU using animal models, but the results are contradictory [2;3;4;5]. Further, a recent clinical study has demonstrated no apparent increase in the number of patients with detectable circulating tumor cells following HIFU treatment, compared to the non-HIFU group [6].

In early clinical applications, HIFU therapy was aimed to produce thermal coagulative necrosis of the tumor while avoiding the induction of cavitation bubbles [7]. This is because of the

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difficulty in controlling cavitation *in vivo* and the desire to minimize mechanical damage outside the target region. However, using modern HIFU systems, cavitation is often unavoidable, and in some instances, may even be beneficial. Indeed, cavitation has been proposed and used to enlarge lesion size during HIFU thermal ablation, and thus enhancing the efficacy of the treatment [7]. In addition, *in situ* cavitation and resultant changes in echogenicity can be used to facilitate tumor targeting in ultrasound image-guided HIFU systems [8]. More recently, our studies have demonstrated that HIFU-induced cavitation can cause lysis of tumor cells with concomitant release of a diverse array of danger signals, leading to a distinct host anti-tumor immune response [9]. These results suggest that HIFU-induced cavitation and associated mechanical damage may be harnessed to improve the clinical outcome of cancer therapy [9;10]. Despite this potential benefit, the risk of mechanical HIFU on distant metastasis has not been investigated.

The goal of this study is therefore to evaluate the risk of distant metastasis induced by both thermal and mechanical HIFU exposures and to monitor the time-course of metastatic development using a bioluminescent murine tumor model. Different HIFU exposure conditions and the resultant anti-tumor immune responses were evaluated. Our results suggest that neither mechanical nor thermal HIFU will increase the risk of distant metastasis. In contrast, HIFU exposures, particularly those that emphasize mechanical damage, can induce a tumor specific immune response that, when properly harnessed, may be beneficial in combating residual cancer cells and reducing the risk of distant metastasis.

Materials and Methods

Animals

C57BL/6 female mice, 5–8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and handled in accordance with the established animal care policy. All animal studies were approved by the Duke University Institutional Animal Care & Use Committee.

Tumor model

Murine melanoma cell line (B16-F10-Luc-G5) with stably transduced firefly luciferase gene was purchased from Xenogen Corporation (Alameda, CA). B16-F10 (the parental counterpart of B16-F10-Luc-G5) and EL4 mouse lymphoma cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA). All the cell lines were maintained in complete Dulbecco's modified eagle medium (DMEM, Hyclone, Logan, UT), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C with 5% CO₂. Since luciferase may present a neo-antigen in the mouse, the parental cell line B16-F10 was used as a control to assess whether the introduction of luciferase gene could affect the host immunity and the metastasis risk following HIFU treatment. The tumor model was prepared by subcutaneous injection of 5×10^5 B16 tumor cells suspended in 50 μ l of PBS into the right hind limb of the C57BL/6 mice. The tumor was allowed to grow for 5–7 days to reach a maximum diameter of 4–6 mm before HIFU treatment. After HIFU treatment, the right hind limb was amputated surgically to ensure total eradication of the primary tumor. To investigate the potential association of metastasis incidence with a HIFU-elicited anti-tumor immune response, amputation procedures were performed either immediately or in 2 days following the HIFU treatment. When the primary tumor was removed immediately after the HIFU treatment, the possibility of DC infiltration and maturation at the damaged tumor site was eliminated.

HIFU Exposure System

The HIFU exposure system (Fig. 1A) consists of a 3.3-MHz focused transducer (H-102, Sonic Concepts, Seattle, WA) mounted at the bottom of a plexiglass test chamber (40×30×15 cm) filled with warm degassed water (37°C), together with a function generator and power amplifier

[9]. The HIFU system was controlled by LabView programs via a GPIB board installed in a PC. During the experiment, the anesthetized tumor-bearing mouse was fixed in a custom-designed animal holder, which was driven by a 3-D positioning system. To facilitate alignment of the tumor with the focus of the HIFU transducer, a portable ultrasound imaging system (Terason 2000, Terason, Inc., Burlington, MA) was employed in conjunction with two orthogonally positioned CCD cameras for visualization of the hind limb tumor (Fig. 1B). The temperature profile near the focus of the HIFU transducer was measured as described previously [9] to determine the temperature elevations inside the tumor tissue under the designated HIFU exposure conditions. Treatment of the tumor was accomplished by progressive scanning of the whole tumor volume point-by-point, translating the tumor-bearing mouse incrementally with the 3-D positioning system. The tumor was scanned from the lower right corner 2 mm inside the tumor boundary, progressing from right to left with a step size of 1.0 mm.

Bioluminescent imaging (BLI)

To assess the occurrence of metastasis, bioluminescent imaging was performed weekly during a 4 week post-operative observation period. Animals were euthanized upon reaching either the observation endpoint at 4 weeks or humane endpoints. BLI was performed with an IVIS system (Xenogen, Alameda, CA), using protocols similar to those previously described [11]. Images were acquired within 15 min following intraperitoneal injection of D-luciferin (Xenogen, Alameda, CA) at 150 mg/kg concentration.

Necropsy

Necropsies were performed in animals reaching humane or observation endpoints. Necropsy provides an appropriate means of metastasis detection by color because the B16 melanoma cell line produces melanin and metastatic tumors. In subsets of the animals, necropsies were also performed at the 1st, 2nd and 4th week after amputation to correlate with metastasis detected by *in vivo* BLI. Heart, lungs, liver, spleen, kidneys, intestine, and lymph nodes were grossly examined, harvested, and then immersed in 4% neutral-buffered formaldehyde before processing for light microscopic examination. Three sections through the center of each selected organ were microscopically evaluated for the presence of metastases.

Animal survival

Kaplan-Meyer analysis was performed to determine animal survival time on the basis that day 0 was set as the day of amputation and the endpoint was the day of euthanasia as determined by humane endpoints (>15% body weight loss, hind limb paralysis or fracture, metastatic tumor size exceeding 1.5 cm in diameter) or at the endpoint of the observation period (28 days).

CTL assay

CTL assays were performed as previously described [12]. Briefly, spleens were harvested from euthanized tumor-bearing mice 10 days after HIFU treatment. Splenocytes were re-stimulated for 5 days *in vitro* with mitomycin C-pretreated B16 cells and used as effector cells. Cytotoxic activity against target B16 (specific) or EL4 (irrelevant) tumor cells was assayed at different effector:target ratios by lactate dehydrogenase (LDH) release assay according to the manufacturer's instruction (Promega, Madison, WI). Three independent experiments were performed with four replicate wells used for each treatment. The percentage of cytotoxicity was calculated as $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})]$ following the manufacturer's protocol.

Statistical analysis

Data are presented as mean value $\pm$ standard deviation. All data was analyzed using the SPSS statistical program (SAS, Cary, NC). Statistical significance was evaluated with the Chi Square, Kaplan Meyer analysis and unpaired t-test, using p values of 0.05.

Results

Concordance of BLI with Necropsy and Metastasis distribution

Since light emission *in vivo* is prone to attenuation with increased tissue depth [13], BLI may not adequately detect micro- or small- metastatic tumors in deep organs. Thus, we first determined if BLI is appropriate for the melanoma model employed in this study. A pilot study was carried out in which both BLI and necropsy were performed to detect tumor metastases 1-, 2-, or 4-weeks after the amputation procedure. BLI easily detected the superficial metastatic lymph nodes in very small tumor sizes (about 1mm $\times$ 1mm in size). Although lung and lumbar lymph node metastases were undetected by BLI during the first week after amputation, results from BLI were consistent with those from necropsy at the 2- and 4-week time points as shown in Table 1. Representative pathological pictures of right inguinal lymph node and lung metastasis are shown in Fig. 2A. In addition to metastases in the lung [14], LN metastases were also commonly observed in this model (Fig. 2B), which is consistent with clinical experience [15]. Furthermore, metastatic tumors in LNs grew rapidly with all involved LNs entirely replaced by melanoma 4 weeks after the amputation. Thus, detailed examination of lymph nodes should be performed to obtain accurate assessment of tumor metastasis.

Determination of metastasis incidence and animal survival rate

When resection of the tumor-bearing leg was performed immediately following HIFU treatment, there was no statistically significant difference in metastasis rates among thermal HIFU, mechanical HIFU, and control groups ($p>0.05$) although the mean value for the mechanical HIFU group is slightly higher (Fig. 3A). However, in mice whose tumors were removed 2 days after HIFU, which provided the possibility for DC infiltration and maturation at the damaged tumor site [9], a 33.3% decrease in metastasis incidence was observed over a 4-week follow-up period ($p<0.05$, Fig. 3B). Furthermore, when amputation was performed 2 days after the treatment, the metastasis incidence in HIFU-treated groups is lower than the groups in which amputation was performed immediately following the same HIFU exposure (Fig. 3C). In contrast, mice in the control groups displayed an increase in metastasis incidence when amputation was performed 2 days instead of 0 days following sham treatment (Fig. 3C). In both comparisons, however, the observed differences were not yet statistically significant at the sample size ($n=15\sim20$) used in this study. Additionally, it was observed that when amputation was performed 2 days following HIFU treatment (Fig. 3C) tumor formation and metastasis incidence with the B16-Luc melanoma model were comparable to those using the parental cell line where the metastasis rates were 38.5%, 14.3% and 7.1%, respectively, in no-HIFU, thermal HIFU and mechanical HIFU groups. This result indicates that the introduction of the luciferase gene does not influence the overall trend in the metastasis results observed in this model.

Kaplan-Meyer analysis demonstrated that in mice treated with HIFU 2 days before amputation, animal survival time was longer in both mechanical and thermal HIFU groups than in the control group, and the difference between mechanical HIFU and control group is of statistical significance ($p<0.05$, Fig. 3D). This result indicates that HIFU may prolong animal survival time by reducing the formation or severity of distant metastasis following surgical removal of the primary tumor.

Alteration of anti-tumor immune response in HIFU-treated mice

When amputation was performed 2 days after HIFU treatment, CTL activity was enhanced in the treatment group, particularly in the mechanical HIFU group when compared to the control group ($p < 0.05$, Fig. 4A). However, the difference of CTL between HIFU treated and control groups were not statistically significant when amputation was performed immediately following HIFU (data not shown). When amputation was performed 2 days following mechanical HIFU, the CTL activity was found to be notably higher than the corresponding data obtained immediately after HIFU treatment (Fig. 4B). EL4 cells were included as nonspecific target cells (10:1 and 5:1) for each cohort and cytotoxicity was $< 5\%$.

Discussion

For years, there is a common concern that exposure of a solid malignancy to HIFU may lead to the dissemination of cancer cells as a result of cavitation damage. This may lead to the spreading of detached cancer cells into the circulation and, subsequently, promote tumor metastasis. On the other hand, recent studies have demonstrated that HIFU treatment, especially mechanical HIFU may boost systemic anti-tumor immunity [9], which may suppress distant metastasis. However, the interplay between these two opposite effects and the impact of mechanical HIFU on the risk of distant metastasis have not been investigated.

The ability for real-time detection of primary and metastatic tumors in live animals is critical for accurate monitoring of neoplastic growth [16]. Recently, *in vivo* imaging based on visible light emission from luciferase-expressing cells or tissues has been developed, and *in vivo* bioluminescent imaging (BLI) has been introduced as a sensitive and efficient method to monitor the dynamics of tumor development and metastasis in live animals [16;17]. *In vivo* BLI has the advantages of being non-invasive and highly sensitive, enabling repeated measurement in the same animal. Our findings indicate that BLI can detect the superficial metastatic lymph nodes even in very small tumor sizes (~ 1 mm in diameter). However, metastases in internal organs or tissues (for example, lung and lumbar lymph nodes) were undetectable among a significant portion of the animals in the first week after amputation because of the limited penetration or strength of the light emitted. Thus, *in vivo* BLI assay is not recommended for early detection of metastasis in deep organs. Yet, on the other hand, *in vivo* BLI assay was found to be reliable and convenient for monitoring late stage metastasis. Based on these observations, we employed weekly BLI analysis after the second week during a 4-week post-operative observation period in order to monitor metastatic tumor growth.

It is noteworthy that while B16-F10 is regarded as an aggressive cell line that predominantly metastasizes to the lung [14], our results also indicate that spontaneous metastasis occurs frequently in the lymph nodes. The incidence of metastasis in the draining lymph node was observed to be as high as that in the lung, and lumbar and mediastinal lymph nodes were the 2nd and 3rd most involved organs, respectively (Fig. 3 C). Thus, when utilizing this cancer model, examination of lymph nodes should be included for accurate assessment of spontaneous metastatic status.

Recent studies have provided increasing evidence to suggest that HIFU can induce a systemic anti-tumor immune response [9;10;18;19;20]. Our previous study demonstrated that HIFU-induced *in situ* tumor cell damage and associated release of endogenous danger signals can lead to the activation of DC and a subsequently elicited anti-tumor immune response [9]. DCs were observed to infiltrate into HIFU-treated tumors and migrate to the draining lymph node within 2 days after the therapy [9]. In this study, amputation of tumor-bearing right legs was performed in one group 2 days after HIFU treatment allowing the altered tumor microenvironment to elicit an anti-tumor immune response and thus impacting on distant metastasis. Compared to the control group, HIFU-treated mice in this group was found to have

lower metastasis incidence with concomitantly prolonged survival time and elevated CTL activity (Fig. 3C, Fig. 4A), and this difference was especially pronounced ($p<0.05$) in mice treated with mechanical HIFU. However, in another group when amputations were performed immediately following HIFU treatment, no statistically significant difference in metastasis rate or CTL activity was observed compared to the control group.

It should be emphasized that in order to elicit a significant anti-tumor immune response, surgical removal of the primary tumor must be performed at a delayed time-point following the HIFU treatment. In the B16-F10 model, resection of the primary tumor via amputation 2 days after HIFU treatment led to reduced metastatic spread of melanoma, increased cytotoxic activity, and prolonged survival of the animal. However, the delayed time-point of surgery also imposes a limitation for an exact parallel comparison. Specifically, the primary tumor in the control mice might grow larger than that in the HIFU-treated mice, which may affect the baseline metastasis status before surgery. On the other hand, even in mice that were all treated with mechanical HIFU, it was found that mice with a two-day delay in amputation (with a larger tumor mass) exhibited a lower mean value in the incidence of metastasis than their counterparts with a zero-day delay in amputation (with a smaller tumor mass), although the difference is not of statistical significance yet with the sample size used in this study (Fig. 3C). Moreover, a higher CTL activity was observed in mice undergoing surgery 2 days after mechanical HIFU therapy (Fig. 4B). Altogether, these results further support the notion that a stronger anti-tumor immunity could be induced by mechanical HIFU.

The strong dependence of HIFU-elicited anti-tumor immunity on the timing between the HIFU therapy and tumor resection may also explain at least partially the contradictory results observed previously [2–5]. In one study when amputation was performed one day following non-thermal, high-amplitude ultrasound exposures used for gene delivery, a higher incidence of metastasis was observed [4]. In contrast, our data with a two-day delay between mechanical HIFU and amputation revealed a significant anti-tumor immune response with lower incidence of distant metastasis. Our finding is consistent with previous reports of HIFU-boosted anti-tumor immunity [9], and the reduced production of tumor-secreted immunosuppressive cytokines in cancer patients following HIFU therapy [21]. Future studies should be carried out to explore strategies to enhance HIFU-elicited anti-tumor immunity and to improve the overall efficacy and quality of HIFU cancer therapy.

In summary, our result from the B16-F10 melanoma model suggests that HIFU treatment with emphasis on producing either thermal or mechanical damage does not increase the potential risk of metastasis. In contrast, HIFU therapy, particularly those that emphasize mechanical damage, can elicit a significant anti-tumor immune response through the simultaneous release of tumor specific antigens and endogenous danger signals. When properly harnessed, these unique features of HIFU therapy may be beneficial in combating residual cancer cells and reducing the risk of distant metastasis.

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Figure 1.

Experimental setup. (A) Schematic diagram, (B) photograph showing a B-mode ultrasound image probe and two orthogonally placed CCD cameras, and (C) alignment of the target tumor (indicated by arrow) under the guidance of B-mode ultrasound image.

Figure 2.

Metastasis detection and tumor distribution in the B16-F10-Luc melanoma model. (A) Right inguinal lymph node and lung metastasis in control and mechanical HIFU groups are indicated by black arrows in representative pathological images (200 $\times$). (B) The frequency of tumor distribution was determined by necropsy after 4-week observation. Note: RI: Right inguinal, LI: Left inguinal, RA: Right axillary, LA: Left axillary, MT: Mediastinal, LB: Lumbar, P: Pyloric, M: mesenteric, PA: para-arterial.

Figure 3.

Dynamic monitoring of metastasis incidence and survival time in B16-F10 tumor-bearing mice treated with HIFU. The metastasis rates during 4-week observation were comparable in the groups undergoing amputation (A) immediately or (B) 2 days after receiving mechanical HIFU, thermal HIFU and no treatment. (C) Observation endpoint data shows no significant difference in metastasis incidence for 0 day amputation, but a significant reduction in metastasis for mice treated with mechanical HIFU 2 days before surgery. (D) Mechanical HIFU treatment prolonged survival time of mice whose tumor-bearing legs were removed 2 day after HIFU when compared with mice that did not receive HIFU ($p < 0.05$). Note: ($n = 13-20$ /group); *, compared with control $p < 0.05$.

Figure 4.

Comparison of CTL in mice receiving amputation immediately or 2 days after HIFU treatment. (A) CTL activity in different groups when amputation was performed 2 days after HIFU treatment, (B) Comparison of CTL activities in mice receiving amputation on zero-day vs. two-day after mechanical HIFU treatment. Note: M. HIFU: Mechanical HIFU, T. HIFU: Thermal HIFU, compared with control (0 day), #, $p < 0.001$; *, $p < 0.05$.

Table 1

Concordance of BLI with Necropsy*

Examination Time (After amputation)	Sample size (n)	Detection of metastases	
		<i>In vivo</i> BLI	Necropsy
1 week	20	4/20 (20%)	5/20 (25%)
2 weeks	10	3/10 (30%)	3/10 (30%)
4 weeks	20	8/20 (40%)	8/20 (40%)

* Detection of metastasis by BLI was found to be concordant with necropsy 2 weeks after amputation.