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BRIEF ARTICLE

Enhancement of antitumor vaccine in ablated hepatocellular carcinoma by high-intensity focused ultrasound

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Abstract

AIM: To investigate whether tumor debris created by high-intensity focused ultrasound (HIFU) could trigger antitumor immunity in a mouse hepatocellular carcinoma model.

METHODS: Twenty C57BL/6J mice bearing H22 hepatocellular carcinoma were used to generate antitumor vaccines. Ten mice underwent HIFU ablation, and the remaining 10 mice received a sham-HIFU procedure with no ultrasound irradiation. Sixty normal mice were randomly divided into HIFU vaccine, tumor vaccine and control groups. These mice were immunized with HIFU-generated vaccine, tumor-generated vaccine, and saline, respectively. In addition, 20 mice bearing H22 tumors were successfully treated with HIFU ablation. The protective immunity of the vaccinated mice was investigated before and after a subsequent H22 tumor challenge. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, the cytotoxicity of splenic lymphocytes co-cultured with H22 cells was determined *in vitro* before the tumor challenge, and tumor volume and survival were measured *in vivo* after the challenge in each group. The mechanism was also explored by loading the vaccines with bone marrow-derived dendritic cells (DCs).

RESULTS: Compared to the control, HIFU therapy, tumor-generated and HIFU-generated vaccines significantly increased cytolytic activity against H22 cells in the splenocytes of the vaccinated mice ($P < 0.001$). The tumor volume was significantly smaller in the HIFU vaccine group than in the tumor vaccine group ($P < 0.05$) and control group ($P < 0.01$). However, there was no tumor growth after H22 rechallenge in the HIFU therapy group. Forty-eight-day survival rate was 100% in mice in the HIFU therapy group, 30% in both the HIFU vaccine and tumor vaccine groups, and 20% in the control group, indicating that the HIFU-treated mice displayed significantly longer survival than the vaccinated mice in the remaining three groups ($P < 0.001$). After bone marrow-derived DCs were incubated with HIFU-generated and tumor-generated vaccines, the number of mature DCs expressing MHC-Ⅱ$^+$, CD80$^+$ and CD86$^+$ molecules was significantly increased, and interleukin-12 and interferon-γ levels were significantly higher in the supernatants when compared with immature DCs incubated with mouse serum ($P < 0.001$). However, no differences of the number of mature DCs and cytokine levels were observed between the HIFU-generated and tumor-generated vaccines ($P > 0.05$).

CONCLUSION: Tumor debris remaining after HIFU can improve tumor immunogenicity. This debris releases tumor antigens as an effective vaccine to develop host antitumor immune response after HIFU ablation.

Key words: Hepatocellular carcinoma; High-intensity focused ultrasound; Immune response; Immunogenicity; Immunotherapy; Thermal ablation; Tumor vaccine

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INTRODUCTION

As a non-invasive thermal ablation, high-intensity focused ultrasound (HIFU) therapy has received increasing attention for the clinical management of patients with solid tumors, including those of the prostate, liver, pancreas, breast, kidney, uterus, bone and soft tissue[1-3]. Under real-time imaging guidance, this technique uses ultrasound energy locally to ablate a targeted tumor at depth, with no damage to overlying tissues. In addition, it has been shown that HIFU ablation can enhance host antitumor immune response[4-13], which may be of benefit in local recurrence and metastasis control in cancer patients who have previous dysfunction of antitumor immunity.

Selective recognition and destruction of tumor cells by the host immune system is a major role of antitumor immunity, and tumor antigens expressed by tumor cells are essential to achieve this antitumor immune response specific to tumor cells. After HIFU ablation, large amounts of tumor debris remain *in situ,* and the host gradually reabsorbs them as a normal process of the healing response. Our previous findings revealed a variety of tumor antigen expressions on HIFU-ablated breast cancer cells[15]. Some tumor antigens disappeared completely, others remained in their entirety such as heat shock protein (HSP) 70, while most remained partially in the tumor debris after HIFU ablation. However, it is still unknown whether the remaining tumor debris may be a potential antigen source available for the induction of host antitumor immunity. To test HIFU effects on the inherent immunogenicity of the tumor debris, we performed HIFU to ablate *in vivo* hepatocellular carcinoma (HCC), and then used the tumor debris to inoculate naïve animals against subsequent tumor challenge. The purpose of this study was to investigate whether the remaining tumor debris created by *in situ* HIFU ablation could be strongly immunogenic as an effective tumor vaccine to stimulate host antitumor immunity, and to provide potential benefit in long-term survival in a murine tumor model.

MATERIALS AND METHODS

Animals

The animal study was approved by the Chongqing Experimental Animal Committee (Chongqing, China). Male and female C57BL/6J mice (6-8 wk old) were obtained from the Experimental Animal Center of Chongqing Medical University (Chongqing, China), and housed in microisolator cages in a laminar flow unit under ambient light in the same animal center. All animal experiments adhered to the Animal Welfare Committee guidelines.

Tumor and vaccine generation

The H22 HCC cell line was provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). 2×10^6 H22 cells (0.02 mL) were injected subcutaneously into the right flank of syngeneic C57BL/6J mice to establish a tumor model. Palpable tumors started to develop after 3 d, and reached the size of 5-7 mm after 7 d.

Twenty C57BL/6J mice bearing H22 tumors were used to generate antitumor vaccines at day 7 after tumor implantation. Under general anesthesia 10 mice underwent HIFU ablation, and the remaining 10 mice received a sham-HIFU procedure with no ultrasound irradiation. HIFU energy was produced by a 2-cm diameter transducer with a focal length of 8 mm, operating at a frequency of 9.5 MHz. Acoustic power was 5 W, and median exposure time was 220 s (range: 180-240 s). All mice were sacrificed 1 d after treatment, and tumor samples were harvested. The tissues were brought up to the same weight in both treated and untreated tumors, and they were respectively minced and homogenized. Repetitive freeze-thaw cycles were performed for the preparation of cellular lysate. Using the Bradford assay (Bio-Rad, Hercules CA, USA), the same protein concentration (0.5 mg/mL) was also achieved in both lysates, where the treated and untreated tumor tissues had the milligram per milliliter protein concentration in RPMI 1640.

Immunization

Sixty C57BL/6J mice were randomly divided into three groups: control group, tumor vaccine group and HIFU vaccine group. Each group had 20 mice, including 10 for cytotoxic T lymphocyte (CTL) assay 15 d after vaccination and 10 for long-term follow-up after tumor challenge. By using subcutaneous injection, the mice in the tumor group and HIFU group received either 10 μg untreated H22 vaccine or 10 μg HIFU-treated H22 vaccine in the left flank of each mouse. Those in the control group received only injection with same amount of saline solution. The vaccination times were 2 sessions, once a week for 2 consecutive weeks.

Cytotoxicity assay of CTL

Ten mice were sacrificed 7 d after the final vaccination in each group, and spleens were harvested to assess the activity of splenic CTL. Single cell suspensions were generated by passage through a metallic mesh. Erythrocytes were lysed with 0.87% ammonium chloride for 1 min, and macrophages were removed by exposure to plastic plates for 2 h. The nonadherent lymphocytic population was collected, washed, and resuspended at 2×10^6 cells/mL as CTL

effectors. H22 and B16 (a mouse melanoma cell line) cells were used as targeted cells, and the effector/target cell ratio was 10:1, which was the best cellular ratio in our pre-experimental study. The splenic lymphocytes were then co-cultured with either H22 or B16 cells in 96-well plates for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to determine the cytotoxicity of the CTLs in each group. This is a standard colorimetric assay for determining *in vitro* cytotoxicity of CTLs against tumor cells in experiments[16,17]. As negative control groups, an equal number of target cells were cultured alone and an equal number of effector cells were cultured without target cells in a total of 200 μL. Using a multiwell spectrophotometer reader (Molecular Devices, Menlo Park, California, USA), the optical intensity at 570 nm was measured. Each assay was performed in triplicate, and RPMI 1640 medium was used as a blank control. According to the optical intensity in each group, the cytotoxicity calculation was determined by the following equation: Cytotoxicity (%) = (Effector spontaneous + Target spontaneous - Experimental)/(Target spontaneous - Target maximal) × 100%.

Tumor challenge

Seven days after the final vaccination, 10 mice were challenged in each group by subcutaneous injection of 2×10^6 H22 cells (0.02 mL) in the right flank of each mouse. The tumor growth was detected every 3 d by measuring its diameter with a Vernier caliper. Tumor volume was calculated using the following formula: tumor volume (mm^3) = $d^2 \times D/2$, where d and D are the shortest and longest diameters of the measured tumor, respectively. All mice were followed up for 48 d to observe the survival data.

Immune response after HIFU

Twenty C57BL/6J mice bearing H22 tumors were treated with HIFU at day 7 after tumor implantation to determine whether specific antitumor reactivity could be detected directly after HIFU ablation. These mice were classified as the HIFU therapy group in this study, and HIFU therapeutic parameters were similar to those used in generating the HIFU-related tumor vaccine. Fifteen days after HIFU ablation, 10 mice were sacrificed for the assessment of *in vitro* CTL cytotoxicity as described above, and the remaining 10 were rechallenged with 2×10^6 H22 tumor cells to follow long-term survival.

Isolation and culture of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (DCs) were isolated from C57BL/6J mice, as described by Inaba and colleagues[18]. Briefly, DCs were obtained from bone marrow precursors by flushing mouse femur and tibia bones with cold PBS. After erythrocytes were lysed with ammonium chloride, erythrocyte-depleted bone marrow cells (4×10^6/2 mL per well) were cultured in 6-well plates (Nunc, Roskilde, Denmark) in complete medium (RPMI 1640 supplemented with 10% FBS, L-glutamine, 5 mmol/L 2-mercaptoethanol, and antibiotics) at 37℃ in a humidified atmosphere with 50 mL/L CO_2. The culture medium also contained 20 ng/mL mouse recombinant granulocyte macrophage colony-stimulating factor (mrGM-CSF; PeproTech, London, UK) and 20 ng/mL mouse recombinant interleukin-4 (mrIL-4; PeproTech, London, UK). The cultures were fed every 2 d with fresh medium containing 10 ng/mL mrGM-CSF and 10 ng/mL mrIL-4. On day 7, nonadherent and loosely adherent cells were collected, washed, and resuspended in PBS at 1×10^6/mL. These cells presented the typical morphological characteristics of immature DCs, and flow cytometry analysis showed that a majority (75%-80%) of them had positive expression of CD11c and CD205 molecules.

DCs loaded with vaccines

2×10^6/mL immature DCs were primed with either 5 μg H22 tumor vaccine (tumor vaccine group) or 5 μg HIFU-generated vaccine (HIFU vaccine group) in complete medium at 37℃. DCs co-cultured with the same amount of mouse serum alone were classified as the control group. These cells were incubated for 5 d in a humidified atmosphere with 50 mL/L CO_2. Using flow cytometry (FACSCalibur™ Flow Cytometer, BD Biosciences, San Jose, CA, USA), the cells in each group were then analyzed for the expression of MHC class Ⅱ, CD80, and CD86 molecules. Culture supernatants were harvested in each group, and cytokine production was determined in the supernatants by enzyme-linked immunosorbent assay using murine kits from R&D Systems (Minneapolis, MN, USA) for interleukin (IL)-12 and interferon (IFN)-γ, according to the manufacturer's recommendations. Each assay was performed in triplicate with separate DC preparations.

Statistical analysis

All observed data are displayed as mean ± SD. Statistical analysis was performed using the Student's *t* test. A cumulative survival rate was calculated by using the Kaplan-Meier method, and the statistical significance of any survival difference was evaluated by the log-rank test. Differences were considered statistically significant when the *P* value was less than 0.05.

RESULTS

HIFU-generated vaccine induces CTL cytotoxicity against H22 Cells

As shown in Figure 1 and Table 1, HIFU therapy, tumor- and HIFU-generated vaccines significantly increased cytolytic activity against H22 cells in the splenocytes of the vaccinated mice when compared with the activity in mice vaccinated with saline alone ($P < 0.001$). None of the vaccines elicited CTL cytotoxicity to control target B16 cells ($P > 0.05$), suggesting that HIFU therapy and the HIFU-generated vaccine could induce specific antitumor immunity. The splenocytes isolated from the HIFU-treat-

Table 1 Cytotoxicity rate of splenic lymphocytes against H22 and H16 tumor cells in the vaccinated and high-intensity focused ultrasound-treated mice (mean ± SD)

	Cytotoxicity rate (%)	
	H22 tumor cells	B16 tumor cells
Control group	15.9 ± 3.6	13.5 ± 2.3
Tumor vaccine group	30.7 ± 2.7[d]	14.1 ± 1.9
HIFU vaccine group	37.5 ± 4.5[a,d]	13.8 ± 2.2
HIFU therapy group	62.7 ± 6.5[d,f]	18.1 ± 3.9

[a]$P < 0.05$ *vs* the tumor vaccine group; [d]$P < 0.001$ *vs* the control group; [f]$P < 0.001$ *vs* the HIFU vaccine and tumor vaccine groups. HIFU: High-intensity focused ultrasound.

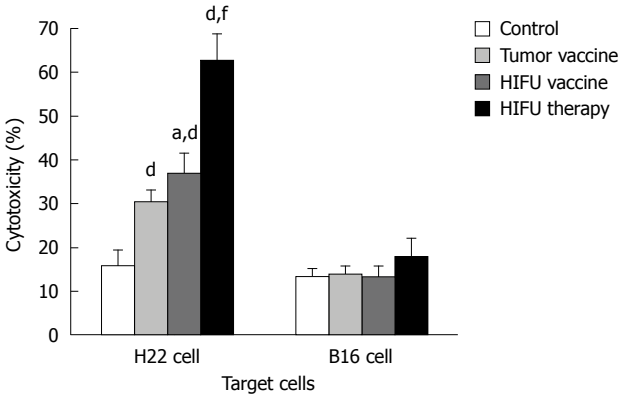

Figure 1 Cytotoxic activity of cytotoxic T lymphocytes against either H22 or B16 tumor cells *in vitro* at 10:1 effector:target ratio in the vaccinated and high-intensity focused ultrasound-treated mice. Naïve mice were vaccinated with high-intensity focused ultrasound (HIFU)-generated and tumor-generated vaccines, and saline alone once a week for 2 wk. The mice bearing H22 tumors were also treated with HIFU ablation. The vaccinated animals were sacrificed 7 d after the 2nd vaccination, and the HIFU-treated mice were sacrificed 15 d after HIFU therapy. The spleens were harvested, and single cell suspensions were generated. The splenic lymphocytes were then co-cultured with either H22 or B16 cells for 24 h. The cytotoxicity of the cytotoxic T lymphocytes was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in each group after 2 h co-incubation. [a]$P < 0.05$ *vs* the tumor-generated vaccine; [d]$P < 0.001$ *vs* the control; [f]$P < 0.001$ *vs* the HIFU- and tumor-generated vaccines.

ed mice produced significantly stronger anti-H22 CTL activity than that of either the HIFU vaccine or tumor vaccine group ($P < 0.001$). The HIFU-generated vaccine was significantly better than the tumor-generated vaccine at increasing the cytotoxicity of CTLs ($P = 0.013$).

HIFU-generated vaccine mediates inhibition of tumor growth

The tumors were monitored with the caliper for 4 wk until mean tumor size was too variable due to death of the mice. As shown in Figure 2 and Table 2, vaccination with HIFU-treated tumor had a marked inhibitory effect on tumor growth in the 3rd and 4th wk of the tumor challenge when compared with the control (saline) ($P < 0.01$) and tumor-generated groups ($P < 0.05$). The strongest inhibition was observed in the HIFU therapy group because after H22 rechallenge no tumor growth was detected during the follow-up period. An inhibition of tumor growth was

Table 2 Tumor volume of the vaccinated and high-intensity focused ultrasound-treated mice after a subsequent H22 tumor challenge (mean ± SD)

	Average tumor volume (mm^3) after H22 tumor challenge		
	2 wk	3 wk	4 wk
Control group	343.3 ± 129.5	829.3 ± 316.4	1953.0 ± 848.2
Tumor vaccine group	504.1 ± 173.7	733.4 ± 301.3	1760.2 ± 1075.1
HIFU vaccine group	279.7 ± 117.2	382.8 ± 170.6[a,d]	914.3 ± 474.2[a,d]
HIFU therapy group	0	0	0

[a]$P < 0.05$ *vs* the tumor vaccine group; [d]$P < 0.01$ *vs* the control group. HIFU: High-intensity focused ultrasound.

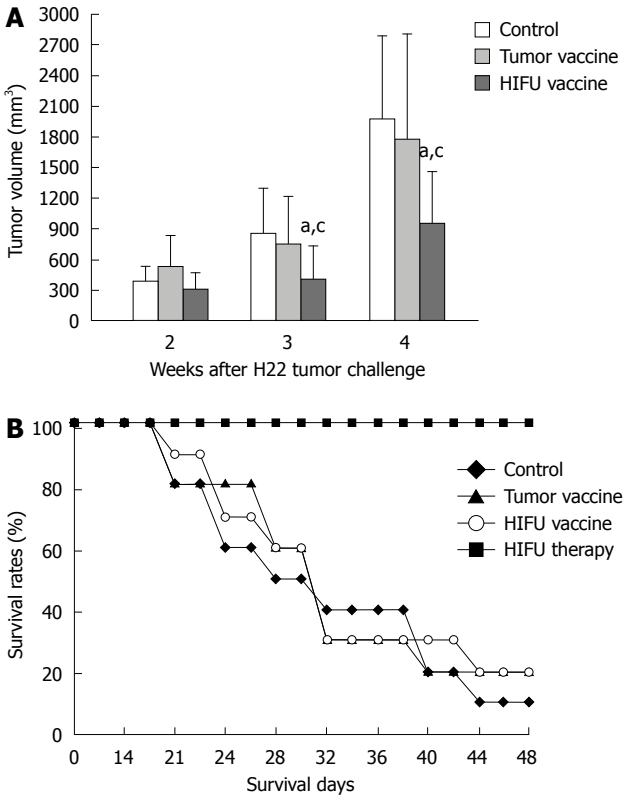

Figure 2 High-intensity focused ultrasound-generated vaccine inhibits tumor growth after a subsequent tumor challenge in a mouse H22 tumor model. Naïve mice were vaccinated with high-intensity focused ultrasound (HIFU)-generated and tumor-generated vaccines, and saline alone once a week for 2 wk. The mice bearing H22 tumors were also treated with HIFU ablation. 7 d after the 2nd vaccination, the vaccinated animals were challenged with 2×10^6 viable H22 cells, and the HIFU-treated mice received a second tumor challenge with the same number of H22 cells 15 d after HIFU therapy. Tumor diameters were measured for 3 wk, and the results were reported as the tumor volume. All mice were followed up for 48 d, and a cumulative survival rate was calculated in each group. A: The tumor volume, measured with a Vernier caliper, in the vaccinated and HIFU-treated mice after a subsequent tumor challenge. [a]$P < 0.05$ *vs* the control; [c]$P < 0.05$ *vs* the tumor-generated vaccine; B: Cumulative survival curves, calculated with the Kaplan-Meier method, in the vaccinated and HIFU-treated mice. Compared to the other groups, HIFU therapy shows a significant increase in survival ($P < 0.001$, the log-rank test).

observed in the tumor-generated vaccine group. However, there was no statistical difference between the control and tumor-generated vaccine groups ($P > 0.05$).

Table 3 CD86, CD80 and MHC-Ⅱ expression by bone marrow-derived dendritic cells after incubation with high-intensity focused ultrasound- and tumor-generated vaccines (mean ± SD, %)

	CD86	CD80	MHC-Ⅱ
Control group	2.05 ± 1.40	4.81 ± 1.56	3.04 ± 0.60
Tumor vaccine group	11.70 ± 0.85[b]	13.50 ± 0.14[b]	13.75 ± 0.59[b]
HIFU vaccine group	14.65 ± 0.49[b]	16.05 ± 0.50[b]	15.90 ± 0.28[b]

[b]$P < 0.001$ *vs* the control. HIFU: High-intensity focused ultrasound.

Table 4 Interleukin-12 and interferon-γ secretion by bone marrow-derived dendritic cells after incubation with high-intensity focused ultrasound- and tumor-generated vaccines (mean ± SD)

	IL-12 (pg/mL)	IFN-γ (pg/mL)
Control group	80.2 ± 4.6	58.0 ± 0.9
Tumor vaccine group	206.8 ± 5.3[b]	207.0 ± 3.4[b]
HIFU vaccine group	264.7 ± 2.0[b]	247.8 ± 9.0[b]

[b]$P < 0.001$ *vs* the control group. HIFU: High-intensity focused ultrasound; IL-12: Interleukin-12; IFN-γ: Interferon-γ.

HIFU therapy results in better survival than tumor or HIFU vaccines

Survival of the vaccinated mice and HIFU-treated mice was also recorded for up to 48 d after tumor challenge with H22 cells. Survival curves (Figure 2) showed that 100% of HIFU-treated mice, 30% of HIFU vaccinated mice, and 30% of tumor vaccinated mice survived for 48 d, whereas 20% of saline vaccinated mice (control) survived 48 d. The HIFU-treated mice displayed significantly longer survival than the vaccinated mice in the remaining three groups ($P < 0.001$). The mice inoculated with either the HIFU-generated vaccine or tumor-generated vaccine survived a little longer than the mice vaccinated with saline (control). However, no statistical differences were observed among them.

HIFU-generated vaccine activates immature DCs

The expression of MHC class Ⅱ, CD80 and CD86 molecules on DCs was determined by flow cytometry after incubation for 5 d. As shown in Figure 3 and Table 3, incubation with either the HIFU-generated vaccine or tumor-generated vaccine significantly increased the number of mature DCs (MHC-Ⅱ$^+$, CD80$^+$ and CD86$^+$) when compared with incubation with mouse serum ($P < 0.001$), suggesting both vaccines could induce phenotypic maturation of DCs. However, no differences in the expression of MHC-Ⅱ, CD80 and CD86 were observed between the HIFU- and tumor-generated vaccines ($P > 0.05$).

HIFU-generated vaccine induces IL-12 and IFN-γ secretion by DCs

As shown in Figure 3 and Table 4, after immature DCs were incubated with HIFU- and tumor-generated vaccines, IL-12 and IFN-γ levels were significantly higher in the supernatants compared to DCs incubated with mouse serum ($P < 0.001$). However, there was no statistical difference in IL-12 and IFN-γ secretion between the HIFU and tumor vaccine groups ($P > 0.05$). These results demonstrated that both HIFU- and tumor-generated vaccines could induce the functional maturation of DCs.

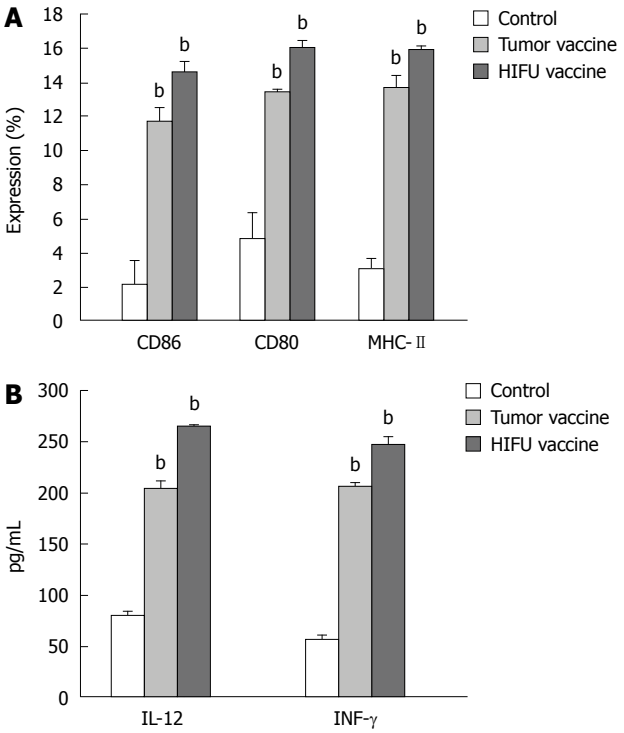

Figure 3 High-intensity focused ultrasound-generated and tumor-generated vaccines activate dendritic cells. Immature dendritic cells (DCs) were isolated from C57BL/6J bone marrow cultures, and then incubated for 5 d with the high-intensity focused ultrasound (HIFU)-generated vaccine, tumor-generated vaccine, and mouse serum alone. After incubation the cells were subjected to flow cytometry. Culture supernatants were harvested, and the enzyme-linked immunosorbent assay method was used to determine the production of interleukin (IL)-12 and interferon (IFN)-γ in the supernatants. [b]$P < 0.001$ *vs* the control. A: HIFU-generated vaccine induces the maturation of bone marrow-derived DCs. Results are reported as percentage of MHC-Ⅱ$^+$, CD80$^+$ and CD86$^+$ cells in the total population; B: HIFU-generated vaccine induces IL-12 and IFN-γ secretion by mature DCs.

DISCUSSION

The concept of HIFU as a noninvasive therapy for the local destruction of diseased tissue dates back more than 60 years. Much of the clinical application is recent, where clinical results are very promising in the treatment of solid malignancies[1-3]. It is obvious that large amounts of *in situ* tumor debris remain after HIFU ablation, however, little is known about whether this debris may be a potential antigen source for triggering host antitumor immune response. Using a newly developed tumor destruction model, we demonstrated that the remaining tumor debris can be immunogenic as an effective vaccine to elicit tumor-specific immune responses, including induction of CTL cytotoxic activity in the spleen, protection against a lethal tumor challenge in naïve mice, activation of immature DCs, and secretion of Th1-associated cytokines

by mature DCs for the development of cell-mediated immune response. To our knowledge, this is the first report of the use of HIFU ablation to generate an *in situ* tumor vaccine, and the first report of the use of this crude tumor vaccine which is functional in stimulating tumor-specific immunity in naïve animals in the absence of immune adjuvant. Our findings may contribute greatly to the understanding of how *in situ* HIFU ablation triggers the host antitumor immune response. However, with the use of flow cytometry, peptide MHC tetramers analysis is needed in the future to measure the number of antigen-specific CD8+ T lymphocytes.

Thermal and nonthermal effects are two major mechanisms related to HIFU-induced coagulation necrosis. During HIFU exposure, the absorption of ultrasound energy in a targeted tumor leads to a rapid temperature rise above 56℃ within the focal volume[19], and thus induces complete coagulation necrosis of the targeted cancer, with no direct evidence of apoptotic cells detected by the TUNEL method in the treated tumors[20].

It is postulated that antitumor immunity enhanced by the ultrasound thermal effect would be similar to those observed in other thermal therapies such as radiofrequency[21-23]. Cavitation is the most important nonthermal mechanism for HIFU-induced tissue destruction. It can cause membranous organelles, including mitochondria, endoplasmic reticulum, cell and nuclear membranes to collapse instantaneously, and thus lead to tumor cells breaking up into small pieces, on which the tumor antigens may remain intact[24]. Recent studies have revealed that acoustic cavitation can upregulate expression of tumor antigens such as heat shock proteins[4,5,8,14,15]. If heat shock proteins (HSPs) remain and upregulate as intracellular molecular chaperones in the tumor debris after HIFU ablation, they may bind tumor peptide antigens, and act as tumor vaccines to produce a potent cellular immune response[25,26]. However, as overexpression of HSPs may have deleterious effects on antitumor immunity after heat treatments such as hyperthermia, further studies are needed to evaluate whether HSPs could play an important role in the induction of host antitumor immune response after HIFU therapy.

Although the mechanism behind this enhanced immune response is still unknown, our findings reveal that it should be specific antitumor immunity. We have demonstrated that the HIFU-generated tumor vaccine can significantly elicit cytotoxicity of CTLs to H22 cells, whereas cytolytic activity against control target B16 cells was not observed in the splenocytes of vaccinated mice. Compared to tumor lysate, *in vitro* anti-H22 CTL activity was stronger in mice receiving the HIFU-generated tumor vaccine. Similar results were also observed in a mouse H22 tumor model after the vaccinated mice were challenged with H22 cells. Vaccination with HIFU-treated tumor caused a stronger inhibition of tumor growth than the control and tumor-generated vaccine groups, indicating the involvement of a tumor-specific immune response. However, this immune protection was still weak, because no significant survival benefit was observed after a lethal H22 challenge in the HIFU-generated vaccine group when compared with the control and tumor-generated vaccine groups.

We have found that the mice bearing H22 tumors, which were treated by HIFU previously, had the strongest protection against a second H22 cell challenge. The most potent cytotoxicity of CTLs against H22 cells *in vitro* was detected in mice who received previous HIFU ablation. Compared to the HIFU- and tumor-generated vaccine groups, *in situ* HIFU ablation of H22 tumors resulted in complete protection against a subsequent H22 tumor rechallenge. All mice survived during the follow-up period, with no evidence of tumor growth. These data suggest that once mice bearing H22 tumors are cured by HIFU treatment, a bona fide systemic memory response may be generated. In addition, tumor debris remaining after *in situ* ablation may continuously stimulate the host immune system during the reabsorption of dead tissue, leading to a stronger antitumor immune response. However, to support our speculation, further studies are needed to investigate the mechanisms behind this. Furthermore, as follow-up time was limited in this study, a longer period is necessary in future studies to observe the survival benefit and tumor development after tumor rechallenge in the HIFU-treated mice. Both the HIFU- and tumor-generated vaccine groups developed a relatively weak immune response, because the vaccination times were very limited, only performed once a week for 2 consecutive weeks. Therefore, further studies are necessary in this mouse H22 tumor model to optimize the vaccination method including the number of sessions, dosage and interval time. In order to induce a stronger immunological response, a longer vaccination time with the HIFU-treated tumor will be investigated, and immunoadjuvants will be used in combination with HIFU therapy.

Similar to other thermal therapies, a marked inflammatory reaction, with abundant leukocytic infiltration, has been histologically observed at the margins of coagulation necrosis in HIFU ablation[27-30]. We have recently found that HIFU ablation can significantly induce local infiltration of activated DCs within the marked inflammatory reaction in patients with breast cancer[31]. DCs are the most potent antigen-presenting cells for induction of adaptive immunity against cancer[32]. They infiltrate local tumors and present tumor antigens to naïve T lymphocytes in a MHC restricted fashion. Activating signals, delivered directly or indirectly by tumor cells including apoptotic and necrotic tumor cells, can induce the progression of infiltrating DCs from an immature to a mature stage[33]. During maturation DCs increase the expression of costimulatory molecules such as CD80 and CD86, and mature DCs secrete Th1-associated cytokines to induce cell-mediated immunity[34]. This study produced direct evidence that with no immune adjuvant, the remaining tumor debris can activate immature DCs, and thus induce secretion of IL-12 and IFN-γ by mature DCs for the development of cellular antitumor immune response. However, it is still

unknown whether the activated DCs could induce an *in situ* antitumor immune response by presenting tumor antigens directly to lymphocytes. Further studies are necessary to investigate the potential role of activated DCs in the induction of specific antitumor immunity *in vivo*.

Our findings indicate that a weak but tumor-specific immune response was produced by the HIFU-generated tumor vaccines after *in situ* tumor destruction. Therefore, active immunological stimulation such as immunoadjuvants, in combination with HIFU, could augment the efficacy of HIFU-induced antitumor immunity specifically against the targeted tumors, if the destruction of tumors releases tumor antigens or improves tumor immunogenicity.

HCC is one of the most common malignancies worldwide. Local tumor recurrence and metastasis are usually the cause of failure of multidisciplinary treatments of HCC in clinical practice. Using a mouse HCC model, we found that HIFU ablation can trigger host tumor-specific immune response. This may decrease or perhaps even eliminate residual and metastatic tumor cells in HCC patients who have had original antitumor immunity dysfunction.

In summary, this study demonstrated that tumor debris remaining after *in situ* HIFU ablation may improve tumor immunogenicity. This debris may release tumor antigens as an effective vaccine to elicit tumor-specific immune responses. However, further studies are needed to explore the nature of the "activation" factors in HIFU-generated tumor debris.

COMMENTS

Background

As a non-invasive thermal ablation, high-intensity focused ultrasound (HIFU) therapy has received increasing attention for the clinical management of patients with hepatocellular carcinoma (HCC). After HIFU ablation, large amounts of tumor debris remain *in situ*, and the host gradually reabsorbs them as a normal process of the healing response. However, it is still unknown whether the remaining tumor debris may be a potential antigen source available for the induction of host antitumor immunity.

Research frontiers

In the present study, the authors investigated whether tumor debris created by *in situ* HIFU could be an effective tumor vaccine to stimulate antitumor immunity in a mouse HCC model.

Innovations and breakthroughs

This study demonstrated that the remaining tumor debris after HIFU can be immunogenic as an effective vaccine to elicit tumor-specific immune responses, including induction of cytotoxic T lymphocyte cytotoxic activity in the spleen, protection against a lethal tumor challenge in naïve mice, activation of immature dendritic cells (DCs), and secretion of Th1-associated cytokines by mature DCs for the development of cell-mediated immune response. These findings may contribute greatly to the understanding of how *in situ* HIFU ablation triggers the host antitumor immune response.

Applications

These findings contribute greatly to the understanding of how *in situ* HIFU ablation triggers the host antitumor immune response. In addition, they suggest that HIFU ablation combined with subsequent immunotherapy such as immunoadjuvants may augment the efficacy of HIFU-induced antitumor immunity specifically against the targeted tumor, leading to a decrease of local recurrence and metastasis in HCC.

Peer review

It is a very interesting paper with adequately described and written all necessary parts of a manuscript.

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