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Host antitumour immune responses to HIFU ablation

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Abstract
The ideal cancer therapy not only induces the death of all localized tumour cells without damage to surrounding normal tissue, but also activates a systemic antitumour immunity. High-intensity focused ultrasound (HIFU) has the potential to be such a treatment, as it can non-invasively ablate a targeted tumour below the skin surface, and may subsequently augment host antitumour immunity. In addition to thermal and cavitation effects, which act directly and locally on the tumour, there is increasing evidence linking systemic anti-tumour immune response to HIFU ablation. This may provide micro-metastatic control and long-term tumour resistance for cancer patients. The goal of this article is to review the emerging pre-clinical and clinical results suggesting that HIFU ablation may enhance host anti-tumour immunity, and to discuss its potential mechanisms. It is concluded that the systemic immune response induced by thermal ablation may play an important role in local recurrence and metastasis control after HIFU treatment.

Keywords: *High intensity focused ultrasound, neoplasm, antitumour immunity, immunotherapy, thermal ablation*

Introduction

Impaired immune function is one of the major factors in the development and progression of human tumours. In most cancer patients, lymphocyte-mediated immunity fails to control the development and growth of the primary tumour, and cannot prevent local recurrence and metastasis after conventional therapies. There are several mechanisms by which tumours resist the immune system, including poor tumour antigen presentation and processing, and release of immunosuppressive cytokines by the tumour. As a result, either there are insufficient initial cancer cells to stimulate the immune response, or the immune response stimulated is unable to prevent the initial establishment of cancer in the patients.

Surgery and ionizing radiation therapy are widely used as local therapies in the treatment of solid cancers. In addition to the positive local effects, a number of studies reported functional suppression of lymphocyte-mediated cellular immunity after surgical procedures [1–4] and local radiation therapy [5–9]. This immunosuppression could exacerbate postoperative dissemination of cancer cells, and the development of tumour metastases in patients with non-functioning antitumour immunity.

In the past two decades, technological advances have meant a change from open surgery to less invasive modalities for the treatment of solid tumours. Minimally invasive ablative techniques, such as radiofrequency (RF), microwave, laser, high-intensity focused ultrasound (HIFU) and cryosurgery, have partially replaced some open surgical procedures in the treatment of cancer, with an associated reduction in cost, morbidity and hospital stay [10–14]. In addition to local destruction of tumours, spontaneous regression of tumour metastases have been reported after ablation of primary lesions, particularly following

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cryosurgical procedures. This suggests that during the process of the necrotic tissue resorption by the host, active immunity to the tumour tissue was developed, and the host immune system was sensitized to the ablated tumour in animals [15–17]. It has also been shown that tumour debris created by RF ablation might be a potential antigen source for the induction of host antitumour immunity. RF ablation may cause marked inflammatory reactions with an influx of immune cells to the periphery of the coagulation zone in normal liver [18]. The reduction in treated-tumour volume, accompanied by circulating T-cells activated specifically toward tumour antigens, were observed after RF ablation of VX-2 liver tumours in rabbits, resulting in longer survival times in the RFA-treated animals than in controls [19]. den Brok and colleagues reported that in mice this antitumour reactivity was transferred to naive animals by splenocytes, and was potentiated by co-administration of cytotoxic T-lymphocyte- associated antigen 4 blocking antibodies [20], which lower the threshold for T-cell activation and lead to increased tumour protection. Similar findings have recently been noted after HIFU tumour ablation. This paper reviews the host immune responses associated with HIFU tumour ablation, and discusses the potential mechanisms and relevance for the treatment of cancer.

Mechanisms of HIFU ablation

The coagulation necrosis induced by HIFU can be considered to be the result of biological effects from the simultaneous combination of heat, mechanical effects, cavitation and vascular destruction in targeted tissues. The absorption of ultrasound energy in living tissue can result in a measurable temperature elevation. In HIFU, the maximum absorption of ultrasound is in the focal volume, where mechanical energy is converted into heat in targeted tissue. The thermal effect is dependent on the temperature achieved in the focal volume of targeted tissue during HIFU exposure. If the temperature rise is above a threshold of 56°C and the exposure time is 1 s, cell death will be induced through coagulation necrosis. In fact, the temperature within the focal volume may rise rapidly above 80°C during HIFU treatments [21, 22]. A steep temperature gradient is induced between the focus and normal extra-focal surrounding tissue, and therefore sharp demarcation between the treated and untreated tissue is seen under histological examination [23].

In an ultrasound field, particle movement can result in mechanical stress and strain, which can cause biological effects in living tissue. At high-intensity levels, mechanical effects are usually caused by the formation of small bubbles or the oscillation of those small bubbles which are already present, a phenomenon referred to as acoustic cavitation [24]. The source of cavitation in living tissue is the presence of small gaseous nuclei existing in subcellular organelles and fluid in the tissue. During HIFU exposure these bubbles can expand and contract in the acoustic pressure field, resulting in the collapse of the bubbles and the destruction of nearby tissue.

Tumour blood vessel damage caused by HIFU may also account for the ablative nature of HIFU treatment [25–29]. It can directly cut off the tumour blood supply through the destruction of the feeder vessels, thus causing nutrition and oxygen deprivation, resulting in indirect coagulation necrosis.

Changes in cellular antigens after HIFU ablation

Selective recognition and destruction of tumour cells by components of the host immune system is a major component of antitumour immunity. Achievement of this effect requires tumour antigens to be expressed by tumour cells. Studies have revealed that large amounts of tumour debris can be released in situ and reabsorbed after HIFU ablation. However, it is unclear whether ablated tumour debris contains tumour antigens.

Kennedy and colleagues [30] reported that the blood vessels were well preserved in the central portion of the HIFU-treated tumour in a patient with liver metastases, but that endothelial cells lacked factor VIII antigen evident by immunohistochemistry staining. However, van Leenders et al. [31] found expression of the prostate-specific antigens, pancytokeratin and Ki67 in the treated region in prostate cancer patients after transrectal HIFU treatment, also lack of cytokeratin 8. Our initial study demonstrated that on some occasions tumour antigens (proliferating cell nuclear antigen, cell adhesion molecule CD44v6, and matrix metalloproteinase-9) completely disappeared [32], but in other cases were partially present after HIFU ablation in patients with breast cancer [33] (including carbohydrate antigen 15–3 (52% of positive expression) and vascular endothelial growth factor (30% of positive expression)). This finding is of interest because the remaining tumour antigens can have the potential to produce an antitumour response. Further studies showed nuclear positivity of breast cancer cells for estrogen receptor and progesterone receptor in 9% of the treated samples. The positive rate of intense cytoplasmic staining for transforming growth facter-β_1, transforming growth facter-β_2, interleukin 6, and interleukin 10 was 57%, 70%, 48% and 61% in the

treated cancer cells respectively. The positive rate of cytoplasmic membrane staining for epithelial membrane antigen and heat shock protein 70 was 100% in the zones of treated cancer cells [34].

Recently, more evidence has been found to support that the debris of tumour ablated by RF therapy [18–20] or laser-based cancer therapy [35–38] releases tumour antigens or improves tumour immunogenicity, and subsequently enhances host immune response. However, further studies for various kinds of carcinoma are needed to evaluate whether the remaining tumour antigens after HIFU ablation are able to stimulate host antitumour immunity, and to provide benefit from local recurrence and metastasis control.

Evidence of immune response after HIFU ablation

In addition to the direct destruction of tumours, several studies have suggested that HIFU may modulate host systemic antitumour immunity. Yang and colleagues [39] treated C1300 neuroblastoma implanted in mouse flanks using HIFU. After HIFU exposure, C1300 neuroblastoma was re-implanted into mice whose tumours had previously been successfully ablated by HIFU. A significantly slower growth of re-implanted tumours was observed in these pre-clinicals compared with the controls. HIFU caused both thermal and mechanical necrosis of the tumour cell line MC-38 *in vitro*, leading to the release of endogenous danger signals from the damaged tumour cells, including ATP and heat shock protein 60. These signals subsequently activated antigen presenting cells (APCs), and led to an increased expression of co-stimulatory molecules (CD80 and CD86), with enhanced secretion of IL-12 by the dendritic cells and elevated secretion of TNF-α by the macrophages. The potency of APC activation from mechanical lysis was much stronger than that from thermal necrosis, suggesting that optimization of treatment strategy may help to enhance immune response after HIFU ablation [40].

Our study revealed *in vivo* evidence of specific immune protection induced after HIFU ablation in H22 tumour bearing mice (unpublished data). Compared with either sham-HIFU or control group, the lymphocyte cytotoxicity was significantly increased in HIFU group ($p = 0.0001$). Immune protection of the peripheral blood lymphocytes purified from HIFU-treated H22 tumour-bearing mice was also observed. Long-term follow-up results, such as survival rate, tumour extinction rate and metastatic rate, were significantly better in the HIFU group than in the sham-HIFU and control groups. Furthermore, we found that HIFU-activated cytotoxic T lymphocytes (CTLs) could have therapeutic potential as adoptive immunotherapy for homogeneous tumours in mice (unpublished data). In addition to CTL cytotoxicity, TNF-α and IFN-γ levels *in vitro* were significantly higher in the HIFU group than in the sham-HIFU and control groups, and tumour extinction and 6-week survival rates were significantly higher in the HIFU group than in the sham-HIFU and control groups. Compared with the other groups, significantly lower metastatic rates were observed in the HIFU-exposed mice. It is concluded that T lymphocytes activated by HIFU ablation may provide specific antitumour immunity, leading to micro-metastatic control and long-term tumour resistance in mice after HIFU treatment.

Emerging clinical results revealed that systemic immune response was observed in cancer patients after HIFU treatment. Rosberger and colleagues [41] have reported five consecutive cases of posterior choroidal melanoma treated with HIFU. Patient immune function was monitored by determination of T-cell helper/suppressor (CD4/CD8) ratios immediately before, and approximately 1 week after HIFU treatment. Three patients had abnormal, and two patients normal CD4/CD8 ratios before treatment. One week after treatment, the ratio in two patients reverted to normal, while another was noted to have a 37% increase in his CD4 T-cells relative to his CD8 cells. Two patients with initially normal CD4/CD8 demonstrated no significant change postoperatively. Wang and Sun [42] used multiple-session HIFU (average: 8.1 sessions; range: 2–12 sessions) to treat 15 patients with late-stage pancreatic cancer. Changes in natural killer (NK) cell activity and T lymphocyte and subset were observed in 10 patients before and after HIFU treatment. The results showed that the average values of NK cell, $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ ratios in peripheral blood increased after HIFU in 10 patients, a significant statistical difference was observed in only NK cell activity before and after HIFU treatment ($p < 0.05$).

We observed changes in circulating NK, T lymphocyte and subsets in patients with solid malignancy treated with HIFU [43]. All patients received one HIFU treatment session, and follow-up contrast-enhanced CT or MRI demonstrated clear evidence of tumour ablation. Six of these patients had osteosarcoma (Enneking Stage, II_B 4, III_B 2); five had hepatocellular carcinoma (TNM Stage, III 3, IV 2), and five had renal cell carcinoma (TNM Stage, III 2, IV 3). Using flow cytometry techniques, T lymphocyte and subset, B lymphocyte, and NK cell in the peripheral blood were measured in these patients on the day before HIFU and 7–10 days after HIFU. The results showed a significant increase in the population of $CD4^+$ lymphocytes ($p < 0.01$)

and the ratio of $CD4^+/CD8^+$ ($p < 0.05$) in the blood circulation of cancer patients after HIFU treatment. The abnormal levels of $CD3^+$ lymphocytes returned to normal in two patients, $CD4^+/CD8^+$ ratio in three, $CD19^+$ lymphocytes in one, and cytotoxic NK in one respectively, in comparison to the values in the control group.

In addition to a systemic immune response, clinical evidence suggests that HIFU may locally enhance antitumour immunity in cancer patients. Madersbacher and colleagues [44] used immunohistochemistry to examine heat shock protein 27 (HSP27) expression in nine benign prostatic hyperplasia (BPH) patients treated with transrectal HIFU 3 h–8 days prior to surgical removal. They found that in 10 untreated BPH specimens, muscle cells stained HSP27-positive in all samples, while epithelial cells were negative in 6 out of 10 specimens. At the border of the coagulation necrosis induced by HIFU, increased HSP27 expression was consistently demonstrated ($n = 9$). HSP27 upregulation was strongest 2–3 h after HIFU but still demonstrable after 5–8 days. In this border zone, basal and secretory epithelial cells as well as muscle cells stained strongly for HSP27. Further study revealed that HIFU treatment may alter the presentation of prostate tissue and tumour antigens, and that this presentation is most likely to be stimulatory [45]. Histological examination showed significantly upregulated expression of HSP72, HSP73, glucose regulated protein (GRP) 75, GRP78 at the border zone of HIFU treatment in BPH patients. Remarkably, even untreated regions of BPH specimens revealed relative over-expression of HSP72, HSP73, GRP75, and GRP78. Heated prostatic cancer cells exhibited increased Th1-cytokine (IL-2, IFN-gamma, TNF-alpha) release but decreased Th2-cytokine (IL-4, -5, -10) release of TIL.

In our clinical studies, a controlled clinical trial was performed to explore the possibility of using HIFU for the treatment of patients with localized breast cancer [46]. Forty-eight women with biopsy-proven breast cancer (T1–2, N0–2, M0) were randomized to either the control group in which modified radical mastectomy was performed, or the HIFU group in which an extracorporeal HIFU ablation of breast cancer was followed by modified radical mastectomy. Pathologic examination was performed to assess the therapeutic effects on the tumours. The results showed that HIFU-treated tumour cells underwent complete coagulation necrosis, and tumour blood vessels were severely damaged.

Using the histological blocks from this study, we used immunohistochemical staining to investigate immune response in both the HIFU-treated region and axillary lymph nodes histologically. The results showed that antigen presenting function and anti-tumour immunity increased considerably within the treated-tumour and axillary lymph nodes after HIFU ablation in patients with breast cancer (unpublished data). In both the primary breast cancer and the axillary lymph nodes, positive expression of DC, macrophages, and B lymphocyte were significant higher in the HIFU group than those in the control group ($p < 0.01$). The positive rates of HLA-DR, CD80 and CD86 expressed on DC, macrophage, and BL were all increased in the HIFU group ($p < 0.01$). Furthermore, locally in the breast tumour, the average numbers of $CD3^+$, $CD3^+HLA\text{-}DR^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $CD57^+$ cells were significantly higher in the HIFU group than those in the control group ($p < 0.01$). In the axillary lymph nodes, $CD3^+$, $CD3^+HLA\text{-}DR^+$, $CD4^+$ positive cells in the paracortical area, and $CD57^+$ cells were significantly higher in the HIFU group than in control group ($p < 0.01$). $CD3^+$ cells and $CD20^+$ positive cells in medullary cords were also significantly higher in the HIFU group than in the control group. These findings were encouraging, and implied that lymphocyte-mediated immune reaction may be induced in cancer patients after HIFU treatment.

Potential mechanisms of immune response to HIFU ablation

As one of the mechanisms responsible for host immune suppression associated with cancer, immunosuppression in the tumour-bearing host is a major obstacle to cancer treatment. Recent evidence indicates that cancer cells can secrete factors that downregulate and paralyse the immune system of the host, contributing to the growth and progression of tumour. We investigated changes in the circulating level of immunosuppressive cytokines in patients with solid malignancy before, and after, HIFU treatment, and the results showed that serum levels of immunosuppressive cytokines were significantly reduced in peripheral blood after HIFU treatment for patients with malignancy (unpublished data). In this study the ELISA method was used to measure serum levels of VEGF, TGF-β1, TGF-β2, IL-6, and IL-10 in 15 patients with malignancies before, and 1 week after, a single HIFU treatment. Serum levels of the immunosuppressive factors were decreased after HIFU treatment, and there were significant differences of VEGF, TGF-β_1, and TGF-β_2 before and after ($p < 0.05$). This change may lessen tumour-induced immunosuppression, renew host antitumour immunity, and improve the prognosis for the cancer patient.

As the body gradually resorbs the necrotic tissues following HIFU treatment, the effects of host antitumour immunity largely depend on the antigenicity of tumour cells ablated by focused ultrasound. Previous results have shown that some antigens disappear completely, others remained in their entirety, while most remained partially in the tumour debris after HIFU ablation. Although the mechanism for the expression of tumour antigens after HIFU ablation is still unknown, it is postulated that acoustic cavitation could be responsible for the antigen presentation in contrast with other thermal ablation techniques. This mechanical force leads to tumour cells breaking up into small pieces, on which the tumour antigens may remain intact. Furthermore, although the critical temperature needed for denaturation varies among proteins, high temperatures appear to result in the denaturation of protein constituents, which can be defined as the unfolding of proteins from the native state to a more random state of lower organization [47]. The unfolding of the three-dimensional protein structure can lead either to loss or to preservation of antigenic determinants.

The most striking change seen after HIFU ablation is the upregulated expression of HSPs including HSP60 [40], HSP27 [44], HSP72 and HSP73 [45], and HSP70 [34] in tumour debris, indicating that HIFU may modify tumour antigenicity to produce a host immune response. As intracellular molecular chaperones, HSPs can bind tumour peptide antigens, and enhance tumour cell immunogenicity [48]. Antigen presenting cells take up HSP-tumour peptide complex, and present the chaperoned peptides directly to tumour-specific T-cells with high efficiency, resulting in potent cellular immune responses against tumour cells [49]. Pre-clinical studies have revealed that autologous HSP-peptide complexes generated from each individual's tumour could generate a therapeutic immune response [50]. As random mutations in cancer cells usually produce unique tumour antigens in each patient, HSP vaccination may be a rational personalized approach that may obviate the requirement to identify the unique antigens. Recently, autologous HSP-based immunotherapy phase I and II clinical trials have been performed in patients with eight different types of cancer, and positive response was observed in some patients after HSP vaccination [51]. Furthermore, Schueller and colleagues [52] found that RF ablation could induce the HSP70 formation of human hepatocellular carcinoma in nude rats, and the maximum level of HSP70 expression was increased from 0% to 60% after RF treatment. However, further studies are necessary to provide evidence about to whether the active HSP70-peptide complexes which are increased by HIFU ablation could subsequently enhance host antitumour immunity.

The tissue destruction that occurs during in situ HIFU ablation is usually accompanied by an inflammatory response with an influx of immune cells to the margin of the targeted tumour. This active inflammation is a normal process of wound healing in which many different cytokines will be released. It may present an environment that contributes to the development of mature CTLs, and actually abrogates the requirement for antigen-specific CD4 releasing specific cytokines upon antigen stimulation. The HIFU-induced cytokine production is not a specific reaction. However, it may appear specific as it acts on tumour-reactive immune cells already present in the patient, resulting in the increased possibility that the tumour cells can serve as targets by enhancing the expression of the MHC molecules, which are often down-regulated in tumours. On the other hand, it is still unknown what kinds of cytokine are locally secreted after the inflammation caused by HIFU, and whether HIFU-induced cytokines could stimulate tumour growth if it is a normally wound healing.

Massive amounts of cellular debris are gradually phagocytized after HIFU ablation. As a result, macrophages and other cells with distinct endosomal-enzyme compositions take part in phagocytosis of cellular debris. They can function as APCs, leading to a major alteration in the processing of cellular proteins of HIFU-ablated tumour from an endogenous pathway to an exogenous pathway. It is possible that this process could not only result in the creation of large numbers of class II peptide complexes, but also the production of new peptides from the tumour antigens. Therefore, the maturation of CTL precursors may be induced, resulting in the destruction of tumour metastases far from the ablation site. However, further studies are needed to analyse the specificities and activities of CTLs isolated from patients before and after HIFU.

Discussion and conclusion

The concept of HIFU as a non-invasive tool replacing surgical resection 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 encouraging. In addition to local ablation of tumours, preliminary evidence in pre-clinical and clinical studies suggests that HIFU could elicit a systemic antitumour immune response. This finding may lead to a procedure that reduces or perhaps eliminates metastases, and prevents local recurrence in cancer patients who have had original dysfunction of antitumour immunity after

HIFU treatment. The immune responses are related to various factors, particularly to HIFU exposure levels, temperature rise and cavitation. However, the proper control and enhancement of immune stimulation for a systemic antitumour immunity, as well as complete destruction of local tumour are important topics deserving further studies.

Although the mechanism for the HIFU-induced immunologic changes and clinical significance of these changes are unknown, several possibilities are put forward in this review based on recent study results. First, host immune suppression induced by tumour cells could be lessened or relieved after HIFU as the tumour is completely ablated, leading to renewed host antitumour immunity. Second, tumour antigens remain partially or entirely in the tumour debris after HIFU ablation owing to acoustic cavitation. The most important observation after HIFU ablation is that HIFU may modify tumour antigenicity, resulting in the upregulated expression of HSPs, which act as tumour vaccines to produce potent cellular immune responses. Third, cytokines are secreted by immune cells at the inflammatory margin of the HIFU-treated region, presenting a milieu for the development of mature CTLs. Finally, large amounts of cellular debris are gradually phagocytized by macrophages and other cells that can function as APCs. However, further studies are needed to determine the mechanisms associated with the systemic immune response after HIFU.

Local tumour recurrence and metastasis are the cause of failure in many multidisciplinary treatments of cancer. Clinical results have shown that local recurrence and metastasis after HIFU treatment persist, indicating that the antitumour immune response induced by HIFU is not sufficient to eliminate residual tumour cells, and to control metastases in some cancer patients. Therefore, active immunological stimulation such as immunoadjuvants, in combination with HIFU, could augment the efficacy of HIFU-induced antitumour immunity specifically against the targeted tumours, if the destruction of tumours releases tumour antigens or improves tumour immunogenicity.

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