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● *Original Contribution*

ACTIVATED ANTI-TUMOR IMMUNITY IN CANCER PATIENTS AFTER HIGH INTENSITY FOCUSED ULTRASOUND ABLATION

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Abstract—T cell-mediated immune responses represent the main cellular antitumor immunity in cancer patients. Recent studies have shown that that both surgical procedure and radiation therapy could cause the functional suppression of lymphocyte-mediated cellular immunity. The purpose of current study is to evaluate whether high intensity focused ultrasound (HIFU) might change a systemic antitumor immunity, particularly T lymphocyte-mediated immunity in cancer patients. A total of 16 patients with solid malignancies were treated with HIFU. Among them, six patients had osteosarcoma (Enneking stage, II_B4, 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 technique, T lymphocyte and subset, B lymphocyte and natural killer cell (NK) in the peripheral blood were measured in these patients on the day before HIFU and 7 to 10 d after HIFU. The statistical significance of any observed difference is evaluated by Student's *t*-test. The results showed a significance increase in the population of $CD4^+$ lymphocytes ($p < 0.01$) and the ratio of $CD4^+$ /$CD8^+$ ($p < 0.05$) in the circulation of cancer patients after HIFU treatment. The abnormal levels of $CD3^+$ lymphocytes returned toward the normal range in two patients, $CD4^+$/$CD8^+$ ratio in 3, $CD19^+$ lymphocytes in one and cytotoxic NK in one, respectively, in comparison to control values. It is concluded that HIFU could enhance a systemic antitumor cellular immunity in addition to local tumor destruction in patients with solid malignancies. (Email: mfengwu@yahoo.com) © **2004 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **High intensity focused ultrasound, Focused ultrasound surgery, Antitumor immunity, T lymphocyte and subsets, Natural killer cell, Immunotherapy.**

INTRODUCTION

Recent studies have indicated that host immune system provides protection of an organism from abnormal growth of cells and lymphocyte-mediated immunity is believed to protect host against the outgrowth of primary tumors and metastases. The development and progression of tumor result from a failure of the immune system either to recognize or to mediate destruction of tumor cells. However, animal studies showed that this failure could be corrected by immunotherapy, which may help to restore and maintain host antitumor responses to tumor cells.

The ideal cancer treatment is not only to cause destruction of local tumors, but also to activate a systemic antitumor immunity, which may provide the micrometastatic control and long-term tumor resistance for cancer patients. A few studies in recent years reported an interesting feature of high intensity focused ultrasound (HIFU) treatment, in addition to the direct destructive effect on tumors. Yang et al. (1992) treated C1300 neuroblastoma implanted in mouse flank using HIFU. After ultrasound exposure, C1300 neuroblastoma was reimplanted into previously HIFU-cured tumor-bearing mice. A significantly slower growth of reimplanted tumor was observed in these animals as compared with controls. Rosberger et al. (1994) reported five consecutive cases of posterior choroidal melanomas treated by HIFU. Patient immune function was monitored by determination of T-cell helper/suppressor (CD_4/CD_8) ratios immediately before and approximately one week following HIFU treatment. Three patients had abnormal CD_4/CD_8 ratios before treatment, while two patients had normal ratios. One week following treatment, the ratio in two reverted

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to normal; another case was noted to have a 37% increase in his CD_4 T cells relative to his CD_8. Two patients with initially normal CD_4/CD_8 demonstrated no significant change postoperatively. These findings were encouraging and implied that lymphocyte-mediated immune reaction may occur in tumor tissue after ultrasound treatment.

In this study, flow cytometry technique was used to observe changes in lymphocytes and natural killer cell (NK) of treated patients with solid malignancies before and after HIFU treatment. It is our purpose to evaluate whether HIFU might activate a systemic antitumor immunity, particularly lymphocyte-mediated immune response in patients with solid malignancies.

MATERIALS AND METHODS

Patients

From September 2001 to March 2002, a total of 16 patients (eight men, eight women; mean age, 40.7 y; age range, 9 to 74 y) with biopsy-proven solid malignancies were enrolled into this study. Among them, six patients had osteosarcoma (Enneking stage, II_B4, 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). The TNM staging system is usually used in clinical practice to indicate the progressive extent of most malignant carcinomas. It is based on the extent of the primary tumor (T), the absence or presence and extent of regional lymph node metastasis (N) and the absence or presence of distant metastasis (M). The use of numerical subsets of the TNM components reveals the clinical stage of the malignant disease. The most common staging system used to stage bone sarcoma is that developed by Enneking et al. (1980). This staging system is based on the histologic grade, anatomic extent and presence of metastatic disease at presentation. Patients with a metastasis to any site, most commonly lung, are placed in the highest stage III. Seven patients had lung metastases before receiving HIFU treatment. The primary tumor size ranged from 5 to 12 cm in diameter, with average size of 8.17 cm.

The protocol design was approved by the ethics committee at our university before the beginning of this study. At the time of enrollment, each patient signed an informed consent form.

HIFU treatment

The same therapeutic HIFU system [Chongqing Haifu (HIFU) Tech Co., Ltd, China] was used to treat all patients, as described in detail previously (Kennedy et al. 2004; ter Haar et al. 2004; Wu et al. 2001, 2002, 2004). Briefly, therapeutic ultrasound energy is produced by a 12-cm diameter transducer with a focal length of 135 mm, operating at a frequency of 0.8 MHz. The focal region is 9.8 mm along the beam axis and 1.3 mm in the transverse direction, assuming it to correspond with pressure ful- width half maximum at −6dB.

HIFU treatment was performed under general anesthesia in nine patients or epidural anesthesia in seven patients, respectively. All patients received one session of HIFU treatment for primary cancer. Acoustic focal peak intensities ranged from 5000 to 20000 W cm^{-2}. Multiple single exposure and linear scanning exposure were usually used in the treatment. Scanning speed ranged from 1 to 3 mm s^{-1}, and the track length from 10 to 30 mm. A total of therapeutic time including anesthesia for the patients ranged from 2.5 to 8 h (median mean, 5.2 h).

Table 1. Monoclonal antibodies for analysis of lymphocytes and subsets

Monoclonal antibody	Specificity
Anti-CD3	$\alpha\beta+$ T cells
Anti-CD4	T helper cells
Anti-CD8	T suppressor/cytotoxic cells
Anti-CD19	B cells
Anti-CD16/Anti-CD57*	Cytotoxic natural killer cells subset

* Two monoclonal antibodies used for subset analysis.

Sample collection

Two ml peripheral venous bloods were obtained from the enrolled patients on the day before and 7 to 10 d after HIFU. They were used to assess T lymphocytes and subset, B-lymphocytes and natural killer cells (NK). As negative controls, 20 peripheral blood samples were collected from healthy subjects. Each blood sample was placed into glass tubes containing sodium heparin and then transported in a chilled container to laboratory. White cell counts and Wright-stained differential counts were conducted to calculate a total number of white cells and the absolute number of cells per liter of blood.

Monoclonal antibodies

Simultest IMK-Lymphocyte kit (Becton Dickinson, San Jose, CA) was used in all patients. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies reactive to lymphocytes and NK are shown in Table 1.

Staining

Cell staining was conducted as described following instructions given by the supplier. Briefly, T lymphocyte and subset were stained with FITC-conjugated antibodies against human CD3, CD4, and CD8 respectively. B lymphocyte was stained with FITC-conjugated antibod-

ies against human CD19. For double staining, cytotoxic NK cells were stained with FITC-conjugated antibodies against human CD16 and then stained with anti-CD57.

Flow cytometry

Flow cytometry (FACScalibur, Becton Dickinson, San Jose, CA) was performed to analyze immunofluorescence of cell surface. For each stained sample, 10000 cells were analyzed for staining positivity after linear light scatter gating on lymphocytes. Using Simulsetv 3.0 software the fluorescent distribution was analyzed and results were expressed as the proportion of positively stained cells.

Statistical analysis

All the data are reported as the mean ± standard deviation. The statistical significance of any observed difference is evaluated by Student's *t*-test. Statistical significance is defined as a p value of less than 0.05.

RESULTS

Alteration of lymphocyte and subsets in each patient

Table 2 shows the changes of lymphocytes and NK in the peripheral blood of 16 patients with solid malignancies, before and after HIFU treatment. The mean percentages of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, cytotoxic NK and CD4/CD8 ratio in the peripheral blood of 20 healthy adults were 67.34 ± 9.03, 41.2 ± 7.25, 28.7 ± 6.18, 11.00 ± 1.64, 13.00 ± 1.84, and 1.15 ± 0.22, respectively. Compared with the normal values, the percentage of $CD3^+$ lymphocytes before HIFU was lower in five patients, $CD4^+/CD8^+$ ratio in 11, $CD19^+$ lymphocytes in 10 and cytotoxic NK in nine in comparison with control values. The abnormal level of $CD3^+$ lymphocytes in five patients was increased postoperatively and two of them returned toward the normal range. $CD4^+/CD8^+$ ratio was increased in 10 of 11 patients and three reverted to normal value after HIFU treatment. The percentage of $CD19^+$ lymphocytes was increased postoperatively in seven of 10 patients, including one up to normal value, but diminished $CD19^+$ level, less than the normal value, were detected postoperatively in four patients with normal level preoperatively. The percentage of cytotoxic NK cells was increased in four patients with abnormal level before HIFU, and one of them returned toward control value after HIFU treatment.

T lymphocytes and subsets

The mean percentages of $CD3^+$, $CD4^+$, $CD8^+$ and CD4/CD8 ratio preoperatively in the peripheral blood of patients with solid malignancies were 74.75 ± 15.35, 36.75 ± 10.19, 34.81 ± 11.02, and 1.15 ± 0.45, respectively;

Table 2. Alteration of lymphocyte subsets in each patient before and after HIFU treatment

	No. of patients	$CD3^+$		$CD4^+$		$CD8^+$		CD4/CD8		$CD19^+$		CD ($16^+ + 56^+$)	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Osteosarcoma	1	75	79	35	40	32	32	1.09	1.25	18	6	13	19
	2	90	90	30	48	60	36	0.5	1.33	1	2	8	7
	3	58	69	23	32	28	33	0.82	0.97	13	1	31	27
	4	83	90	31	52	45	34	0.69	1.53	3	4	12	5
	5	90	94	52	53	34	33	1.53	1.61	4	0	5	4
	6	80	84	28	39	43	44	0.65	0.89	3	2	15	14
HCC	7	93	75	37	35	52	40	0.71	0.88	4	12	4	7
	8	64	74	38	45	27	29	1.41	1.55	7	5	25	19
	9	79	73	34	29	39	40	0.87	0.72	13	12	11	13
	10	84	85	44	45	37	40	1.19	1.12	6	6	9	11
	11	62	69	42	42	23	21	1.83	2.0	22	10	13	19
RCC	12	80	79	48	50	30	29	1.6	1.72	10	6	8	9
	13	36	54	22	33	16	21	1.38	1.57	6	10	62	35
	14	80	84	46	52	29	31	1.59	1.68	11	8	7	7
	15	57	62	24	28	32	35	0.75	0.8	5	8	35	26
	16	85	83	54	52	30	28	1.8	1.86	6	10	7	6
Mean ± SD		74.75 ± 15.35	77.75 ± 10.75	36.75 ± 10.19	42.19 ± 8.74 **	34.81 ± 11.02	32.88 ± 6.49	1.15 ± 0.45	1.34 ± 0.40*	8.25 ± 5.84	6.38 ± 3.86	16.56 ± 15.15	14.25 ± 9.15

* Compared with pretreatment level, $P < 0.05$;
** Compared with pretreatment level, $P < 0.01$.

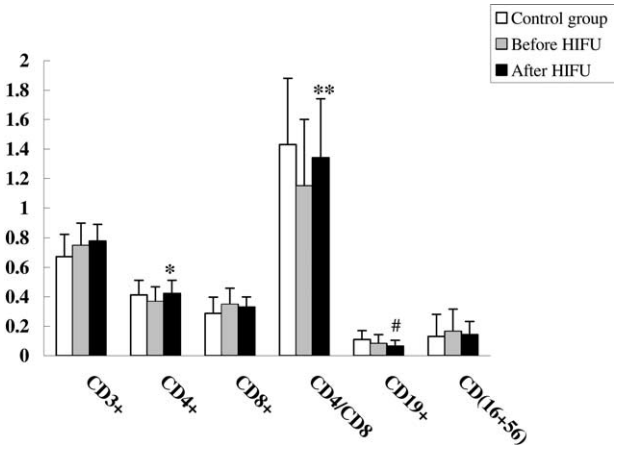

Fig. 1. Effect of HIFU treatment on lymphocyte and subsets in cancer patients. Compared with preoperative group: * $p < 0.05$; ** $p < 0.01$; # compared with control group, $p < 0.05$.

while the postoperative values were 77.75 ± 10.75, 42.19 ± 8.74, 32.88 ± 6.49 and 1.34 ± 0.40, respectively. Compared with the normal values, a significant decrease of peripheral blood in $CD4^{\pm}$ ($p < 0.01$) and CD4/CD8 ratio ($p < 0.05$) was observed in the patients before HIFU treatment. Average $CD4^+$ level and CD4/CD8 ratio increased in the peripheral blood of the patients postoperatively. There were significant differences of $CD4^+$ percentage ($p < 0.01$), and CD4/CD8 ratio ($p < 0.05$) before and 7 to 10 d after HIFU (Table 2 and Fig. 1).

B lymphocytes

The mean percentages of $CD19^+$ were 8.25 ± 5.84 on the day before HIFU and 6.38 ± 3.86 7 to 10 days after HIFU, respectively (see Table 2 and Fig. 1). $CD19^+$ percentage was decreased postoperatively, and there was no significant difference before and after HIFU. But, compared with the normal level in control group, a significant decrease was statistically seen in the patients after HIFU treatment ($p < 0.05$).

Cytotoxic NK cells

The mean percentages of cytotoxic NK cells were 16.56 ± 15.15 preoperatively and 14.25 ± 9.15 postoperatively, which were slightly higher than the value in the control group ($p > 0.05$). There was a slight decrease in cytotoxic NK cells postoperatively, but no statistical difference was observed before and after HIFU treatment (Table 2) and (Fig. 1).

DISCUSSION AND SUMMARY

$CD3^+$ T lymphocytes are the major lymphocyte subset in the peripheral circulation, and T cell-mediated immune responses represent the main cellular antitumor immunity in cancer patients (Whiteside and Heberman 2003). T lymphocytes are divided into $CD4^+$ subset (T helper cells) and $CD8^+$ subset (T suppressor/cytotoxic cells) and, therefore, $CD4^+$ /$CD8^+$ ratio is associated with T lymphocyte-mediated function. $CD4^+$ /$CD8^+$ ratio is usually used in clinical practice as an indicator of patient's antitumor immunity (Mafune and Tanaka 2000; Tsutsui et al. 1996) and as prognostic markers for cancer patients receiving immunomodulative therapy (Hernberg 1999; Muhonen et al. 1994). In a randomized trial, patients with renal cell carcinoma were treated with vinblastine alone or in combination with interferon and peripheral blood lymphocyte subsets before and during treatment. The results showed that increasing $CD4^+$/$CD8^+$ ratios were observed in 10 of 17 patients in the combination group and those treated with vinblastine plus IFN who showed an increasing ratio had a better median survival compared with those with a decreasing ratio (Hernberg et al. 1997). Similar results were also revealed in other clinical trials (Gez et al. 1999; Gohring et al. 1996; Hernberg et al. 1996) and these studies implied that this change could have an impact on clinical outcome and prognosis. Furthermore, NK cells, functionally defined as cells with the spontaneous cytolytic activity (Whiteside et al. 1998), is correlated with both $CD16^+$ and $CD57^+$ positive cells, which can be used as an indicator of cellular immunity in cancer patients. In this study, 11 of 16 patients had originally abnormal $CD4^+$ /$CD8^+$ ratios, and nine patients had abnormal percentages of NK respectively in the circulation before HIFU treatment. This high frequency of reduced cell-mediated immunity observed in our series possibly contributed to the fact that most patients (12/16) with malignancies are classified as stages III and IV. However, the mechanism for the development of this immunosuppression is still unknown.

The presence of impaired immune function is a serious problem in cancer patients. As local therapies, surgery and radiation therapy play an important role in the treatment of solid cancer. In the past decades, a number of reports have demonstrated that the functional suppression of lymphocyte-mediated cellular immunity was observed after both surgical procedures (Hansbrough 1984; Mafune et al. 2000; Vallejo 2003; van Sandick et al. 2003; Yoshihara et al. 1986) and local radiotherapy (Chamberlain et al. 1980; Rotstein et al. 1985; Shukla et al. 1986; Toivanen et al. 1984; van Rijswijk et al. 1984). It has been widely believed that this immunosuppression may cause the possibility of postoperative dissemination of cancer cells and development of tumor metastases in cancer patients having original dysfunction of antitumor immunity. However, in this study, we observed a significance increase in the population of $CD4^+$ lymphocytes ($p < 0.01$) and the ratio of $CD4^+$

/$CD8^+$ ($p < 0.05$) in the circulation of cancer patients after HIFU treatment. Immunologic abnormality caused by tumors in some patients, particularly T lymphocyte-mediated antitumor immunity, was reversed after HIFU ablation. This result suggests that HIFU could activate T lymphocyte-mediated cellular immunity and lead to a long-term defense against cancer of same origin, while primary cancer is successfully treated.

While the underlying mechanisms remain unknown, the factors correlated with the reversal of impaired antitumor immunity in the cancer patients treated by HIFU are no doubt very complicated. However, it seems clear that HIFU ablation induces coagulative necrosis of carcinoma, thereby allowing immune system to recover. We have speculated that acoustic energy somehow affects host immunologic system, although at this point we cannot document specific details. One possible mechanism is that immunosuppressive factors secreted by tumor cells are immediately reduced or disappear following tumor destruction induced by HIFU. Another hypothesized mechanism is an acoustic effect on the tumor cells. High-power ultrasonic radiation may alter the biophysical structures of tumor cells (Burov and Dmitrieva 2002), which have the capability to impair the function of tumor-specific T cells in some tumors. This may result directly from cavitation, excluding thermal effect, and may produce a specific immune response through disrupting the cancer cells.

Our results showed some significant changes of antitumor immunity in patients before and after HIFU. However, they were limitations for evaluating clinical benefit of patient's immunity alteration because this study was a preliminary and nonrandomized clinical trial. The solid malignancies in our series originated from bone, liver and kidney and most of them were classified as stages III and IV. It is difficult to evaluate the potential benefit of reversed antitumor immunity that was abnormal before HIFU treatment, on the basis of small clinical samples. Many factors, such as nutritional status, tumor type and pathologic stage, also affected the prognosis of the patients who received HIFU ablation. Therefore, a randomized clinical trial is necessary to perform for the assessment of clinical benefit of antitumor immunity activated by HIFU in near future.

In conclusion, the results from this study are very encouraging. This preliminary study suggests that HIFU could enhance a systemic antitumor cellular immunity in addition to direct tumor destruction. As antitumor immune system is very complicated, it is still unknown whether antitumor immunity obtained after HIFU treatment is caused by tumor breakdown products or directly by ultrasound ablation. Therefore, further work will be done to determine how long the activated immunity lasts after HIFU, whether the enhanced immune response is specific or not, and the underlying mechanisms.

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