Cytotoxic Changes of Lymphocytes in Axillary Lymph Nodes in Patients with Breast Cancer Treated by High Intensity Focused Ultrasound

Xueqiang Zhu, feng Wu[1], Yuelei Wu[2], qiang Zhou[3], Jun Zhang[4], Zhibiao Wang[1]

Department of oncology of Sichuan provincial people hospital, Chengdu, 610072, China
[1] Medical ultrasound engneering institute of Chongqing Medical University, Chongqing 400015
[2] The first hospital of Chengdu, Chengdu 610016, Sichuan
[3] Suining people hospital, Suining 629000, Sichuan
[4] The third hospital of Chengdu, Chengdu 610031, Sichuan

Abstract **Objective** To investigate the changes of lymphocytes respectively expressing FasL, granzyme, B(GzB) and perforin (Pf) in axillary lymph nodes of breast cancer after in situ treatment with high intensity focused ultrasound (HIFU). **Methods** Expression of FasL, GzB and Pf of lymphocytes in axillary lymph node were detected by SP immunohistochemical stain. **Results** In HIFU group, the number of positive lymphocytes expressing FasL, GzB and Pf in metastasis-positive and negative axillary lymph nodes was obviously higher than these of control group respectively ($P<0.01$). **Conclusions** The positive lymphocytes expressing FasL, GzB and Pf in tumor-draining lymph node (TDLN) (whether it had metastases or not) was significantly increased after HIFU treatment for primary breast cancer., which could cause the enhancement of local antitumor cytotoxicity and prevent tumor from metastasis.

Key Words High intensity focused ultrasound; Breast cancer; Tumor draining lymph node; Fas ligand; Granzyme B; Perforin.

High intensity focused ultrasound (HIFU) was a local tumor treatment currently used in clinic for its efficacy. The studies of medical ultrasound engneering institute of Chongqing medical university found that the number of T, CD4, CD8 and NK cells increased, and function of T lymphocytes up-regulated after HIFU treatment for the primary breast cancer [1]. These indicated HIFU had anti-tumor immunological effection in the whole body. Nowadays there were lack of advanced evidence that increased immuological effect-lymphocytes has direct cytotoxicity to tumor cells after HIFU treatment. So it was important to investigate the changes of FasL, Granzyme B (GzB) and Perforin (Pf) of lymphocytes in axillary lymph node in the patients with breast cancer treated by HIFU,which were major effective molecules of cytotoxic T cells.

Correspondence to: Xueqiang Zhu, doctor-in-charge, M.D, 13183880109, 028–87394139
zhuxueqiang1234@163.com

MATERIALS AND METHODS

Clinical materials

A total of 48 femal patients with biopsy-prove breast cancer were divided into HIFU group and control group. Among them, 23 patients underwent HIFU treatment 1~2weeks before modified radical mastectomy (HIFU group). the other 25 patients received surgical operation alone (control group). The specimens of axillary lymph nodes were collected, fixed in 10% neutral buffered formalin.

In HIFU group, the average age of patients was 46.5 ranged from 23 to 65 years old. The tumor diameter was from 2.0cm to 4.7cm, averaging 3.1cm. And 11 cases of metastasis-positive axillary lymph node which occupied 47.8% in total (11/23). Among them, 5 patients were stages N1, and 6 patients were stage N2. In control group, the average age was 47.4 (ranged from 29 to 70 years) years old. The diameter of tumors average 3.2cm (the range was 1.5~6.1cm). In 12 cases, occupied 48.0% in total, the axillary lymph node were tu-

mor-metastasized. The cases both in stage N1 and in stage N2 were 6 evenly. There was no significant difference in both groups (probability $P<0.05$).

Treatment

JC-high intensity focused ultrasound treatment system was made in Chongqing HiFu Technology Limited Corporation. The targets ablated by HIFU in epidural anesthesia, based on tumor surgical principle, included the primary tumor and the normal tissues around the tumor. The main parameters of HIFU were as follow, frequency was 1.6MHz, focal length was 120mm, focal region size was 1.1mm×1.1mm×3.3mm, acoustics-intensity in focal region was 5000-15000w/cm^2 and therapy time was 1200-4000s.

Detecting methods

SP immunohistochemical stain (DAB stain and haematoxylin counter stain) was used to detecte FasL, GzB and Pf. Poly-clonal antibody of FasL and monoclonal antibody of GzB were purchased from Maxim Biotechnology corporation in USA. Monoclonal antibody of Pf was purchased from Novocastra Laboratories Ltd. The operating dilution were 1:80, 1:30 and 1:20 respectively. There were both positive control and negative control from beginning to end. we counted positive lymphocytes of 10 fields at random in ×400 microscope field with micrometer counter, and the average of them was taken for each specimen with the unit pc/mm^2.

Statistics

The results were expressed by means(x)±standard deviation(s). The data were analyzed by using student t test of two-specimen mean. There was a significant difference of these data when the $P<0.05$.

RESULTS

Immunohistochemical stain

Regardless of metastases or nonmetastases in axillary lymph nodes, the positive lymphocytes of FasL, GzB and Pf in HIFU group were significantly increased respectively. They were concentrated to form nests and mainly distributed into paracortical zone of axillary lymph nodes without metastases. Moreover a large numbers of positive cells were observed on margin of normal tissues and axillary lymph node with metastases in HIFU group. Contrast to control group, it was obvious that more positive lymphocytes were seen in medullary sinus and cords, especially for FasL positive cells in HIFU group.

The changes of lymphocytes expressing FasL, GzB and Pf in two groups listed in table 1, 2.

Self-comparison of positive cells between axillary lymph node with metastases and those without metastases in same group. All of t values were listed in table 3

There was no significant difference between FasL、GzB and Pf positive cells in axillary lymph node with metastases and those without metastases in both groups. ($P>0.05$), which indicated that number of lymphocyte expressing FasL、GzB and Pf in axillary lymph nodes were no relation to metastases in these lymph nodes.

Table 1 number of FasL, GzB and Pf pisitive lymphocytes in axillary lymph node with metastases (pc/mm^2)

groups	FasL	GzB	Pf
HIFU	55.9±15.8	52.0±15.5	25.4±8.1
control	32.5±13.1	32.1±16.6	12.2±5.4
t value	3.914	2.963	4.647
P value	㊀0.001	㊀0.01	㊀0.001

Table 2 number of FasL, GzB and Pf pisitive lymphocytes in axillary lymph node without metastases (pc/mm^2)

groups	FasL	GzB	Pf
HIFU	71.1±36.0	70.5±31.2	30.6±16.8
control	26.1±11.1	36.2±12.5	12.9±6.8
t value	4.395	3.783	3.445
P value	㊀0.001	㊀0.005	㊀0.005

Table 3 self-comparison of positive cells in same group

Groups	HIFU		ontrol		P
	LN(+)	LN (−)	LN(+)	LN (−)	
FasL	t=1.375		t=1.509		all of them
GzB	t=1.882		t=0.698		>0.05
Pf	t=0.966		t=0.297		

DISCUSSION

The immune reactive state of anti-tumor to host was closely related with her changes in tumor-draining lymph node(TDLN). Cellular immunity in TDNL, especially its cytotoxic lymphocyte including CTL and NK., which induced cancer cells to apoptosis mediated by cytotoxicity was the most important mechanism for immune defense and anti-tumor immunity in organism. Medical ultrasound engineering institute of Chongqing medical university found that lymphoid follicle and lymphatic sinus histocyte had proliferated in various degree, but the reaction was not obvious in paracortical zone of axillary lymph node in control group.[2] And in HIFU group, no matter whether there was metastatic breast cancer cells in axillary lymph nodes, lymphocytosis deep lymphoid follicle and in paracortical zone increased thickness, sinus deep capsule of lymph node and medullary sinus expanded, and sinus histocytes proliferated obviously. Simultaneously number and function of antigen −presenting cell (APC), T-lymphocytes and NK cells in axillary lymph node was increased significantly after primary tumor treated by HIFU compared with control group. Furthermore, $CD4^+$ T cells and $CD4^+/CD8^+$ ratio raised[1−3]. These investigation showed that cellular immunity function in TDLN, following antigen-presenting function up-regulation, was reinforced after HIFU treatment for primary breast cancer. Cytolytic lymphocyte (including CTL and NK) sould has directly killed tumor cells after local cellular immunity function of host was activated by tumor cells. Among them, Fas-FasL and perforin−granzyme pathway were generally accepted as two major mechanisms of CTL mediated cytotoxicity, which could directly cause lysis of tumor cells by inducing apoptosis[4,5]. Smyth[6] had revealed that tumor metastases was controlled by perforin-dependent cytotoxicity mediated by NK cells, and perforin-deficient mice were 10-100 fold less than normal mice in protection from tumor metastases using experimental and spontaneous models of metastases in prostate and mammary cancer. Asano[7]found that immunostimulation therapy with anti-CD3 monoclonal antibodies and recombinant interleukin-2 enabled effectively to reduce lung metastases and elongate survival-time of murine with renal cell carcinoma owing to heightening in vivo lung expression of cytotoxic molecules such as perforin, granzyme and FasL so on. The above results showed that active cytotoxic lymphocytes could protect the host from tumor metastases because more cytotoxic molecules of them expressed in metastases tissues.

This investigation found that lymphocytes of axillary lymph node expressing of FasL, GzB and Pf were significantly increased in HIFU group. They were mainly distributed on not only paracortical zone, the border between normal lymph tissues and metastases but also medullary sinus, medullary cord of axillary lymph nodes. Contrasted with control group, it was outstanding that more positive lymphocytes were seen in medullary sinus and cords, especially for FasL positive cells in HIFU group. The result showed that the activated cytotoxic lymphocytes expressing of FasL, GzB and Pf in TDLN (whether it had metastases or not) were significantly increased after HIFU treatment for primary breast cancer, which could cause the enhancement of local specific antitumor immune cell. The outcome had advantage of removal and diminution of metastases in TDLN. Thus host effectively prevented tumor from metastases in all over the body.

At present, it was unclear what were causes of enhancement the specific anti −tumor immunity in TDLN after HIFU treatment for the primary tumor. We supposed it was concerned with below factors. First, because of tumor tissues coagulative necrosis caused by HIFU, the tumor loads of host decreased, the ratios of host to tumor improved, thereby tumor inhibition to the host immunological function relieved. Second, tumor cells generated the specific antigenic peptides after teatment by HIFU hyper-

thermal effect, which became targets of immunocyte [8]. Third, the heat shock proteins elicited by heating effect of ultrasound was combined with the tumor antigen to form polypeptide compounds which were able to stimulate the host immune system to yield specific anti-tumor immunity[9]. The last, non-thermal mechanisms of HIFU, such as cavitation, disrupted tumor cell membrane, so it resulted in sufficient exposure and release of membrane, cytoplasm and nucleus antigens. Therefor tumor cell antigenicity was reinforced, quantity of antigen and immunogen increased, and finally the host specific immunity function was activated. Nevertheless, we needed advanced studies to confirme whether there was a relationship between increased cytotoxic lymphocyte and increased apoptosis of tumoer cells in TDLN after HIFU treatment.

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