Microwave Ablation of Hepatocellular Carcinoma:the Effect on Immune Response of Tumor-bearing Mice

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Abstract Objective To investigate the effect of microwave ablation (MA) of mouse hepatocellular carcinoma (HCC), which may alter the host´s immune response. **Methods** A HCC model of C57BL/6J mouse was established by s.c. injection of Hepa1-6 cells, the tumors were treated by MA or resection, before and after the treatments, the proportions(%)of $CD8^+$ T, $NK1.1^+$ cells of peripheral blood, and the concentrations(pg/ml) of Th1, Th2 cytokines (IFN-γ, IL-4) of plasma of the hosts were determined with flow cytometry and ELISA, respectively. **Results** Compared with group normal, in group tumor-bearing, the proportion of $CD8^+$ T cells was decreased, $NK1.1^+$cells increased, and the concentrations of IL-4 and IFN-γ were reduced (all $P>0.05$). For comparisons between groups MA and tumor-bearer, $CD8^+$ T, $NK1.1^+$cells, and IFN-γof the former were higher than that of the latter ($P>0.05$, $P<0.05$, $P<0.01$,respectively), but IL-4 was evidently lower than that of the latter ($P<0.05$). Compared with the group tumor-bearer , the $CD8^+$ T cells and IFN-γof the group resection were enhanced, $NK1.1^+$ cells and IL-4 lowered (all $P>0.05$). The $CD8^+$T cells and IFN-γ of group MA were higher, but IL-4 lower than group resection (all $P>0.05$), and the $NK1.1^+$cells of MA obviously higher than resection ($P<0.01$). **Conclusion** The MA of the mice HCCs may promote the immune response towards Th2/Th1 shift, and markedly enhance the proportion of NK 1.1^+cells of the hosts.

Key words: Hepatocellular carcinoma; Cytokine; Microwave ablation;Th2/Th1 shift; NK1.1+ cell

Thermal ablation is, now, one of the important treatments of HCC, besides directly killing the tumor cells, it can also stimulate the host´s immunity against the tumor, but the results from different authors are always different[1,2]. Here, the present study was done to investigate the effect of MA of mice HCCs on the immune response of the hosts.

MATERIAL AND METHODS

Animals and tumor cell line

5~8 weeks old C57BL/6J female mice weighing 19±1g were obtained from the division of animals of Sun Yat-sen Medical College of Sun Yat-sen University, and maintained under specific pathogen-free conditions, the syngeneic HCC cell line of Hepa1-6 was kindly presented by Institute of Oncology of Second Military Medical University of PLA, and grown in DMEM complete medium (10% FCS, 100 U /ml penicillin, 100μg/ml streptomycin, 0.45% glucose and 2 mmol/L L-glutamine)under standard conditions (37℃, 5% CO_2). All experiments were conducted according to local animal protection guidelines.

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HCC model

The tumor model was established by s.c.injection of Hepa1-6 cells at right back of the mouse at the dose of 2×10^6 cells in 100μl PBS for each animal, when the tumors reached a long diameter of 10~15mm (about 2 weeks after inoculation of the tumor cells), the mice were eligible for experiment.

Resection and MA

For resection, the tumor-bearing mouse was anesthetized by i.p. injection of 10% chloral hydrate (1.5ml/kg), the tumor area was disinfected with 70% alcohol and underwent a skin incision, the tumor was dissected from surrounding tissue and completely

Table 1 Comparisons of lymphocytes and cytokines between tumor-bearer and normal mice

Group	N	Proportion of lymphocytes(%, $\bar{x}\pm s$)		Level of cytokines(pg/ml, $\bar{x}\pm s$)	
		CD8+	NK1.1+	IL-4	IFN-γ
Tumor-bearer	6	19.1±8.4	9.9±1.8	24.1±6.7	5.5±1.2△
Normal	6	22.9±7.2	7.2±1.5	33.0±12.3	7.4±2.7

t -Test: △.compared with normal, $P>0.05$.

removed, after the bleeding was thoroughly stopped, the wound was immediately closed. For MA, anesthesia, disinfection, and skin incision were the same as mentioned above, the tumor was sufficiently exposed to avoid the skin being burned, a FORSE-MTC -3 type, 2450MHz monopolar microwave generator (Qinghai Institute of microwave electronics, Nanjing, China)capable of producing 5 ~12W of power, with it´ s 15mm MA-probe(0.3mm active tip) inserting into the parenchyma of the tumor, was used to ablate the tumor, in the same time, a RKC-C9000 digital temperature -mearuring divice (PKC,Osaka, Japan) with the needle puncturing into the ablated area was applied to mearure the temperature, a term of 90± 5℃, 2 min was utilized to ensure the HCC tissue entirely coagulated (our preliminary experiment result not published), then the hemostasis and incision suture were also completed at once. After the treatments, the animals were housed and bred as before so that they recovered.

Flow cytometry and ELISA

Four groups animals (each group of 6 mice), that is, normal (without intervention and tumor), tumor-bearing (tumor´ s long diameter of 10 ~15mm), resection and MA (3 weeks after the treatments, no palpable residual tumor, without canker and infection in the local site), were included for detection. Blood sample was collected from each mouse by extraction of the eyeball, the sample was added EDTA.K2 for anticoagulation, thereafter , a part of the sample was stained, and left at room temperature for 30 min, with mAbs against CD8 (PE-Cy5,53-6.7) and NK1.1 (PerCP-5.5,PK136) (BD PharMingen, San Diego, USA), and then flow cytometry was carried out using a two-color ELITE flow cytometer(Beckman Coulter, FL, USA), another part of the anticoagulated blood underwent centrifugate and was analyzed for concentrations of IL-4 and IFN-γ(sensitivities were 4 pg/ml, 0.781pg/ml, respectively) by ELISA kits (eBioscience,San Diego, USA) according to the instructions of the manufacturer.

Statistics

The data were standed for $\pm s$, comparisons between two groups by using *t*tests, and among three groups by one-way ANOVA or rank sum tests, all analysis were carried out by using a SPSS10.0 statistical software, $P<0.05$ was considered to be statistic significance.

RESULTS

The lymphocytes and cytokines of normal and tumor-bearing mice

Compared with normal, in mice with the HCCs, $CD8^+$ T cells, IL-4 and IFN-γ were descending, $NK1.1^+$ cells ascending, but all the differences were not statistically significant(all $P>0.05$)(table 1.)

Changes of lymphocytes and cytokines after the treatments

For comparisons between MA and tumor-bearer, $NK1.1^+$ cells and IFN-γ of the former were obviously higher, but IL-4 markedly lower than that of the latter ($P<0.05$, $P<0.01$, $P<0.05$,respectively).

Table 2 The lymphocytes and cytokines after the treatments

Group	N	Proportion of lymphocytes(%, $\bar{x}$±s)		level of cytokines(pg/ml, $\bar{x}$±s)	
		CD8+	NK1.1+	IL−4	IFN−γ
M A	6	26.2±7.2	19.7±6.9△,★	13.7±2.6△	31.1±16.5◆
Resection	6	19.4±3.2	9.3±1.4	23.6±12.2	11.7±3.7
Tumor−bearer	6	19.1±8.4	9.9±1.8	24.1±6.7	5.5±1.2

One way ANOVA: comparisons of CD8+ between each two−group among the three groups, all P>0.05 .Rank Sum Test:
△. compared with tumor−bearer, P < 0.05;
★.compared with resection, P <0.01;
◆. compared with tumor−bearer, P <0.01.

The $CD8^+$ T cells and IFN−γ in resection group were increased, and $NK1.1^+$ cell and IL−4 decreased compared with that of tumor−bearing, however, there were no statistic significances in these differences(all P >0.05).

Compared with resection, $NK1.1^+$cells of MA was evidently enhanced (P <0.01), other differences in $CD8^+$T cells, IFN−γ and IL−4 between the two groups were not statistically significant(all P >0.05) (Table 2).

DISCUSSION

$CD4^+$ T, $CD8^+$ T and $NK1.1^+$ cells are the main immunocytes in anticancer immunity, the $CD4^+$ T cells´ (T helper lymphocyte, Th) responses to the stimulus of antigen, according to the cytokines secreted, may be classified into two types of Th1 and Th2. IFN−γ,IL−12 ,etc, are the representative cytokines of Th1 immune response, whereas IL−4, IL−10 represent Th2 immune response [3]. Th1 cytokines may induce $CD8^+$ T cells´ differentiation into cytotoxic T lymphocytes(CTLs) and activate NK cells, CTLs can specifically lyse tumor cells, and NK cells can unspecifically destroy cancer cells. Increase of Th1 or decrease of Th2 cytokines called Th2/Th1 deviation or shift, vice versa, Th1/Th2 deviation [4], Th1/Th2 shift may promote the tumor [5], and Th2/Th1 shift check the carcinoma [6].

Tumor growing in the body leaded to the decreases of $CD4^+$ and $CD8^+$T cells in both of peripheral blood and spleen of the host [7], our results that the $NK1.1^+$ cells of tumor−bearing mice was higher, and Th2 cytokine(IL−4) lower than that of normal mice, but the important $CD8^+$ T cell and Th1 cytokine(IFN−γ) in anticancer of tumor−bearing were decreased though the differences no statistic significance, also indicated the declining trend of anticancer immune components of the hosts.

Some experiments showed that 10 days after the tumors treated by local thermal ablation, there were weak enhancements of $CD8^+$ T cells and IFN−γ of the hosts, and adding antibodies of anti−CTLA−4 resulted in obvious rise of $CD8^+$ T cells[2], and treating carcinomas by combination of MA and local injection of alcohol induced visible increases of T cells number and NK cell activity[8]. In the present study, we used only MA not combining any other procedures to ablate the HCCs, also induced increase of $CD8^+$T cells, evident enhances of $NK1.1^+$ cells and IFN−γ, and marked decrease of IL−4 in the hosts. Therefore, MA of tumors may stimulate the hosts´ immunocytes and Th1 cytokine, and promote the immune response towards Th2/Th1 deviation.

For the mice of resection group in our experiment, $CD8^+$T cells and IFN−γ were higher, and $NK1.1^+$ cells and IL−4 lower than that of tumor

bearer, showing resection of tumor may boost the Th1 immune response of the host though this effect was not statistically significant. The growing tumor can curb the host´ s immunity[9], thus, a mild stimulating effect of immuninity by resection in our study may be related to removing of the tumor.

In the pesent experiment, the NK1.1$^+$ cells of MA group was greatly increased, IL−4 decreased, CD8$^+$ T cells and IFN−γ were enhanced compared with that of resection, so the immune stimulating effect in MA group was stronger than that in resection group, this may be due to in situ MA of tumor creating a antigen resource for generation of anticancer immunity[2], and the stimulus of antigen is a premise to bring about the immune response. In this trial, the ablated HCC tissue needed undergoing 1 ~2 weeks to be, largely or completely absorbed, so one of the mechanisms for producing stronger Th2/Th1 shift of immune response in group MA ,may be the lasting stimulation of ablated tumor tissue in the body of the host, another reason may be the increase of NK1.1$^+$ cells, because NK1.1 antigen is a marker of mouse NK cells, but also expressed on a special kind of T cells−NKT cells [10], NK cells can secrete Th1 and Th2 cytokines[11], and after being activated, the NKT cells can also excrete Th1 or Th2 cytokine [12]. Contrast to MA, the resection group produced a weaker immune response probably because of lack of the stimulation of the coagulated tumor tissue. In conclusion, the MA of implanted HCCs may result in Th2/Th1 deviation of immune response and obvious increase of NK1.1$^+$ cells of the hosts, and has a stronger stimulation of immunity than resection, these results may suggest us how to apply immunotherapies after MA or resection of the tumors, however, the immune responses of MA of HCCs at other time points, the actual influence of this immune response on the growth of tumor, and so on, need further investigating.

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REFERENCES

1.Isbert C, Ritz JP, Roggan A, et al. Enhancement of the immune response to residual intrahepatic tumor tissue by laser−induced thermotherapy compared to hepatic resection. Lasers Surg Med, 2004, 35: 284−292.

2.Den Brok MH, Sutmuller RP, van der Voor R, et al. In situ tumor ablation creats an antigen source for the generation of antitumor immunity. Cancer Res, 2004, 64 : 4024−4029.

3.Hsu TL, Wu YY, Chang YC, et al. Attenuation of Th1 response in decoy receptor 3 transgenic mice. J Immunol, 2005, 175: 5135−5145.

4.Shibata Y, Henriksen RA, Honda I, et al. Splenic PGE2−releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat−killed BCG. J Leukoc Biol, 2005, 78: 1281−1290.

5.Loskog A, Dzojic H, Vikman S, et al. Adenovirus CD40 ligand gene therapy counteracts immune escape mechanisms in the tumor microenvironment. J Immunol, 2004, 172: 7200−7205.

6.Matar P, Rozados VR, Gervasoni SI, et al. Th2/Th1 swtich induced by a single low dose of cyclophosphosaminde in arat metastatic lymphoma model. Cancer Immunol Immunother, 2002, 50: 588−596.

7.Mandal D, Bhattacharyya A, Lahiry L, et al. Failaure in peripheral immuno−surveillance due to thymocyte and apoptosis in adult tumor−bearer. Life Sci, 2005, 77: 2703−2716.

8.Nakayama J, Kokuba H, Kobayahshi J, et al. Experimental approaches for the treatment of murine B16 melanomas of various sizes injection of ethanol with combinations of beta−interferon and microwave hyperthermia for B16 melanomas with a size of greater than 11 mm in diameter. J Dermatol Sci, 1997 ,15: 82−88.

9.Scholl SM, Michaelis S, and Mcdermott R. Gene therapy application to cancer treatment. J Biomed Biotechnol, 2003, 2003: 35−47.

10.Cardillo F, Cunha FQ, Tamashiro WM, et al. NK1.1 cells and T−cell activation in euthymic and thymectomized C57BL/6 mice during acute Trypanosoma cruzi infection. Scand J immunol, 2002, 55:96−104.

11.Kimura MY, Nakayama T. Differentiation of NK1 and NK2 cells. Crit Rev Immunol, 2005, 25: 361−374.

12.Lee PT, Benlagha K, Teyton T, et al. Distinct functional lineage of human V (alpha)24 natural killer T cells. J Exp Med, 2002, 195: 637−641.