Effects of intratumoral injection of immunoactivator after microwave ablation on antitumor immunity in a mouse model of hepatocellular carcinoma

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Abstract. This study investigated the effects of intratumoral injection of immunoactivator after microwave ablation on antitumor immunity in a mouse model of hepatocellular carcinoma. Hepatocellular carcinoma cell line Hepa1-6 was subcutaneously injected into C57/B6 mice to establish a mouse model of hepatocellular carcinoma. When tumor diameter reached 8 mm, microwave ablation was performed for 3 min with temperature controlled at 55˚C. Cytokine sustained-release microspheres (CytoMPS) containing human interleukin-2 (hIL-2) and mouse granulocyte macrophage colony-stimulating factor (mGM-CSF) were injected into the tumor of mice in the experimental group (n=5) at 3, 7 and 14 days after ablation, while sustained-release microspheres containing no cytokine were used in the control group (n=5). Mice were sacrificed on the 17th day after ablation, and CD4$^+$ and CD8$^+$ T cells in peripheral blood were counted by flow cytometry. Spleen was collected from the mice to isolate lymphocytes. Lactate dehydrogenase (LDH) release assay was used to determine the cytotoxicity of spleen cells to Hepal-6 cells. Injection of CytoMPS after ablation increased the percentage of CD4$^+$ and CD8$^+$ T cells in peripheral blood. Cytotoxicity of CD8$^+$ CTL to Hepal-6 is significantly higher in experimental group than in control group (P<0.01). The results showed that intratumoral injection of CytoMPS containing hIL-2 and mGM-CSF can significantly increase the proportion of CD4$^+$ and CD8$^+$ T cells in blood and increase the cytotoxicity of CTL cells to tumor cells in mice with hepatocellular carcinoma.

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Introduction

As the most common cancer in the digestive system, the incidence of hepatocellular carcinoma is ranked in the fifth position among all malignant tumors. More than 500,000 patients develop hepatocellular carcinoma every year, and China accounts for about one-third of them (1). Surgical resection, thermal ablation and liver transplantation are primary means of radical treatment of hepatocellular carcinoma, and surgical resection is the gold standard. More than 70% of patients are diagnosed too late for surgical resection due to tumor progression or liver dysfunction. In addition, the recurrence rate after surgery is high. Therefore, the application of surgical treatment is limited (2). With the advantages of small trauma, less complications and high repeatability, thermal ablation has become the main treatment for hepatocellular carcinoma patients who did not have surgical treatment and patients with recurrent hepatocellular carcinoma (3). However, recurrence rate of hepatocellular carcinoma after ablation is still high (4). Therefore, how to reduce the recurrence rate after ablation is the key to improve the treatment outcomes of ablation.

The failure in producing efficient antitumor immune response is the main reason for tumor metastasis and recurrence (5). Antitumor immunity requires the participation of tumor antigens, antigen presenting cells and effector cells (6). Previous studies have shown that local ablation can increase the production of tumor antigens to induce antitumor immune response in the body (7). However, the failure of immune system in removing tumor cells is not due to the lack of tumor antigen, but the weak function of tumor antigen, which cannot be used to stimulate effective antitumor immune response (8).

Previous studies have found that local high concentrations of cytokines can significantly amplify antitumor immune responses (9). Human interleukin (hIL-2) can enhance the cytotoxic activity of cytotoxic T cells (CTL) and natural killer cells (NK) to tumor cells, and hIL-2 is the first cytokine that was approved by the US Food and Drug Administration (FDA) to be used clinically. Injection of hIL-2 into tumor of mice can induce protective immunity to inhibit tumor growth (10). Mouse granulocyte-macrophage colony-stimulating

factor (mGM-CSF) can promote the proliferation, differentiation, maturation and migration of antigen-presenting cells, and induce the production of CTL. mGM-CSF is an immune cytokine with the strongest ability in stimulating antitumor immune responses (11-13). We have successfully constructed CytoMPS containing hIL-2 and mGM-CSF, which can serve as immune activator to stimulate immune response in the body (14). Based on our previous studies, in this study, a mouse model of hepatocellular carcinoma was constructed by subcutaneous injection of hepatocellular carcinoma cell line Hepa1-6, and CytoMPS injection was performed after ablation to observe the effects on immune response, especially cellular immunity.

Materials and methods

Animals and cells. C57/B6 mice (4-6 weeks old) were raised in our laboratory. Hepatocellular carcinoma cell line Hepa1-6 derived from C57/B6 mice was purchased from the Institute of Physical and Chemical Research (Yokohama, Japan). Human laryngeal carcinoma epithelial cell line Hep2 was from the stock of our laboratory. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Gibco, Grand Island, NY, USA) in incubator (37˚C, 5% CO_2).

Establishments of a mouse model of hepatocellular carcinoma. Adherent Hepa1-6 cells were digested with trypsin to make single cell suspension. After centrifugation at 5,000 x g for 8 min, supernatant was removed and cells were counted. Cells ($2x10^6$) were subcutaneously injected into unilateral loin and rib of mice (n=10). The diameter of the subcutaneous tumor was measured every day. The study was approved by the Ethics Committee of Guangzhou Twelfth People's Hospital.

Microwave ablation. Microwave ablation was performed using FORSEA MTC-3 microwave ablation therapy instrument (Qinghai Microwave Electronic Institute, Nanjing, China) when the diameter of tumor reached 8 mm. The power of microwave ranged from 5 to 100 W, and the frequency was 2450 Hz. Mice were intraperitoneally injected with chloral hydrate (1.5 ml/kg) for anesthesia before ablation. Under sterile conditions, skin was cut 1 cm away from the tumor to fully expose the tumor. Microwave ablation electrode needle was inserted into the tumor along the longitudinal axis to start the ablation. Ablation was performed for 3 min with the power of 5 W. The temperature inside the tumor reached 90˚C, and temperature outside the tumor reached 55˚C. The ablation was successful if the mice were still alive at 3 days after ablation and the skin around the incision area grew well. Tumor was collected from one mouse of each group at 3 days after ablation. Tumor tissue was fixed in formalin solution, followed by paraffin-embedding and section. After a series of treatment, hematoxylin and eosin (H&E) staining was performed.

Injection of CytoMPS. Preparation of CytoMPS: sterile saline was used to prepare 2.5% human serum albumin (HSA), and PH was adjusted to 3.0. One milliliter of HIL-2 ($2x10^4$ U/ml) and 1 ml of mGM-CSF ($2x10^4$ U/ml) were mixed with 2 ml of HSA containing 1,000 units of heparin. Coupling agent EDAC was then added, followed by oscillation for 15 min. Glycine

(0.1 mol/l) was added, followed by oscillation for 15 min to make suspension. Suspension (50 μl) was mixed with 50 μl of phosphate-buffered saline (PBS) containing 1,000 units hIL-2 and 1,000 units mGM-CSF to make a dose of CytoMPS. One dose of CytoMPS was injected into the tumor of mice in experimental group (n=5) at 3, 7 and 14 days after ablation, while 100 μl of sustained-release microspheres containing no hIL-2 and mGM-CSF was used in the control group (n=5).

Lymphocyte count. Mice were sacrificed on the 17th day after microwave ablation, and peripheral blood was collected after removing the eyeball. Ficoll density gradient centrifugation was performed to isolate peripheral blood mononuclear cells (PBMC). PBMCs were divided into two equal volumes. After staining with PE-labeled anti-mouse CD3 antibody and FITC-labeled anti-mouse CD4 antibody or FITC-labeled anti-mouse CD8 antibody (all from Abcam, Cambridge, MA, USA), the proportions of CD4+ T cells and CD8+ T cells in PBMC were detected by flow cytometry.

Lactate dehydrogenase (LDH) assay. Mice were sacrificed on the 17th day after microwave ablation, and spleen was collected under sterile conditions. Spleen was ground to make single cell suspension, and sterile Tris-NH4Cl was used to remove red blood cells, and DMEM medium was used to make spleen cell suspension ($1x10^7$ cells/ml). Hepa1-6 or Hep2 cells ($4x10^4$) were added into each well of 96-well plate, and $8x10^5$ spleen cells were also added to make an effector-target ratio of 20:1. Effector cells natural release control, target cells natural release control, target cells max release control, culture medium control and cell culture medium correction control were all set. Three repeats were set for each sample. After incubation at 37˚C in an incubator for 4 h, 10 μl of cell lysate was added into the target cells Max release control wells and culture medium control wells. After incubation for another 1 h, 50 μl of supernatant was collected from each well and transferred to another 96-well plate. LDH substrate (50 μl) was added into each well, followed by incubation at room temperature for 30 min in the dark. Stop solution (50 μl) was then added and OD value at 490 nm was measured using a microplate reader. Killing activity = (OD value of experimental group-OD value of effector cell natural release control-OD value of target cells natural release control + OD value of cell culture medium control)/(OD value of target cell Max release control A490-OD value of target cell natural release control + OD value of cell culture medium correction control) x100%. The average value of three repeat wells was calculated.

Statistical analysis. SPSS 13.0 statistical software was used for statistical analysis. Data are expressed as mean ± standard deviation, and comparison between groups were performed by Student's t-test. $P<0.05$ was considered to be statistically significant.

Results

Microwave ablation of a mouse model of hepatocellular carcinoma. Mice were subcutaneously injected with $2x10^6$ Hepa1-6 cells, and palpable grain size nodules with a diameter of ~8 mm were observed on the 14th day. Microwave abla-
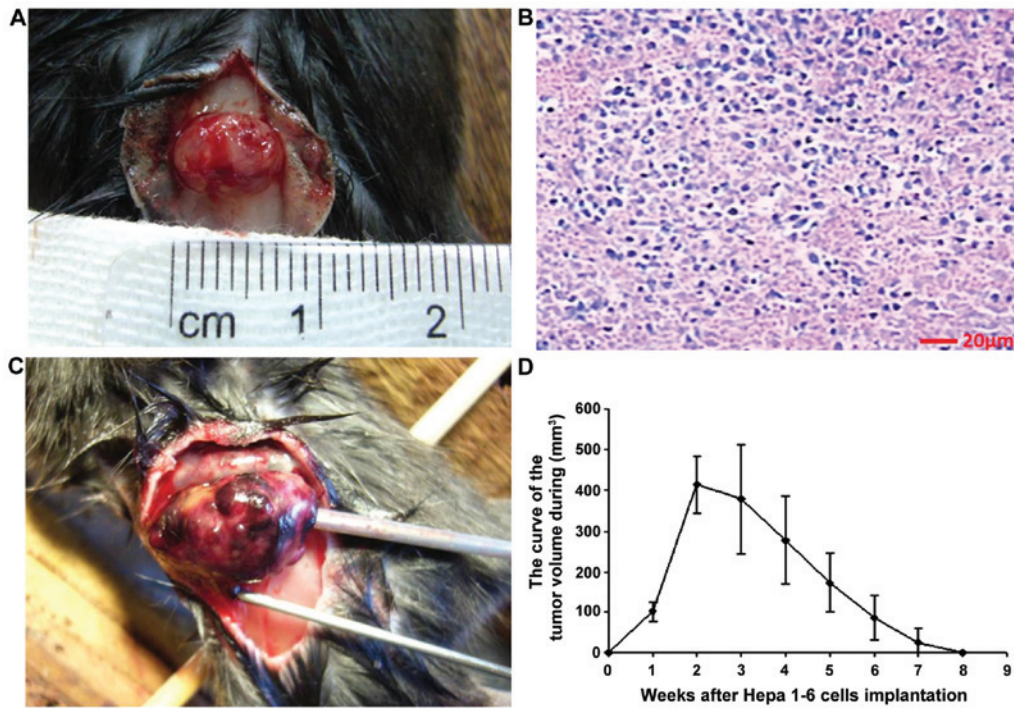
Figure 1. Microwave ablation of a mouse model of hepatocellular carcinoma. (A) Tumor diameter reached 8 mm at 14 days after subcutaneous injection of Hepa1-6 cells. (B) Tumor tissue was collected 3 days after ablation. Pathological staining was performed. Coagulation necrosis was observed in ablation area, cell structure was destroyed and no live tumor cells were found. (C) Intracranial microwave ablation was performed for 3 min with the power of 5 W. Temperature inside the tumor reached 90℃, temperature outside the tumor reached 55℃. Tumor necrosis was observed after oblation. (D) The curve of the tumor volume during the 8 weeks after Hepa1-6 cell implantation.
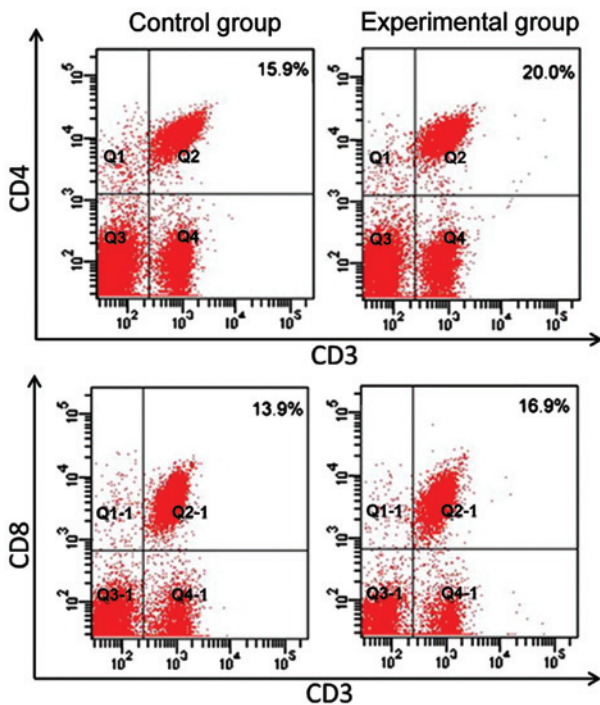

Figure 2. Percentage of CD4+ T cells and CD8+ T cells in the peripheral blood after the injection of cytokine sustained-release microspheres (CytoMPS). peripheral blood mononuclear cells (PBMCs) were isolated and the percentage of CD4+ T cells and CD8+ T cells in PBMC was detected by flow cytometry.

Table I. Percentage of CD4+ T cells and CD8+ T cells in the peripheral blood after the injection of CytoMPS.

Group	n	CD4+ T cells	CD8+ T cells
Experimental group	3	16.0±1.1%	21.1±1.1%
Control group	3	13.5±2.0%	17.0±0.9%
t-value		7.616	3.488
P-value		<0.05	<0.05

CytoMPS, cytokine sustained-release microspheres.

necrosis was observed in ablation area, cell structure was destroyed and no live tumor cells were found. Tumor volume reached the maximum value at day 14, and the volume gradually decreased after ablation. Tumor disappeared completely at week 8 (Fig. 1).

Lymphocytes after the injection of CytoMPS. CytoMPS containing hIL-2 and mGM-CSF was injected into mice at 3, 7 and 14 days after ablation. Percentage of CD4+ T cells and CD8+ T cells in the peripheral blood of experimental group was significantly higher than that of control group at 3 days after the third injection (P<0.05) (Table I and Fig. 2).

The in vitro cytotoxicity of spleen cells to tumor cells. LDH release assay showed that cytotoxicity of splenocytes to Hepa1-6 cells was significantly stronger than that of control mice after the injection of CytoMPS containing hIL-2 and

tion was performed on the tumor site and tumor tissue was collected for pathological staining 3 days later. Coagulation
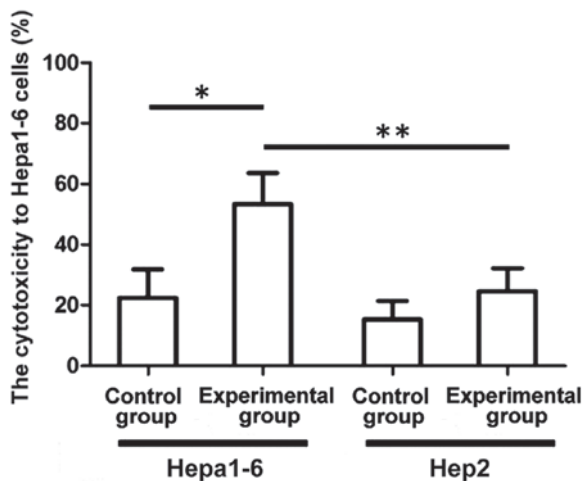
Figure 3. Cytotoxicity of spleen cells to tumor cells (%). Lactate dehydrogenase (LDH) release assay was used to detect the cytotoxicity of spleen cells to Hepa1-6 cells and Hep2 cells. *P<0.05 compared with control group; **P<0.05 compared with the cytotoxicity to Hepa1-6 cells.

mGM-CSF (P<0.05). In the experimental group, cytotoxicity of spleen cells to Hepa1-6 cells was also significantly higher than the cytotoxicity to Hep2 cells (P<0.05) (Fig. 3).

Discussion

In this study, Hepa1-6 cells were implanted subcutaneously in mice to construct a mouse model of hepatocellular carcinoma. After tumor formation, microwave ablation was performed to induce tumor necrosis. After three injections of CytoMPS containing hIL-2 and mGM-CSF after operation, number of CD4^{+} T cells and CD8^{+} T cells in the peripheral blood of mice was significantly increased, and the cytotoxicity to Hepa1-6 cells was also enhanced. The results suggest that injection of CytoMPS containing hIL-2 and mGM-CSF after microwave ablation can significantly promote cellular antitumor immune function in mice.

Antigen-presenting cells present tumor-associated antigens to lymphocytes, so as to stimulate the proliferation, and differentiation of lymphocytes, and induce specific targeting antitumor immune responses, eventually leading to the death of tumor cells (15). Microwave ablation can lead to tumor cell necrosis, heat shock proteins (Hsp70 and Hsp90) will be accumulated on the cell surface to sensitize dendritic cells (DCs), which in turn promote the antitumor immunity of CD4^{+} and CD8^{+} T cells (16). It has been reported that the DC cell vaccine prepared using tumor tissue antigen induced by ablation has certain antitumor effects (17). In most cases, ablation failed to induce specific antitumor cellular immunity in mice, which is consistent with the phenomenon that microwave ablation therapy cannot be used to control distant metastasis of the tumor (18).

One of the reasons is that hepatocellular carcinoma is relatively weak in immunogenicity, leading to failure in inducing effective antitumor immunity. To this end, we prepared CytoMPS containing hIL-2 and mGM-CSF precoated with HSA to reach long-term reactivity. HIL-2 can enhance the cytotoxicity of CD8^{+} T cells, and MGM-CSF can promote the proliferation, differentiation, maturation and migration of

antigen-presenting cells. The synergistic effect of HIL-2 and MGM-CSF can persistently promote the antitumor immune response. Results of this study showed that the injection of CytoMPS containing hIL-2 and mGM-CSF after microwave ablation can significantly enhance antitumor immune response in a mouse model of hepatocellular carcinoma.

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