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Immunological response induced by cryoablation against murine H22 hepatoma cell line *in vivo*

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ABSTRACT

Objectives: To describe immunological consequences induced by cryoablation against H22 cells *in vivo*.
Methods: Adult BALB/c mice underwent subcutaneous implantation of H22 cells. All of them were assigned into three groups randomly: group A (false surgery), group B (cryoablation) and group C (cryoablation plus Freund's adjuvant). Animals were sacrificed 1, 2 and 3 weeks after treatment. Serum IFN-γ and IL-4, Th1/Th2 in spleens and cytotoxicity were detected.
Results: Compared with that of group A, (1) INF-γ of group B was higher, but IL-4 was lower; cryoablation plus Freund's adjuvant enhanced these effects. (2) Th1/Th2 rose significantly in both group B and group C. (3) Strong cytolytic activity against H22 cells of group B and group C was found on day 7, 14 and 21.
Conclusions: Our study showed a marked shift toward Th1 and IFN-γ expression after cryoablation, with an immuno-stimulatory effect against murine H22 hepatoma Cell.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers [5,18], especially in adults in China [21]. Based on GLOBOCAN estimates [18], an estimated 782,500 new liver cancer cases and 745,500 deaths occurred worldwide during 2012, with China alone accounting for about 50% of the total number of cases and deaths. Although surgical resection and liver transplantation are the curative treatments, they only apply to patients with limited tumor burden. Thus, therapies like radio frequency ablation (RFA), cryoablation, and microwave ablation are currently important modes of HCC treatment. As is known, cryoablation has the advantages of minimal invasiveness, better repeatability, and immune regulation. Hence, cryoablation has been used to treat many types of inoperable tumors, including prostate cancer [6,19], kidney cancer [15], liver cancer [14] and metastatic liver carcinoma [4,22].

Cryoablation has been used for HCC in China and some other countries and regions [9] and it was shown to be highly effective in local tumor control with an acceptable safety profile and survival benefits. To our knowledge, it has been observed for a long time that large amounts of tumor debris remain *in-situ* after thermal ablation [20] or cryoablation [7]. Our recent studies also found that cryoablation in patients with high-risk prostate cancer and advanced renal cancer could induce an immunological response [13,17]. We speculated that it may also be possible to achieve effective immunotherapy using cryoablation for HCC. However, little is known about this topic and further studies are needed before this goal can be achieved.

Our current study was designed to investigate the characteristics of the antitumor response induced by cryoablation in a mouse model of HCC. Therefore, we studied the alterations in serum cytokine levels (IFN-γ), T-cell responses to HCC derived antigens, and the cytolytic activity of lymphocytes from the spleen against the murine HCC cell line, H22. Our study will increase pre-clinical evidence linking antitumor immune response to cryoablation.

2. Materials and methods

2.1. Mice and cell lines

Specific-pathogen-free Institute of Cancer Research (SPF ICR) C57BL/6 mice, aged 6–8 weeks old and weighing 18–22 g, were purchased from the Laboratory Animal Science Department of Capital Medical University (Beijing, China). Mice were bred, housed, and treated according to approved institutional animal protocols and were

Abbreviations: Hepatocellular carcinoma, HCC; interferon-y, INF-γ; Interleukin–4, IL-4; enzyme-linked immunosorbent assay, ELISA; natural killer cell, NK; cytotoxic T lymphocyte, CTL

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used for experiments no earlier than 5 days after their arrival.

The H22 hepatoma cell line was purchased from Bio-Rad Life Sciences Development Co., Ltd. (Beijing, China). H22 cells were cultured in RPMI-1640 medium (SH30809.01B; HyClone) containing 10% fetal bovine serum (SH30084.03; HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml L-glutamine in a humidified incubator at 37 ˚C with 5% CO2. H22 cells (1×10^7) were intradermally injected into the left flank of each mouse. For the tumor challenge, mice (24 per group) were randomly assigned to receive sham operation (group A), cryoablation only (group B), or cryoablation plus Freund's adjuvant injection (group C). In total, about 72 mice were used in this study. Cryoablation and Freund's adjuvant injection were conducted on day 1, when the primary tumors reached about 8–10 mm in the longest diameter, about 14 days after tumor inoculation.

2.2. Cryoablation and Freund's adjuvant injection

Cryoablation was conducted on day 1, when the primary tumors reached about 8–10 mm at the longest diameter, about 14 days after tumor inoculation. The mice were anesthetized by an intraperitoneal injection of a ketamine/xylazine mixture (23.75 mg/ml ketamine + 1.25 mg/ml xylazine; 100 μl/25 g). Mice from groups B and C were prepped by shaving the targeted area and cleansing it with alternating 70% alcohol rinse and povidone-iodine scrub before surgery. A 1.7-mm cryoablation probe (Endocare Per Cryo) was inserted into the tumor, and freezing was administered for 60–90 s under a working pressure of 17,225 kPa (2500 psi) and a temperature of −110˚C–-125 ˚C at the needle hub. Standard recovery procedures were implemented. Mice in group C received intratumoral injection with 0.01 ml Freund's adjuvant (F5881; Sigma) on the same day.

2.3. Specimen collection

On days 0 (the day before treatment), 7, 14, and 21, six mice in each of the three groups were anesthetized by an intraperitoneal injection of a ketamine/xylazine mixture (23.75 mg/ml ketamine + 1.25 mg/ml xylazine; 100 μl/25 g). Peripheral blood was collected by removing the eyeball. Subsequently, all mice were sacrificed by high-concentration carbon dioxide (CO_2). These blood samples were spun at 3000 rpm for 5 min, and the serum was collected and stored at −20 ˚C. Spleens were also collected. Lymphocytes from spleens were separated with mouse lymphocyte separation medium (DKW33-R0100).

2.4. Measurement of serum interferon-y (INF-γ) and interleukin – 4 (IL-4) levels

Serum samples were collected from the peripheral blood of mice as described above. Serum INF-γ and IL-4 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Dakewe Biotech Co., Ltd, China) to determine the concentration of these cytokines following the manufacturer's instructions.

2.5. Flow cytometry

Lymphocytes from spleens were resuspended in staining buffer (PBS containing 3% fetal bovine serum) and stained for 30 min at 4 ˚C with FITC-conjugated anti-CD4 antibody (Lot 100,405; BioLegend) as well as their isotype control antibody (Lot 400,633; BioLegend) as a negative control. Cells were fixed and permeabilized using a fix/perm kit (Lot 88-8823-88; eBioscience) according to the manufacturer's instructions. Intracellular IFN-γ and IL-4 staining was performed on the cells stained with labeled PE-conjugated anti-IL-4 (Lot 504,103; BioLegend) and APC-conjugated anti-IFN-γ (Lot 505,809; BioLegend) antibodies, and their isotype control antibody (Lot 400,407; Lot 400,411; BioLegend) as a negative control. Flow cytometry was performed using a *BD Canto II* flow cytometer (BD Biosciences).

2.6. In vitro cytotoxicity assay

Lymphocytes from spleens (effector cells) were incubated with H22 HCC cells (target cells). Cytotoxicity was tested with a standard 4-h CytoTox 96 non-radioactive cytotoxicity assay (G1780; Promega, USA) with a 40:1 effector cell-to-target cell ratio according to the manufacturer's instructions.

2.7. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL). Differences in the number of cells at each time point for each treatment group were compared using pairwise comparisons from the mixed effects analysis of variance (ANOVA) model. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Serum INF-γ and IL-4 detected by ELISA

3.1.1. IFN-γ

Compared with group A, a significant increase in serum INF-γ levels of group B on day 7 (38.84 ± 3.612 pg/ml vs 30.89 ± 5.602 pg/ml $P < 0.05$), day 14 (45.26 ± 4.512 pg/ml vs 20.48 ± 2.340 pg/ml $P < 0.05$), and day 21 (33.39 ± 5.720 pg/ml vs 17.68 ± 3.831 pg/ml $P < 0.05$) was observed; cryoablation plus Freund's adjuvant enhanced this effect, which was also found in group C on day 7 (40.25 ± 3.541 pg/ml), day 14 (48.89 ± 4.631 pg/ml), and day 21 (50.59 ± 3.752 pg/ml) ($P < 0.05$). However, a significant difference between group B and group C ($P < 0.05$) was only observed on day 21. Compared with that observed for day 0 (26.35 ± 3.131 pg/ml) and day 7 (30.89 ± 5.602 pg/ml), the IFN-γ level of group A showed a significant decline on day 14 (20.48 ± 2.340 pg/ml)($P < 0.05$) and 21 (17.68 ± 3.831 pg/ml) ($P < 0.05$). In group B, the IFN-γ level showed a moderate increase from baseline (day 0: 26.56 ± 3.730 pg/ml, day 7: 38.84 ± 3.612 pg/ml, day 14: 45.26 ± 4.512 pg/ml, $P < 0.05$), but declined on day 21 (33.39 ± 5.720 pg/ml, $P < 0.05$). In group C, the IFN-γ level showed an obvious increase from the baseline (day 0: 25.79 ± 3.253 pg/ml, day 7: 40.25 ± 3.541 pg/ml, day 14: 48.89 ± 4.631 pg/ml, $P < 0.05$) and continued to increase on day 21 (50.59 ± 3.752 pg/ml) ($P < 0.05$) (Fig. 1a and b).

3.1.2. IL-4

Compared with group A, a significant decline in the serum IL-4 level of group B and group C was detected on day 7 (group A: 45.48 ± 3.163 pg/ml; group B: 32.89 ± 2.191 pg/ml; group C: 29.69 ± 2.752 pg/ml, $P < 0.05$), day 14 (group A: 52.54 ± 4.262 pg/ml; group B: 29.24 ± 3.572 pg/ml; group C: 26.57 ± 3.220 pg/ml, $P < 0.05$) and day 21 (group A: 56.72 ± 3.801 pg/ml; group B: 32.84 ± 4.063 pg/ml; group C: 25.87 ± 2.961, $P < 0.05$). Moreover, cryoablation plus Freund's adjuvant (group C) aggravated this effect on days 7, 14, and 21 ($P < 0.05$). A significant difference between group B and group C was found only on day 21(32.84 ± 4.063 pg/ml vs 25.87 ± 2.961 pg/ml, $P < 0.05$). Compared to that observed on day 0 (41.18 ± 3.010 pg/ml) and day 7 (45.48 ± 3.163 pg/ml), the IL-4 level showed a significant increase on day 14 (52.54 ± 4.262 pg/ml) ($P < 0.05$) and day 21 (56.72 ± 3.801 pg/ml) ($P < 0.05$) in group A. In group B and group C, a significant decline was observed on days 7, 14, and 21 ($P < 0.05$). On day 21 (32.84 ± 4.063 pg/ml), the IL-4 level in group B showed a slight recovery ($P > 0.05$), which was different from that observed for group C (See Fig. 2a and b).