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Heat shock protein 70/alpha-fetoprotein epitope peptide induced specific immunity against hepatocarcinoma

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Abstract. To study anti-tumor immunity mechanism induced by a reconstructed peptide complex-heat shock protein 70 (HSP70) and alpha-fetoprotein (AFP), Mice were immunized with AFP peptide, HSP70 and HSP70-AFP peptide complexes respectively. The killing effects of IFN- γ , TNF- α , perforin, granzyme B and CD8⁺ T cells on H22 cells were detected in each group of CD8⁺ T cell culture supernatants and investigate the immune effect of heat shock protein 70 (HSP70)-alpha-fetoprotein (AFP) epitope peptide complex on mouse H22 hepatoma cells.

1. Introduction

Liver cancer treatment mainly uses radiotherapy, chemotherapy, and surgery, but because of its large side effects, the survival rate is low after 3 years and does not achieve the desired effect[1]. In recent years, the research and development of tumor vaccines targeting AFP have attracted much attention. AFP is a glycoprotein of embryonic liver cells, highly expressed in embryonic stage and low expression after birth, when AFP expression is elevated after hepatocellular carcinogenesis[2], 70% of primary liver cancer patients may have high AFP expression. Studies have shown that alpha-fetoprotein has different epitopes and can provide a solution to the problem of hepatocellular carcinoma immunotherapy[3]. However, AFP can not be effectively presented in the patient's body to activate immune cells. Another convenient and effective method is the use of immune adjuvants. Heat shock protein 70 has a good "immunoadjuvant" effect. Especially for antigens with weak antigens or difficult to induce immunity, HSP70 exhibits a good intrinsic immune adjuvant effect[4]. In this experiment, AFP epitope peptide was combined with adjuvant HSP70 to form a recombinant vaccine to immunized mice[5]. The mechanism of specific cellular immunity against AFP tumors induced by recombinant vaccines in tumor-bearing mice was observed.



2. Material and methods

2.1 Animals and cell line. 40 6-8-week-old healthy C57BL/6 mice (females) with a body weight of (20 ± 2) g. Offered by the Experimental Animal Center of Medicine School of Xi'an Jiaotong University, license number: SCXK (Shaanxi) 2007-001; The mouse H22 liver cancer cell line was purchased from Laboratory of Immunology, Shaanxi University of Traditional Chinese Medicine. MTT, purified heat shock protein 70 and lymphocyte separation fluid (Sigma); fetal bovine serum and RPMI1640 culture fluid (Hyclone); ELISA kit and immunomagnetic beads MicroBead CD8⁺ T cell sorting resistance (Miltenyi Biotec); PHA (BD Biosciences); Shanghai Ziyu Biotechnology synthesizes AFP epitope peptide-FMNKFIYEI 9 amino acid oligopeptides, and the HSP70-AFP epitope peptide complex connects the carboxyl terminus of heat shock protein 70 with the amino terminus of AFP epitope peptide by amino acid coupling method. Lyophilized powder was purified by high performance liquid chromatography and stored at -20°C .

2.2 Cell Culture and Amplification. H22 hepatoma cells were inoculated into RPMI1640 culture medium containing 10% fetal bovine serum and double antibiotics (100 U/penicillin, 100 U/streptomycin) and cultured at 37°C 、5% CO₂ saturation humidity, and using trypan blue staining to detect cell viability.

2.3 Establishment of tumor model mice and in vivo tumor burden test. 40 mice were randomly divided into HSP70-AFP peptide vaccine group, HSP70 group, AFP group, and model group. The amplified H22 hepatocarcinoma cells were prepared into a cell suspension with a density of 2×10^5 /ml and inoculated into the right forefoot of the mice, which was 0.1 ml/body. After 3 days, the HSP70-AFP peptide vaccine, AFP, and HSP70 were diluted with physiological saline to 1 ug/ul and the amount of 0.1 ml/body inoculated into the tibia in the right upper quadrant of the mouse equivalents to 10 ug/body, and inject an equal volume of saline on the same side of the model group. After the first immunization, it was repeated once every 1w and repeated with the same method for 3 times. The survival and tumor growth of the mice were observed. The size of the solid tumor block was measured three times a week and the mean value was calculated. The volume of the tumor block was calculated according to the formula: $\frac{4}{3} \pi r^3$ (r=radius).

2.4 Isolation of mouse spleen mononuclear cells (PBMCs). All mice were sacrificed 3 days after the last immunization. The spleen was removed from the clean bench and rinsed twice with sterile saline. The pieces were chopped and ground with a sterile homogenizer. The filtrate was filtered through a sterile 200 mesh filter to obtain 1 ml of the filtrate (Ficoll-Hypaque Method) and separated PBMCs with lymphocyte separation fluid.

2.5 CD8⁺T Cell Sorting Purification - Immunomagnetic Bead Separation (MACS). Each group of PBMCs was counted, and the supernatant was discarded by centrifugation (1500 r/min 10 min). Each 10^7 cells were resuspended in 40ul buffer (PBS containing 0.5% EDTA, 0.5% bovine calf serum) and 10ul of CD8⁺ T cell biotin labeling was added to antibody, then mixed and incubated for 5 min at 4°C . Then 20ul avidin beads were added to the mixture and incubated at 4°C for 10 min. The separation column was placed on the sorter, the separation column was rinsed with 3 ml of sterile PBS buffer, and the cell suspension that had been labeled with magnetic beads was passed through the column; the

effluent unlabeled cells were collected and used. The column was further washed with 3 ml of PBS buffer and the flow-through was continued to collect, which contained the desired CD8⁺ T cells[6]. Flow cytometry was used to test the cell purification rate.

2.6 In vitro expansion of CD8⁺T cells. The purity of CD8⁺T cells was more than 90% detected by flow cytometry. The cells were stored in 10% fetal bovine serum RPMI1640 medium containing PHA 5ug/ml for 12 hours, and the cells were further expanded for 3 days at 37°C and 5% CO₂ saturation humidity. After centrifugation, supernatant cells were counted, and the cell density was adjusted to 5×10^6 /ml for use.

2.7 Detection of TNF- α , perforin, IFN- γ and granzyme levels in culture supernatants of mouse CD8⁺T lymphocytes (ELISA). The supernatant was centrifuged to test the TNF- α , perforin, IFN- γ and granzyme levels by referring to ELISA kits such as TNF- α and perforin.

2.8 Killing effect of CD8⁺ T lymphocytes on H22 hepatoma cells. After 72 h amplification, H22 hepatoma cells will be expanded to a density of 5×10^4 cells/ml and inoculated in 96-well cell culture plates at 100 ul/well. After 24 hours, the culture plate was centrifuged at 1000r/min for 10 minutes and the supernatant was discarded, ie target cells. In vitro expansion of cultured CD8⁺T cells was added as effector cells to the corresponding target cell wells (effector: target cells were 10:1, 20:1, 40:1, respectively), and 10ul of 5 mg/ml MTT solution was added to each well. Set up 3 replicates in each group and incubate at 37°C for 4 hours. Remove the culture medium, add DMSO 150ul/well, vibrate for 10 minutes at 37°C, fully dissolve the crystals; measure the optical density of each well (wavelength 490nm) with a microplate reader. The percentage of cell survival was calculated (average OD of experimental group/average of OD of control group \times 100%).

2.9 Statistical analysis. Using SPSS 10.0 package for statistical analysis. Using the Student-Newman-Keuls method and analysis of variance for comparison, $P < 0.05$ was considered statistically significant.

3. Results

3.1 Experimental results of tumor burden in mice in each group

After each group of mice was inoculated with H22 hepatoma cells, the survival and tumor growth of the mice were observed. It was found that the HSP70-AFP peptide vaccine group had better food intake, free movement, and better coat gloss than other groups. After 30 days, 8 deaths occurred in the blank groups; 6 deaths occurred in the AFP group; 7 deaths occurred in the HSP70 group; 3 died in the HSP70-AFP group, and the rest survived. It was demonstrated that the protective effect of the HSP70-AFP peptide vaccine group was dramatically greater ($P < 0.01$) than that of other groups. The volume of tumor in this group was obviously smaller ($P < 0.01$), as shown in Table 1.

Table 1. Comparison of tumor growth in various mice inoculated with hepatocellular carcinoma cell H22

Groups	n	No. of tumor-bearing/ No. of mice challenge	15 days after tumor challenge / Volumn size of tumor (mm ³)	30 days after tumor challenge / Volumn size of tumor (mm ³)
HSP70-AFP	10	3/10	35.01±5.48 ^{1,2}	46.21±4.65 ^{1,2}
HSP70	10	7/10	142.25±9.78 ³	839.53±12.27 ³
AFP	10	6/10	139.38±7.86 ³	836.41±9.61 ³
Empty	10	8/10	144.15±10.63	841.37±13.43

¹P<0.01; ²P<0.01, compared with AFP group; ³P>0.05.

3.2 Flow Cytometry Tests CD8⁺ T Cell Purity

The purity of the CD8⁺T-lymphocytes isolated from magnetic beads was as high as 90% by flow cytometry.

3.3 TNF- α , IFN- γ , perforin, and granzyme B levels in culture supernatants of mouse CD8⁺T lymphocytes

The levels of IFN- γ , TNF- α , perforin, and granzyme B in the culture supernatant of mouse CD8⁺T-lymphocytes in the HSP70-AFP peptide vaccine group were significantly higher than those in other groups, as shown in Table 2.

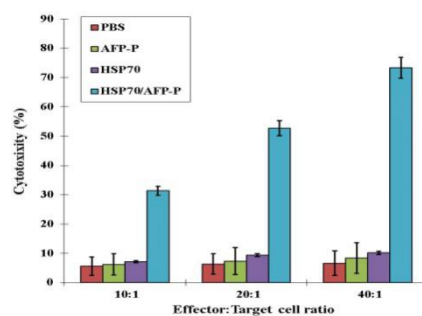
Table 2. Level of TNF- α , IFN- γ , perforin and granzyme B in various mice serum ($\bar{x} \pm s$, pg/ml)

Groups	HSP70-AFP	HSP70	AFP	Empty
TNF- α	465.32±13.15 ^{1,2}	153.26±7.59 ³	151.34±7.06 ³	143.61±8.24
perforin	223.52±13.17 ^{1,2}	116.09±8.17 ³	118.25±7.63 ³	105.39±8.25
IFN- γ	423.72±9.34 ^{1,2}	181.51±8.14 ³	187.61±8.79 ³	171.19±9.68
granzymeB	101.28±11.13 ^{1,2}	58.31±7.09 ³	60.39±7.38 ³	54.87±8.79

¹P<0.01; ²P<0.01, compared with AFP group; ³P>0.05.

3.4 Killing effect of CD8⁺T lymphocytes on target cells (H22)

The killing effect of mouse CD8⁺T cells in HSP70-AFP peptide vaccine group on H22 hepatocarcinoma cells in mice was higher than that in other groups (P < 0.01), as shown in Figure 1.

**Figure 1** The killing effect of CD8⁺T lymphocyte cells from different mice on H22 cells of hepatocellular carcinoma in mice The ratio of Effector/Target cell: 10:1,20:1,40:1

4. Discussion

Tumor immunotherapy has the characteristics of high efficiency, low side effects, etc., and can initiate or restart independent tumor immune response, while its own tissue cells are not damaged. AFP, as a related antigen of liver cancer, can promote the proliferation of hepatoma cells, and thus become a new target for liver cancer immunotherapy[7]. Because of the generation of AFP in the embryonic stage, the body has a certain degree of immune tolerance and cannot start a strong immune response[8].

The immunization of mice with the HSP70-AFP recombinant polypeptide vaccine constructed by the amino acid coupling method demonstrated that the recombinant vaccine has better immunity, which helps maintain the functional structure of the protein polypeptide, and maximizes the activity of the epitope peptide[9,10]. Direct coupling of epitope peptides to HSP70 promotes the presentation of antigens, which in turn activates effector cells to exert a tumoricidal effect. The results also confirmed that the HSP70-AFP epitope peptide vaccine constructed by the amino acid coupling method induced a stronger immune attack effect than the glutaraldehyde coupling method, and activated CD8⁺ T cells to release higher levels of cytokines[11]. The in vitro lymphocyte toxicity test further confirmed that the recombinant protein vaccine had a kill rate of 60% on mouse hepatoma cells. Lan[12] et al. reconstructed AFP and HSP70 at the gene level to construct eukaryotic expression vectors and performed in vivo experiments in mice to induce certain immune effects against AFP-expressing tumors. Compared to amino acid coupling methods, DNA vaccines constructed at the gene level are less expressed and the immune effects are relatively limited. In this study, a simple amino acid coupling method was used to construct a protein peptide vaccine. The direct coupling of HSP70 and AFP epitope peptide facilitates antigen presentation and activation of effector cells. In vitro and in vivo cytotoxicity experiments further confirmed that the amino acid coupling method for reconstitution of HSP70-AFP epitope peptide vaccine can more effectively induce specific CTL to attack liver cancer cells effectively[13].

In addition, in vivo tumor load experiments confirmed that the rate of tumor-bearing mice in the recombinant vaccine group was low, the survival time was longer, and the tumor volume was small. Through the detection of cytokines such as TNF- α and perforin in the culture supernatant of CD8⁺ T lymphocytes, it was found that all indicators in the recombinant vaccine group were significantly increased. Studies report that DC-activated AFP-specific T lymphocytes can up-regulate various cytokines, such as TNF- α , granzymes, and perforin, and have significant antitumor immunity in vitro and in vivo, which is supported by this study. The results of this study suggest that HSP70 can effectively enhance the immunogenicity of AFP, and after immunization of mice, a protective cellular immune response against AFP antigen-producing killer tumor cells is generated in vivo, and extending the survival time of tumor-bearing mice provides an important theoretical basis for the preclinical study of tumor vaccines.

Acknowledgments

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