Study on the Anti-tumor Mechanisms of Heat Shock Protein 72/alpha-fetoprotein Epitope Peptide Complex Against Hepatocellular Carcinoma Cell Hepa1-6

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Abstract Objective: Mice immunized with heat shock protein 72 (HSP72)–alpha-fetoprotein (AFP) epitope peptide complex were used to study whether the complex has specific anti-tumor immune effect against AFP tumor. Methods: Kunming mice were immunized subcutaneously with HSP72-AFP peptide complex, and mice were immunized with AFP polypeptide and HSP72, respectively. The serum IFN-γ levels in mice after immunization were detected by ELISA. The killing effect of lymphocytes on hepatic cancer Hepal-6 cells was detected by MTT assay. The immune effect of protein complex was evaluated by tumor burden test in mice. Results: The serum IFN-γ levels and the killing effect of lymphocytes on Hepal-6 cells in HSP72-AFP peptide complex group were significantly higher than those in AFP polypeptide and HSP72 group (P<0.01). The tumor volume in HSP72-AFP polypeptide complex group was also significantly smaller than that of the AFP polypeptide and HSP72 immunized groups (P<0.01). Conclusion: The HSP72-AFP polypeptide complex vaccine can induce tumor-specific mice to produce specific cellular immunity against AFP tumors, and its killing effect on tumor cells is significantly better than that of the single polypeptide purified vaccine. The surface HSP72-AFP polypeptide complex can induce effective anti-tumor immunity in mice.

1. Introduction

The traditional method of cancer treatment is radiotherapy and chemotherapy. Although it can effectively kill tumor cells, normal cells are also killed at the same time. Tumor immunotherapy is an ideal tumor therapy[1]. In particular, tumor vaccines based on tumor-associated antigens have received much attention. It produces specific responses to tumors and even removes residual tumor lesions[2]. Alpha-fetoprotein (AFP) is a tumor-associated antigen, and studies have confirmed that it can be used as a target protein for tumor therapy, but its application is limited due to its weak immunogenicity[3]. Heat shock proteins (HSP) are currently used as adjuvants to enhance their immunogenicity. HSP are widely distributed in prokaryotic and eukaryotic cells. They are a class of highly conserved stress protein involved in various biological functions in cells and participate in protein synthesis, processing, folding and transport by molecular chaperones[4]. HSP binds to a variety of peptides in the cell to induce a specific tumor immune response. For example, heat shock protein 72 together with tumor antigen peptide can induce tumor-specific active immunity[5]. In this experiment, mice were
immunized with HSP72 as an immunoadjuvant and AFP polypeptide recombinant protein complex to observe whether it can induce specific cell immunity against AFP tumors in tumor bearing mice.

2. Material and methods

2.1 Reagents and Instruments. IFN-\(\gamma\) quantitative sandwich ELISA kit was purchased from Gibco Company, MTT and lymphocyte separation solution from Sigma Company, RPMI 1640 culture medium and fetal bovine serum from Hyclone Company, and mouse AFP epitope peptide and HSP72 were provided by Gene Company. Super Clean Workbench (Suzhou Purification Equipment Company), Inverted Microscope (Auter BDS-200), Carbon Dioxide incubator (Germany BindCD-150), Electronic Precision Balance (Odoris), Automatic High Pressure Sterilizer (Japan Sanyo MLS-3780), RT-2100C Enzyme-Linked Immunoassay Instrument (Shenzhen Leishe Technology Co., Ltd.)

2.2 Animals and cell line. 48 6-8-week-old healthy BALB/C mice (females) with a body weight of \((20\pm2)\) g. were purchased from Chengdu Dashuo Experimental Animal Co., Ltd., license number: SCXK (Chuan) 2015-030; The mouse Hepa1-6 liver cancer cell line was offer by The Second Affiliated Hospital of Shaanxi University of Traditional Chinese Medicine, Translational Medicine Center.

2.3 Cell Culture and Amplification. HEPA1-6 hepatoma cells were inoculated into RPMI1640 culture medium containing 10% fetal bovine serum and 100 U/penicillin and 100 U/streptomycin antibodies and cultured at 37°C,5% CO2 saturation humidity, and using trypan blue staining to detect 95% cell viability.

2.4 Immunization of mice with HSP72-AFP polypeptide protein complex. 40 female Kunming mice were randomly divided into 4 groups: HSP72-AFP peptide complex group, AFP group, HSP72 group and blank group. Except for the blank group, the other different protein groups were diluted to 1 ug/ul with physiological saline, and injected into the left lower limb skin according to 0.1 ml/mouse, equivalent to 100 ug/dose. The blank group was injected with the same amount of normal saline on the same side. Mice were repeated once every 1 week after the first immunization for 3 consecutive times.

2.5 Detection of serum IFN-\(\gamma\) levels in mice by ELISA. One week after the last immunization, the eyeballs were bled and blood was collected from each group. The solution was centrifuged at 3000 r/min for 10 min to separate the serum. The levels of IFN-\(\gamma\) in each group were tested in strict accordance with the instructions of the IFN-\(\gamma\) kit.

2.6 Mouse lymphocyte preparation. After the above groups of mice were sacrificed, the spleen was aseptically taken and placed on a clean bench. Rinse and cut with saline solution, grind with sterile syringe core, 200 mesh sterile filter. Filter and collect the cell filtrate, centrifuge at 1000r / min for 5min, discard the supernatant and precipitate. Add 2ml red blood cell lysate to the cells, mix and let stand for 5min, discard the supernatant, and obtain. The sediment is mixed with lymphocytes and ready to use.

2.7 Killing effect of spleen lymphocytes on Hepa1-6 hepatoma cells. After 48 h amplification at 37°C, 5% CO 2 , and saturated humidity, hepah-6 hepatoma cells will be expanded to a density of 5x10^4 cells/ml and inoculated in 96-well cell culture plates at 100 ul/well. After 24 hours, the supernatant was discarded, ie target cells. The prepared control group, HSP72-AFP polypeptide complex immunization group, AFP group and HSP72 immunized group were added as effector cells to the corresponding target cell wells. (effector: target cells were 10:1, 20:1, 40:1, respectively), and 10ul of 5mg/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 150μl/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. Cell viability was calculated (Experimental group OD average / control group OD average×100%).

2.8 In vivo tumor burden test. Each group of the above immunized animals was inoculated with Hepal-6 cells to the left side of the forelimb of each group of mice 1 week after the last immunization. The trypsin-digested Hepal-6 cells were resuspended in 0.2 ml of PBS buffer by rinsing and counted under a microscope to prepare a monolayer cell suspension having a density of $2\times10^5$, which was inoculated into the left axilla of the forelimb of the mouse. Observing the growth of tumors in each group of mice. At the same time, the HSP72 group and the blank group were set as controls. Measure the size of the tumor 3 times a week, take mean. The tumor mass is calculated by the formula: $4/3\pi r^3$ ($r =$ radius)

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 statistically significant.

3. Results

3.1 Detection of serum INF-$\gamma$ levels in mice by ELISA. Compared with AFP group, HSP72 group and blank group, HSP72-AFP peptide complex Serum IFN-$\gamma$ levels were significantly increased in the mice of the compound group ($P < 0.01$, Table 1)

Table 1. Level of IFN-$\gamma$ in various mice serum ($\bar{x} \pm s$, pg/ml)

| Groups     | HSP72-AFP | HSP72 | AFP    | Empty     |
|------------|-----------|-------|--------|-----------|
| IFN-$\gamma$ | 442.75±12.32<sup>a,b</sup> | 179.57±7.69<sup>c</sup> | 286.37±8.25<sup>c</sup> | 146.47±7.16 |

<sup>a</sup> P<0.01 compared with empty group; <sup>b</sup>P<0.01 compared with AFP group; <sup>c</sup>P>0.05 compared with empty group.

3.2 Toxic effects of lymphocytes from different immunized mice on Hepal-6 cells. Comparison of AFP group, HSP72 group and blank group, the mouse spleen lymphocytes of HSP72-AFP peptide complex group induced a clear anti-tumor effect on mouse hepatoma cells Hepal-6 compared with other immunized groups ($P < 0.01$, Fig. 1)

![Figure 1. The effect of cytotoxicity of various mice splenic lymphocyte cells against mice hepatocellular carcinoma cell hepa1-6. The ratio of Effector/Target cell: 10: 1, 20: 1, 40: 1](image)

3.3 Tumor burden test in mice. Mices were inoculated with Hepal-6 liver cancer cells in HSP72-AFP peptide complex, HSP72, AFP immunization and blank group for tumor burden test. The protective effect of the HSP72-AFP peptide complex group was very significant compared to the other groups. The volume of the HSP72-AFP peptide protein complex group was dramatically smaller than that of the AFP group. And other groups ($P < 0.01$, Table 2)
#### Table 2. Comparison of tumor growth in various mice inoculated with hepatocellular carcinoma cell hepa1-6

| Groups     | n | No. of tumor bearing/15 days after tumor challenge / 30 days after tumor challenge | Tumor size (mm$^3$) |
|------------|---|-----------------------------------------------------------------------------------|---------------------|
|            |   | No. of mice challenge / 35.12±6.34$^{a,b}$ / 43.27±6.05$^{a,b}$                  |                     |
| HSP72-AFP  | 12| 4/12                                                                               | 35.12±6.34$^{a,b}$  |
| HSP72      | 12| 8/12                                                                               | 135.23±9.68$^c$     |
| AFP        | 12| 7/12                                                                               | 93.25±8.19$^c$      |
| Empty      | 12| 9/12                                                                               | 139.72±10.83        |

$^a$P<0.01 compared with empty group; $^b$P<0.01 compared with AFP group; $^c$P>0.05 compared with empty group.

4. Discussion:
Tumor vaccine can use tumor cells or tumor antigen substances to induce specific cellular and humoral immune responses in the body, which can enhance the body's anti-cancer ability. AFP embryonic antigen is highly expressed during embryonic period, and is lowly expressed after birth, but highly expressed in 80% of hepatocellular carcinoma[7]. AFP polypeptides can induce T cells to produce specific CTL effects[8]. Embryonic antigens that induce T cell responses have also been found in the mouse immune system, so AFP can be used as a target protein for immunotherapy. However, immunization of mice with AFP alone induced only low level specific response and weaker protective immunity. At present, AFP plasmid immunization, AFP-mediated dendritic cell immunization, and AFP plasmid-based immunization and AFP adenovirus-enhanced co-immunization are commonly used to solve this defect. The AFP plasmid has a poor immune effect, and the other several immune effects are better, but the application is limited due to complicated operation and complicated process[9].

We used HSP72 and AFP polypeptide recombinant protein complexes to immunize mice and induce stable immunoprotective responses in mice. Among them, HSP72 is an effective molecular chaperone, which can be combined with polypeptides or proteins including tumor antigens to extract HSP/P from tumor cells. After being injected into the body as a vaccine, it was ingested by antigen-presenting cells and combined with MHC. The antigen-presenting cells activate lymphocytes after maturation, and produce an anti-tumor effect[10,11]. The weaker tumor antigen is administered to the host APC to induce a specific T cell response and CTL effect. Studies have shown that the HSP70 and AFP protein complexes are constructed by the simple and convenient glutaraldehyde cross-linking method, and the immunized mice have obvious protective effects against AFP-expressing tumors which is consistent with the results of this study[12]. Sui Xiang et al[13]. Combined with IL-2 and heat shock protein/peptide vaccine for mouse sarcoma can make 30% of mice with tumor regression and long-term survival (average survival period is 22-26d, tumor growth inhibition rate is 65% -76%).
In this experiment, mice immunized with the recombinant protein complex of AFP polypeptide induced a strong anti-tumor effect compared with AFP protein and HSP72 alone. The results suggest that the immunogenicity of AFP is enhanced by HSP72, which fully shows that continuous immunization of HSP72-AFP polypeptide recombinant complex can produce effective AFP protective cell response in mice, and induce obvious anti-tumor effect.

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