Anti-tumor Immunity Induced by Cross-link Complex Alpha-fetoprotein and Glycoprotein 96

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Abstract

Purposes: To study the ability of gp96 to induce specific CTL response and its protective effect against AFP-producing tumor, a recombinant vaccine alpha-fetoprotein (AFP)-glycoprotein (gp96) complex was constructed. Material/Methods: A recombinant peptide vaccine was constructed by conjugating mouse alpha-fetoprotein to glycoprotein 96. By way of intracutaneous injection, mice were primed and boosted with recombinant vaccine mAFP/gp96, whereas single mAFP or gp96 injection as controls. The ELISPOT and ELISA were used to measure the frequency of cells producing the cytokine IFN-gama in splenocytes and the level of anti-AFP antibody in serum from immunized mice respectively. In vivo tumor challenge was carried out to assess the immune effect of the recombinant vaccine. Results: By recombinant mAFP/gp96 vaccine immunization, the number of splenic cells producing IFN-gama and the level of anti-AFP antibody in serum were significantly higher in mAFP/gp96 group than those in mAFP and gp96 groups (122.50±9.30 IFN-gama spots/106 cells vs 46.40±10.32 IFN-gama spots/106 cells, 12.14±7.33 IFN-gama spots/106 cells, P<0.01; 164.52±11.22 μg/mL vs 56.32±8.23 μg/mL, 7.56±3.47 μg/mL, P<0.01). The tumor volume in mAFP/gp96 group was significantly smaller than that in mAFP and gp96 groups (32.46±6.35 mm3 vs 384.16±11.43 mm3, 832.54±12.72 mm3, P< 0.01). Conclusions: The study further confirmed the function of glycoprotein 96’s immune adjuvant. Sequential immunization with recombinant mAFP/gp96 vaccine could generate effective specific antitumor immunity on AFP-producing tumor. The recombined mAFP/gp96 vaccine may be suitable for serving as an immunotherapy for hepatocellular carcinoma.

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Keywords: glycoprotein96 (gp96); alpha-fetoprotein (AFP); recombinant vaccine; immunity

1. Introduction

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The incidence of hepatocellular carcinoma (HCC) is increasing worldwide and accounts for as many as 1.2 million deaths annually. It is also rising rapidly in China because of hepatitis B and C infections [1,2]. Although surgery and liver transplantation are the effective therapy, most patients lost chance due to diagnosis at a late stage or underlying liver insufficiency in the setting of cirrhosis [2]. Novel therapies for HCC should be developed. A combined therapy is likely to prolong patients’ life and living quality.

Much attention is attracted to tumor active immunity which purpose is to induce hosts’ immune attack to tumor cells. 80% of HCC have a high expressing rate of alpha-fetoprotein (AFP), which could serve as a target for immunotherapy [3,4]. AFP is an oncofetal protein during HCC development which could generate weaker and less reproducible antitumor protection. How to enhance its immunity is an interesting issue. A recombinant vaccine approach may be a good method [5,6]. A number of groups have shown that superior levels of T-cell immunity could be generated using a heterogeneous prime-boost strategy, in which animals are primed and boosted with a plasmid vector encoding the stimulating molecule and targeted peptides [4-6]. In many of these vaccine models [7-9], glycoprotein 96 combined with certain antigen prime enhanced immunogenicity, presumably through processing and presenting the antigen to host antigen presenting cells (APCs). In the present study, we investigated whether the immunogenicity of AFP could be improved by presenting to APCs through gp96 molecules. We constructed a recombinant vaccine containing the molecule chaperon-gp96 and AFP protein. Then priming mice with the recombinant vaccine, we elicited robust strong protective immunity.

2. Materials and Methods

2.1. AFP, gp96 and conjugation

Mouse AFP was purchased from the GenWay Biotech, Inc. (San Diego, CA). Lyophilized material was resuspended in sterile distilled water at 10 mg/ml, aliquoted, and stored at 70°C until use. Balb/c mouse glycoprotein 96 (gp96) was expressed from a recombinant Escherichia coli K12 strain harbouring plasmid pUC119, and purified by gp96 polyclonal antibody- affinity chromatography.

One milligram each of AFP was coupled to 1 mg of gp96 in the presence of 0.2% glutaraldehyde for 2 h, and then dialysed against PBS overnight. Aliquots of each conjugate were stored at -70°C until use.

2.2. Mice and cell line

Balb/c mice were provided by Department of Experimental Animal Center at Xian Jiaotong University. The investigation was approved by the Ethics Committee on animal Study at Shaanxi University of Chinese Medicine (2004-4B). mAFP-producing H22 mice hepatocellular carcinoma cells were maintained in RPMI 1640 (life Technologies, Inc.) with 10% fetal bovine serum (Hyclone Technologies, Inc.). The supernatants were detected by AFP immunoradiation (Institute of Nuclear Sciences, Beijing) following the kit’s procedure.

2.3. Mice immunized with recombinant mAFP-gp96 complex

Fifty female Balb/c mice were divided into mAFP/gp96 group, mAFP group, gp96 group and empty group, PBS control group. Every group had 10 mice. Before injection, each group was diluted in saline to 100μg/100μl. Various groups were injected into the left anterior intracutaneous (i.e.) of mice. Priming and boosting was performed with 10μg mAFP or mAFP/gp96, whereas gp96 and PBS were used as controls. A 0.3 insulin syringe with a 25-gauge 0.5-inch-long needle was used for the i.c. injections. Mice were boosted i.c. with above proteins twice at 2 weeks intervals after the first priming.
2.4. ELISPOT and ELISA assay

The ELISPOT was used to measure the frequency of cells producing the cytokine IFN-γ in splenocytes harvested from immunized mice. Two weeks after last immunization, splenocytes were harvested and restimulated directly in anti-IFN-γ monoclonal antibody (PharMingen) coated ELISPOT plate wells in vitro with 5μg/ml of AFP containing 10% fetal bovine serum, 10 units/ml of human interleukin-2. The plates were incubated at 37°C for 24h. The plates were then washed and incubated with a biotin-conjugated secondary antibody and then developed. The color spots, representing cytokine producing cells, were counted under a dissecting microscope. To detect the level of anti-AFP antibody in mice, we examined the serum of mice tail vein after the last immunization by ELISA using alpha-fetoprotein ELISA kits (Biotinge Biomedicine Co, LTD., Beijing) following the manufacturer’s package insert procedure.

2.5. In vivo tumor challenge

Another fifty female Balb/c mice were grouped and immunized as above. Tumor challenge was performed 2 weeks after the last immunization with 1×10⁵ H22 cells in a single cell suspension per animal from tumors progressively growing in syngeneic mice. H22 tumor cells for challenge were washed after enzymatic digestion and resuspended in 0.2 ml of PBS per animal to be injected s.c. into the left flank, while empty and PBS were used as controls. The sizes of tumors were assessed three times a week using calipers. Tumor volume was approximated by the following calculation: 4/3 π r³ (r = radius).

2.6. Statistical analysis

Results were expressed as mean ± SD. The frequencies of IFN-γ-producing splenic cells were valued using χ² test. The Student t test was performed to analyze the significance of differences between final tumor volumes of different groups of animals. P<0.05 was considered statistically significant.

3. Results

3.1. Prime-boost vaccines generate AFP T-cells responses and anti-AFP antibody in Balb/c mice

Immunization of Balb/c mice with recombinant mAFP/gp96 vaccine elicited much more strong T-cells responses than mAFP group, whereas an i.c. vaccination with gp96 and PBS groups produced a small one (122.50±9.30 IFN-γ spots/10⁶ cells vs 46.40±10.32 IFN-γ spots/10⁶ cells, 12.14±7.33 IFN-γ spots/10⁶ cells, P<0.01). Recombinant mAFP/gp96 vaccine immunized mice also produced higher level of anti-AFP antibody than mAFP group, while gp96 and PBS groups produced a lower one (164.52±11.22 μg/mL vs 56.32±8.23 μg/mL, 7.56±3.47 μg/mL, P< 0.01) (Table 1).

Table 1 Spots of IFN-γ-producing splenic cells and level of anti-AFP antibody in mice
3.2. Boost immunization protects mice from in vivo tumor challenge

Balb/c mice were sequentially primed and boosted with mAFP/gp96, mAFP, gp96 and PBS respectively. Then the mice were challenged with H22 cells. Tumor sizes were significantly smaller in mAFP/gp96 immunized mice than in mAFP and gp96 immunized mice (32.46±6.35 mm³ vs 384.16±11.43 mm³, 832.54±12.72 mm³, P<0.01). Although mAFP immunized group produced an obvious tumor, it was still significantly bigger than mAFP/gp96 group (384.16±11.43 mm³ vs 32.46±6.35 mm³, P<0.01) (Table 2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of tumor-bearing/No. of mice challenge</th>
<th>10 days after tumor challenge /Size of tumor (mm³)</th>
<th>20 days after tumor challenge /Size of tumor (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAFP/gp96</td>
<td>1/10</td>
<td>25.21±5.36 a</td>
<td>32.46±6.35 a</td>
</tr>
<tr>
<td>mAFP</td>
<td>8/10</td>
<td>78.34±8.62 a</td>
<td>384.16±11.43</td>
</tr>
<tr>
<td>gp96</td>
<td>10/10</td>
<td>137.32±10.63</td>
<td>832.54±12.72</td>
</tr>
<tr>
<td>Empty</td>
<td>10/10</td>
<td>141.53±11.42</td>
<td>838.36±12.47</td>
</tr>
<tr>
<td>PBS</td>
<td>10/10</td>
<td>142.75±12.13</td>
<td>840.22±13.14</td>
</tr>
</tbody>
</table>

Table 2 Comparision of tumor growth in mice injected with H22 hepatocellular carcinoma cells

4. Discussion

Recent studies on the immunodominant epitopes of AFP have provided a solution to the obstacle of HCC immunotherapy. AFP is produced at low serum levels after birth throughout life [1,2]. The majority of human HCC overexpress the oncofetal antigen AFP, Mr 69 000 glycoprotein. Despite being exposed to high plasma levels of this oncofetal protein during embryonic development, body has a low immunity to it [1,2]. Butterfield et al [10,11] recently found that four peptides of human AFP processed and presented in the context of HLA-A0201, could be recognized by the human T cell repertoire, and could be used to generate AFP-specific CTL in human T cell cultures. It was also found that murine immune system could generate T-cell responses to this oncofetal antigen. Therefore, it may be a better target for immunotherapy. But AFP immunization alone still results in lower levels of specific response and poorly reproducible protective immunity [4-6].

How to enhance host’s active immunity to AFP may be an interesting strategy for HCC therapy. Previous studies on AFP specific immunotherapy for HCC included AFP plasmid immunization, AFP-transduced dendritic cells (DCs) immunization and AFP plasmid prime-AFP adenovirus boost immunization [10-14]. AFP plasmid immunization produced detectable but low levels of AFP specific T cell responses and poorly reproducible protective immunity [10,11]. Additional enhancement of the T-cell stimulatory effect is DCs engineered to express murine AFP demonstrated a powerful ability to generate
tumor-specific immune responses [12,13]. However, the need for costly cell culture procedures limited their wide availability for clinical use, and the unstable culture technique might yield tolerating vaccine [12]. AFP plasmid prime-AFP adenovirus boost immunization could engender significant AFP specific T-cell responses and protective immunity in mice [13,14]. But the miscellaneous procedures precluded their use. In the present study, we tested a novel strategy to induce antitumor immunity by a recombinant vaccine conjugation AFP to gp96 in mice. We found the vaccine could elicit strong AFP-specific T-cell responses and produced a distinctive protective effect on AFP-producing tumor, compared with other immunized groups. We should point out that the single vaccine mAFP also produced a definite antitumor immunity, but the effect was not sufficient and satisfactory than that of recombinant vaccine mAFP/gp96. It is of interest to note that the recombinant vaccine provoked not only the considerable stability of immunoprotective, but also a detectable level of anti-AFP antibody, although humoral immunity alone had a minor limited effect on antitumor.

In the study, we attributed the successful alpha-fetoprotein specific T-cell responses in mice to the gp96 molecule by mediating APCs to efficient uptake and process of AFP. Numerous investigations had shown that gp96 itself had no antigenicity and its immunogenecity has been attributed to the peptides chaperoned carried by itself [7-9]. It has been verified that gp96 was a better molecule chaperon and adjuvant which could process and present weak tumor antigen to MHC-I of host APCs, eliciting specific T-cell response and CTL reaction [8,9]. Several studies have shown that gp96-associated peptides could anchor antigen on the cell membrane and directly present it to nature killer cells or γδ T cells as superantigen without being dependent on the stimulation of MHC-I molecules [15-17]. In the present study, tumor rejection assay demonstrated that recombinant DNA vaccine AFP/gp96 elicited strong specific antitumor immunity against AFP-producing H22 cells than AFP DNA vaccine. Results indicated that AFP immunogenicity can be improved greatly by gp96 molecule and vaccination with DNA encoding gp96 could increase both humoral and T-cell proliferation responses to alpha-fetoprotein. We attributed the successful alpha-fetoprotein specific T-cell responses in mice to the gp96 molecule by mediating APCs to efficient uptake and process of AFP.

In summary, the result suggests that sequential immunization with a recombinant vaccine conjugating alpha-fetoprotein to glycoprotein 96 could generate effective AFP-specific T cell responses and induce definite antitumor effect on AFP-producing tumor, which may be suitable for some clinical testing as a vaccine for HCC.

Acknowledgements

The work was supported by the Research Program of Shaanxi Education Committee (No. 2010JK484) and the national natural science foundation of china (No. 81172135).

References


