Antitumor immunity induced by DNA vaccine encoding alpha-fetoprotein/heat shock protein 70

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INTRODUCTION

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[12]. 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[3]. Novel therapies for HCC should be developed. A combined therapy is likely to prolong patients’ life and living quality.

Much attention has been paid to the induction of host immunity to tumor cells. 80% of HCCs have a high expression of alpha-fetoprotein (AFP), which could serve as a target for immunotherapy[4-8]. AFP is an oncofetal protein during HCC development, which could generate weaker and less reproducible antitumor protection. A DNA-based vaccine may be a good method for enhancing host immunity[9-12]. A number of groups have shown that high levels of T-cell immunity could be generated using a heterogeneous prime-boost protocol, in which animals were primed and boosted with a plasmid vector encoding the stimulating molecules and targeted peptides[8-10]. In many of these vaccine models[11-13], heat shock protein 70 could combine with certain antigen prime enhanced immunogenicity, presumably through processing and presenting the antigen to host APCs. In the present study, we investigated whether the immunogenicity of AFP could be improved by presenting to APCs through HSP70 molecules. We constructed an eukaryotic expression vector containing the molecular chaperon-HSP70 and AFP fragments. Then priming mice with the genetic vaccine, we elicited robust strong protective immunity.

MATERIALS AND METHODS

Mice and cell line

Balb/c mice were provided by Department of Experimental Animal Center at Capital University of Medical Sciences. SP2 mouse myeloma cells were maintained in RPMI 1640 (life Technologies, Inc.) supplemented with 100 mL/L fetal bovine serum (HyClone Technologies, Inc.). The cells were transduced with pBBS212-hAFP or pBBS212-hAFP/HSP70 through calcium phosphate precipitation (Promega Technologies, Inc.). Positive cell clones were screened by conditioned medium and supernatants were detected by AFP radioimmunoassay (Institute of Nuclear Sciences, Beijing) following the manufacturer’s instructions.

Construction of recombinant expression vector

RT-PCR primers were designed to contain the partial hAFP coding region, including the signal sequence. The upper primers were 5’-CCGCTCCAGATGAGGTTGGAATCAA-3’, while the down primers were 5’-CGCGGATCCCTATGAGTGGGCCGTTTTTGATGTTG-3’. RT-PCR template total RNA was isolated from HepG2 hepatocarcinoma cells by TRizol (Life Technologies, Inc.) reagent. Then the 400-bp hAFP cDNA PCR products were cloned into the pBBS212 empty vector and pBBS212-HSP70 eukaryotic expression vector (provided by Dr. Ye L of Zhongshan Medical University, Guangzhou, China). pBBS212-hAFP/HSP70 and pBBS212-hAFP were constructed using the pBBS212 herpes simplex virus expressing vector, in which the backbone
contained the hygromycin resistance gene, being suitable for screening cell clones. The recombinant vectors were identified by restriction enzyme analysis and sequencing. Different plasmid and recombinant expressing vectors were stored at -80°C for intramuscular immunization[10].

Mice immunized with recombinant expression vector
Forty female Balb/c mice were divided into rhAFP/HSP70 group, rhAFP group, HSP70 group and empty vector group, PBS group. Each group had 8 mice. Before injection, plasmid and recombinant expressing vectors were diluted in saline to 1 g/L. Various plasmids were injected into the left anterior tibialis muscle of mice. Priming and boosting with plasmid were performed with 100 µg rhAFP or rhAFP/HSP70 vector, whereas pBBS212-HSP70 and empty vectors were used as controls. A 25-gauge, 0.5-inch insulin syringe was used for intramuscular injection. Mice were intramuscularly boosted with above plasmids twice at intervals of two weeks after the first priming.

ELISPOT and ELISA assay
IFN-γ ELISPOT assay was used to measure the frequency of cells producing cytokine IFN-γ in splenocytes harvested from immunized mice. Two weeks after the last immunization, splenocytes were harvested and restimulated directly in anti-IFN-γ monoclonal antibody (Pharmingen) coated ELISPOT plate wells in vitro with 5 µg/mL of AFP containing 100 mL/L fetal bovine serum, 10 U/mL of human interleukin-2. The plates were incubated at 37°C for 24 h, then washed and incubated with a biotin-conjugated secondary antibody and 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 AFP ELISA kit (Biotinge Biomedicine Co, LTD. Beijing) following the manufacturer’s instructions.

In vivo tumor load
Another 40 female Balb/c mice were grouped and immunized as above. Tumor challenge was performed 2 wk after the last immunization with 1×10⁵ AFP-transfected SP2/0 cells. SP2/0 AFP-transduced tumor cells for challenge were washed after enzymatic digestion and resuspended in 0.2 mL PBS per animal. Another 40 female Balb/c mice were grouped and immunized as above. Tumor challenge was performed 2 wk after the last immunization by ELISA using AFP ELISA kit (Biotinge Biomedicine Co, LTD. Beijing) following the manufacturer’s instructions.

Comparison of tumor growth in mice injected with hAFP-transduced SP2/0 tumor cells (mean±SD)

Table 1 Spots of IFN-γ-producing splenic cells and level of anti-AFP antibody in mice (mean±SD)

| Group                        | hAFP/HSP70 | hAFP | HSP70 | Empty | PBS   |
|------------------------------|------------|------|-------|-------|-------|
| Spots (10⁶ cells)            | 95.3±10.9⁸ | 23.6±11.8⁷ | 9.25±5.4⁴ | 7.17±4.2⁴ | 5.54±2.1⁶ |
| Anti-AFP (µg/mL)             | 126.5±8.2² | 51.7±3.4⁰ | 6.26±4.2⁷ | 5.83±3.7⁹ | 3.42±2.3⁵ |

P<0.01, vs empty group; P<0.01, vs HSP70 group; P<0.01, vs empty group.

Table 2 Comparison of tumor growth in mice injected with hAFP-transduced SP2/0 tumor cells (mean±SD)

| Group                        | No. of tumor-bearing/No. of mice challenge | 10 d after tumor challenge/Size of tumor (mm³) | 20 d after tumor challenge/Size of tumor (mm³) |
|------------------------------|------------------------------------------|---------------------------------------------|---------------------------------------------|
| hAFP/HSP70                   | 2/8                                      | 24.4±5.6±10⁴                                | 37.4±7.3±10⁴                                |
| hAFP                         | 5/8                                      | 73.6±4.8±53                                | 381.1±15.4±53                               |
| HSP70                        | 8/8                                      | 118.2±14.65                                | 785.8±13.87                                 |
| Empty                        | 8/8                                      | 132.2±16.27                                | 817.5±16.25                                 |
| PBS                          | 8/8                                      | 149.7±16.54                                | 860.5±14.72                                 |

P<0.01, vs empty group; P<0.01, vs HSP70 group; P<0.01, vs empty group.

Statistical analysis
Results were expressed as mean±SD. The frequency of IFN-γ-producing splenic cells were valued using χ² test. The Student’s t test was performed to analyze the significance of differences between the final tumor volumes of different groups. P<0.05 was considered statistically significant.

RESULTS
Prime-boost vaccines induced T-cell responses and anti-AFP antibody in Balb/c mice
Immunization of Balb/c mice with recombinant hAFP/HSP70 vector elicited much more strong T-cell responses than rhAFP group (95.50±10.90 IFN-γ spots/10⁶ cells vs 23.60±11.80 IFN-γ spots/10⁶ cells, P<0.01), whereas an intramuscular vaccination with plasmid-HSP70 and empty plasmid produced a weak response (95.50±10.90 IFN-γ spots/10⁶ cells vs 9.25±5.44 IFN-γ spots/10⁶ cells, 7.17±4.24 IFN-γ spots/10⁶ cells, P<0.01). Recombinant hAFP/HSP70 immunized mice also produced a higher level of anti-AFP antibody than rhAFP group (126.5±8.22 µg/mL vs 51.72±3.40 µg/mL, P<0.01), while plasmid-HSP70 and empty plasmid produced a lower level (126.5±8.22 µg/mL vs 6.26±4.27 µg/mL, 5.83±3.79 µg/mL, P<0.01) (Table 1). Boost immunization protected mice from in vivo tumor challenge
Balb/c mice were primed and boosted with rhAFP/HSP70, rhAFP, HSP70 and empty plasmid. The mice were challenged with SP2/0 cells, which were transduced with hAFP. Tumor sizes were significantly smaller in rhAFP/HSP70-immunized mice than in HSP70 and empty plasmid immunized mice (37.41±7.34 mm³ vs 785.8±13.87 mm³, 817.5±16.25 mm³, P<0.01). Although rhAFP immunized group produced an obvious tumor, it was still significantly bigger than rhAFP/HSP70 group (37.41±7.34 mm³ vs 381.1±15.48 mm³, P<0.01) (Table 2).

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[23-5]. The majority of human HCCs could overexpress the oncofetal antigen AFP, M, 70,000 glycoprotein[4-5]. Despite being exposed to high plasma levels of this oncofetal protein during embryonic development, the body has a low immunity to it[6]. Butterfield et al.[17-18] recently found that four peptides of human AFP processed and presented in the context of HLA-A0201, could
be recognized by 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[8]. Therefore, it may be a better target
for immunotherapy. But AFP immunization alone still resulted
in lower levels of specific response and poorly reproducible
protective immunity[3-7].

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 DCs immunization and AFP
plasmid prime-AFP adeno-virus boost immunization[19-22]. AFP
plasmid immunization produced detectable but low levels of
AFP specific T cell responses and poorly reproducible protective
immunity[7,20]. DCs engineered to express murine AFP demonstrated
a powerful ability to generate tumor-specific immune response[20].
However, the need for costly cell culture procedures limited
their wide availability for clinical use, and the unstable culture
technique might yield tolerating vaccines[19,21]. AFP plasmid
prime-AFP adeno-virus boost immunization could engender
significant AFP-specific T-cell responses in mice[19]. But the miscellaneous procedures precluded their
use. In the present study, we tested a novel strategy to induce
antitumor immunity by a DNA vaccine encoding both AFP and
HSP70 in mice. We found that the vaccine could elicit strong
AFP-specific T-cell responses and produce a distinctively
protective effect on AFP-expressing tumors compared with other
immunized groups. We should point out that the DNA vaccine
hAFP also produced a definite antitumor immunity, but the
effect was not sufficient and satisfactory in comparison with
that of recombinant vaccine AFP/HSP70. It is of interest to
note that recombinant DNA vaccines provoked not only the
considerable stability of immunoprotection, but also a detectable
level of anti-AFP antibody, although humoral immunity alone
had a minor effect on antitumor activity[21,24].

In the study, we attributed the successful AFP specific T-
cell responses in mice to the HSP70 molecules by mediating
APCs to efficiently uptake and process of AFP. A number of
investigations have shown that HSP70 itself has no antigenicity
and its immunogenicity can be attributed to the peptide
chaperones carried by itself[18-20]. It has been verified that HSP70
is a better molecular chaperone and adjuvant, which could
process and present weak tumor antigens to MHC-I of host
APCs, eliciting specific T-cell responses and CTL reactions[18-20].
Suzue et al.[20] using a recombinant heat shock fusion protein
containing a large fragment of ovalbumin linked to HSP70
injected without adjuvants into Balb/c mice, CTLs were produced
that recognized an ovalbumin-derived peptide and the mice
were also protected against challenge with ovalbumin-expressing
melanoma tumor cells. Several studies have shown that HSP70-
associated peptides could anchor antigens on the cell membrane
and directly present them to nature killer cells or γδ T cells as
superantigens without dependence on the stimulation of
MHC-I molecules[19-22]. In this experiment, tumor rejection assay
demonstrated that recombinant vaccine AFP/HSP70 elicited
strong specific antitumor immunity against AFP-producing SP2/O
cells than AFP DNA vaccine. The results indicated that AFP
immunogenicity was greatly improved by HSP70 molecules and
vaccination with DNA encoding HSP70 could increase both
humoral and T-cell proliferation responses to AFP.

In summary, sequential immunization with a recombinant
DNA vaccine encoding AFP and heat shock protein70 could
generate effective AFP-specific T cell responses and induce
definite antitumor effects on AFP-producing tumors, which may be
suitable for some clinical testing as a vaccine for HCC.

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