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World J Gastroenterol 2004;10(21):3197-3200
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

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

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Received: 2004-03-18 **Accepted:** 2004-04-07

Abstract

AIM: To construct a DNA vaccine encoding human alpha-fetoprotein (hAFP)/heat shock protein 70 (HSP70), and to study its ability to induce specific CTL response and its protective effect against AFP-expressing tumor.

METHODS: A DNA vaccine was constructed by combining hAFP gene with HSP70 gene. SP2/0 cells were stably transfected with pBBS212-hAFP and pBBS212-hAFP/HSP70 eukaryotic expression vectors. Mice were primed and boosted with DNA vaccine hAFP/HSP70 by intramuscular injection, whereas plasmid with hAFP or HSP70 was used as controls. ELISPOT and ELISA were used to detect IFN-γ-producing splenocytes and the level of serum anti-AFP antibody from immunized mice respectively. *In vivo tumor challenge* was measured to assess the immune effect of the DNA vaccine.

RESULTS: By DNA vaccine immunization, the results of ELISPOT and ELISA showed that the number of IFN-γ-producing splenocytes and the level of serum anti-AFP antibody were significantly higher in rhAFP/HSP70 group than in hAFP and empty plasmid groups (95.50±10.90 IFN-γ spots/10^6 cells *vs* 23.60±11.80 IFN-γ spots/10^6 cells, 7.17±4.24 IFN-γ spots/10^6 cells, $P<0.01$; 126.50±8.22 μg/mL *vs* 51.72±3.40 μg/mL, 5.83±3.79 μg/mL, $P<0.01$). The tumor volume in rhAFP/HSP70 group was significantly smaller than that in pBBS212-hAFP and empty plasmid groups (37.41±7.34 mm^3 *vs* 381.13±15.48 mm^3, 817.51±16.25 mm^3, $P<0.01$).

CONCLUSION: 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.

Wang XP, Liu GZ, Song AL, Li HY, Liu Y. Antitumor immunity induced by DNA vaccine encoding alpha-fetoprotein/heat shock protein 70. *World J Gastroenterol* 2004; 10(21): 3197-3200
http://www.wjgnet.com/1007-9327/10/3197.asp

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[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[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[11-15], 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 a 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. SP2/0 mice 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 phosphorate 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 were5'-CCGCTCGAGATGAAGTGGGTGGAATCAA-3', while the down primers were 5'-CGCGGATCCTTATGGAGTGGGCTTTTTGTGTG-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 ℃ for intramuscular immunization[16].

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 ℃ 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 kits (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 1×10^5 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 to be injected *s.c.* into the left flank, while empty plasmid and PBS were used as controls. The sizes of tumors were assessed 3 times a week using calipers. Tumor volume was approximated by the following calculation: $4/3\ \pi\ r^3$ (r = radius).

Statistical analysis

Results were expressed as mean±SD. The frequency of IFN-γ-producing splenic cells were valued using χ^2 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/10^6 cells *vs* 23.60±11.80 IFN-γ spots/10^6 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/10^6 cells *vs* 9.25±5.44 IFN-γ spots/10^6 cells, 7.17±4.24 IFN-γ spots/10^6 cells, $P<0.01$). Recombinant hAFP/HSP70 immunized mice also produced a higher level of anti-AFP antibody than rhAFP group (126.50±8.22 μg/mL *vs* 51.72±3.40 μg/mL, $P<0.01$), while plasmid-HSP70 and empty plasmid produced a lower level (126.50±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 mm^3 *vs* 785.83±13.87 mm^3, 817.51±16.25 mm^3, $P<0.01$). Although rhAFP immunized group produced an obvious tumor, it was still significantly bigger than rhAFP/HSP70 group (37.41±7.34 mm^3 *vs* 381.13±15.48 mm^3, $P<0.01$) (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[2-5]. The majority of human HCCs could overexpress the oncofetal antigen AFP, M_r 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[3]. Butterfield *et al.*[17-19] recently found that four peptides of human AFP processed and presented in the context of HLA-A0201, could

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 (10^6 cells)	95.50±10.90[bd]	23.60±11.80[f]	9.25±5.44	7.17±4.24	5.54±2.16
Anti-AFP (μg/mL)	126.50±8.22[bd]	51.72±3.40[f]	6.26±4.27	5.83±3.79	3.42±2.35

[b]$P<0.01$, *vs* empty group; [d]$P<0.01$, *vs* HSP70 group; [f]$P<0.01$, *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 (mm^3)	20 d after tumor challenge/ Size of tumor (mm^3)
hAFP/HSP70	2/8	24.43±6.10[bd]	37.41±7.34[bd]
hAFP	5/8	73.64±8.53[f]	381.13±15.48[f]
HSP70	8/8	118.24±14.65	785.83±13.87
Empty	8/8	132.26±17.27	817.51±16.25
PBS	8/8	149.73±16.54	860.53±14.72

[b]$P<0.01$, *vs* empty group; [d]$P<0.01$, *vs* HSP70 group; [f]$P<0.01$, *vs* empty group.

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 adenovirus boost immunization[20-22]. 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 responses[21]. However, the need for costly cell culture procedures limited their wide availability for clinical use, and the unstable culture technique might yield tolerating vaccines[8,21]. AFP plasmid prime-AFP adenovirus boost immunization could engender significant AFP specific T-cell responses and protective immunity in mice[22]. 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[23,24].

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[25-29]. 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[26-28]. Suzue *et al*.[29] 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[30-32]. In this experiment, tumor rejection assay demonstrated that recombinant vaccine AFP/HSP70 elicited strong specific antitumor immunity against AFP-producing SP2/0 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.

REFERENCES

1 **Schafer DF,** Sorrell MF. Hepatocellular carcinoma. *Lancet* 1999; **353:** 1253-1257

2 **Qin LX,** Tang ZY. Hepatocellular carcinoma with obstructive jaundice: diagnosis, treatment and prognosis. *World J Gastroenterol* 2003; **9:** 385-391

3 **Tang ZY.** Hepatocellular carcinoma-Cause, treatment and metastasis. *World J Gastroenterol* 2001; **7:** 445-454

4 **Guo J,** Cai M, Wei D, Qin L, Huang J, Wang X. Immune responses of dendritic cells after loaded with cytotoxicity T lymphocyte epitope based peptide of human alpha-fetoprotein (hAFP). *Zhonghua Ganzangbing Zazhi* 2002; **10:** 178-180

5 **Grimm CF**, Ortmann D, Mohr L, Michalak S, Krohne TU, Meckel S, Eisele S, Encke J, Blum HE, Geissler M. Mouse alpha-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. *Gastroenterology* 2000; **119:** 1104-1112

6 **Hanke P**, Rabe C, Serwe M, Bohm S, Pagenstecher C, Sauerbruch T, Caselmann WH. Cirrhotic patients with or without hepatocellular carcinoma harbour AFP-specific T-lymphocytes that can be activated *in vitro* by human alpha-fetoprotein. *Scand J Gastroenterol* 2002; **37:** 949-955

7 **Hanke P**, Serwe M, Dombrowski F, Sauerbruch T, Caselmann WH. DNA vaccination with AFP-encoding plasmid DNA prevents growth of subcutaneous AFP-expressing tumors and does not interfere with liver regeneration in mice. *Cancer Gene Ther* 2002; **9:** 346-355

8 **Saeki A**, Nakao K, Nagayama Y, Yanagi K, Matsumoto K, Hayashi T, Ishikawa H, Hamasaki K, Ishii N, Eguchi K. Diverse efficacy of vaccination therapy using the alpha-fetoprotein gene against mouse hepatocellular carcinoma. *Int J Mol Med* 2004; **13:** 111-116

9 **Pancholi P,** Liu Q, Tricoche N, Zhang P, Perkus ME, Prince AM. DNA prime-canarypox boost with polycistronic hepatitis C virus (HCV) genes generates potent immune responses to HCV structural and nonstructural proteins. *J Infect Dis* 2000; **182:** 18-27

10 **Kumar V**, Sercarz E. Genetic vaccination: the advantages of going naked. *Nat Med* 1996; **2:** 857-859

11 **Leitner WW**, Ying H, Restifo NP. DNA and RNA-based vaccines: principles, progress and prospects. *Vaccine* 1999; **18:** 765-777

12 **Moelling K**. DNA for genetic vaccination and therapy. *Cytokines Cell Mol Ther* 1997; **3:** 127-135

13 **Srivastava PK**, Udono H. Heat shock protein-peptide complexes in cancer immunotherapy. *Curr Opin Immunol* 1994; **6:** 728-732

14 **Huang XF**, Ren W, Rollins L, Pittman P, Shah M, Shen L, Gu Q, Strube R, Hu F, Chen SY. A broadly applicable, personalized heat shock protein-mediated oncolytic tumor vaccine. *Cancer Res* 2003; **63:** 7321-7329

15 **Casey DG**, Lysaght J, James T, Bateman A, Melcher AA, Todryk SM. Heat shock protein derived from a non-autologous tumour can be used as an anti-tumour vaccine. *Immunology* 2003; **110:** 105-111

16 **Wang XP**, Chen RF, Song AL, Liu YF. Construction and identification of pBBS212-AFP / HSP70 eukaryotic expression vector. *Zhongliu Yanjiu Yu Linchuang* 2002; **14:** 363-365

17 **Butterfield LH**, Koh A, Meng W, Vollmer CM, Ribas A, Dissette V, Lee E, Glaspy JA, McBride WH, Economou JS. Generation of human T-cell responses to an HLA-A2.1-restricted peptide epitope derived from alpha-fetoprotein. *Cancer Res* 1999; **59:** 3134-3142

18 **Meng WS**, Butterfield LH, Ribas A, Heller JB, Dissette VB, Glaspy JA, McBride WH, Economou JS. Fine specificity analysis of an HLA-A2.1-restricted immunodominant T cell epitope derived from human alpha-fetoprotein. *Mol Immunol* 2000; **37:** 943-950

19 **Butterfield LH**, Meng WS, Koh A, Vollmer CM, Ribas A, Dissette VB, Faull K, Glaspy JA, McBride WH, Economou JS. T cell responses to HLA-A*0201-restricted peptides derived from human alpha fetoprotein. *J Immunol* 2001; **166:** 5300-5308

20 **Vollmer CM Jr**, Eilber FC, Butterfield LH, Ribas A, Dissette VB, Koh A, Montejo LD, Lee MC, Andrews KJ, McBride WH, Glaspy JA, Economou JS. Alpha-fetoprotein-specific genetic immunotherapy for hepatocellular carcinoma. *Cancer Res* 1999; **59:** 3064-3067

21 **Banchereau J**, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392:** 245-252
22 **Meng WS**, Butterfield LH, Ribas A, Dissette VB, Heller JB, Miranda GA, Glaspy JA, McBride WH, Economou JS. alpha-Fetoprotein-specific tumor immunity induced by plasmid prime-adenovirus boost genetic vaccination. *Cancer Res* 2001; **61:** 8782-8786
23 **Le Poole IC,** Gerberi MA, Kast WM. Emerging strategies in tumor vaccines. *Curr Opin Oncol* 2002; **14:** 641-648
24 **Reilly RT,** Emens LA, Jaffee EM. Humoral and cellular immune responses: independent forces or collaborators in the fight against cancer? *Curr Opin Investig Drugs* 2001; **2:** 133-135
25 **Milani V,** Noessner E, Ghose S, Kuppner M, Ahrens B, Scharner A, Gastpar R, Issels RD. Heat shock protein 70: role in antigen presentation and immune stimulation. *Int J Hyperthermia* 2002; **18:** 563-575
26 **Harmala LA,** Ingulli EG, Curtsinger JM, Lucido MM, Schmidt CS, Weigel BJ, Blazar BR, Mescher MF, Pennell CA. The adjuvant effects of Mycobacterium tuberculosis heat shock protein 70 result from the rapid and prolonged activation of antigen-specific CD8+ T cells *in vivo*. *J Immunol* 2002; **169:** 5622-5629
27 **Noessner E,** Gastpar R, Milani V, Brandl A, Hutzler PJ, Kuppner MC, Roos M, Kremmer E, Asea A, Calderwood SK, Issels RD. Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. *J Immunol* 2002; **169:** 5424-5432
28 **Feng H,** Zeng Y, Graner MW, Likhacheva A, Katsanis E. Exogenous stress proteins enhance the immunogenicity of apoptotic tumor cells and stimulate antitumor immunity. *Blood* 2003; **101:** 245-252
29 **Suzue K,** Zhou X, Eisen HN, Young RA. Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway. *Proc Natl Acad Sci U S A* 1997; **94:** 13146-13151
30 **Wei Y**, Zhao X, Kariya Y, Fukata H, Teshigawara K, Uchida A. Induction of autologous tumor killing by heat treatment of fresh human tumor cells: involvement of gamma delta T cells and heat shock protein 70. *Cancer Res* 1996; **56:** 1104-1110
31 **Dressel R**, Grzeszik C, Kreiss M, Lindemann D, Herrmann T, Walter L, Gunther E. Differential effect of acute and permanent heat shock protein 70 overexpression in tumor cells on lysability by cytotoxic T lymphocytes. *Cancer Res* 2003; **63:** 8212-8220
32 **Cheng WF**, Hung CF, Lin KY, Ling M, Juang J, He L, Lin CT, Wu TC. CD8+ T cells, NK cells and IFN-gamma are important for control of tumor with downregulated MHC class I expression by DNA vaccination. *Gene Ther* 2003; **10:** 1311-1320

Edited by Wang XL and Chen WW **Proofread by** Xu FM