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Improved efficacy of DNA vaccination against prostate carcinoma by boosting with recombinant protein vaccine and by introduction of a novel adjuvant epitope

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ABSTRACT

DNA vaccine represents an attractive approach for cancer treatment by inducing active immune-deprivation of gastrin-releasing peptide (GRP) from tumor cells, the growth of which is dependent on the stimulation of GRP. In this study, we developed a DNA vaccine using a plasmid vector to deliver the immunogen of six copies of the B cell epitope GRP_{18-27} (GRP6). In order to increase the potency of this DNA vaccine, multiple strategies have been applied including DNA-prime protein-boost immunization and introduction of a foreign T-helper epitope into DNA vaccine. Mice vaccinated DNA vaccine boosting with HSP65-GRP6 protein induced high titer and relatively high avidity of anti-GRP antibodies as well as inhibition effect on the growth of murine prostate carcinoma, superior to the treatment using DNA alone or BCG priming HSP65-GRP6 protein boosting. Furthermore, the introduction of a novel foreign T-helper epitope into the GRP DNA vaccine showed a markedly stronger humoral immune response against GRP and tumor rejection even than the DNA-prime protein-boost strategy. No further stronger immunogenicity of this foreign T-helper epitope modified DNA vaccine was observed even using the strategy of modified DNA vaccine-priming and HSP65-GRP6 boosting method. The data presented demonstrate that improvement of potency of anti-GRP DNA vaccine with the above two feasible approaches should offer useful methods in the development of new DNA vaccine against growth factors for cancer immunotherapy.

1. Introduction

Cancer represents a relevant current health problem. Identification of the hallmarks of cancer has substantiated the development of several targeted therapies that may contribute to its control [1]. Cancer vaccines targeting hormone/growth factor are under evaluation with some success recently [2]. Another feasible approach for cancer treatment involves gastrin-releasing peptide (GRP), which can act as growth factor in many types of cancer and has been regarded as a molecular target in experimental anticancer therapy [3,4]. This study is focused on effective immune-deprivation of GRP for cancer treatment, evaluated using a GRP-dependent tumor model.

GRP is a self-peptide, and appears to be very effective at establishing immunological tolerance. We have previously found that GRP protein vaccine in the presence of adjuvant failed to significantly inhibit the growth of RM-1 tumor xenografts (data unpublished), due to the lack of high levels of humoral response against GRP and the relatively low avidity of antibodies induced. Thus our attention has turned to construct novel DNA vaccines in order to break the already established tolerance of this self-peptide GRP. We have already constructed a DNA vaccine encoding six copies of a B cell epitope GRP_{18-27} (N) [5] as immunogen and HSP65 (H) as carrier protein. But the immunogenicity of this DNA vaccine is still low. For further increasing the immunogenicity of DNA vaccine, a sitimulatory CpG motif within the backone of vector [6] and two foreign T-helper epitopes tetanus $toxoid_{830-844}$ [7] and pan HLA-DR-binding epitope (PADRE) [8] have been introduced into anti-GRP DNA vaccine. However, these multiple strategies have lead to anther depressed result without significantly enhanced immunogenicity.

For further enhancing the potency of anti-GRP DNA vaccine, we have applied a DNA-priming, protein-boosting strategy in the following study to overcome the low and slow development of humoral antibody often observed with DNA vaccines. At the same time, a novel T-helper epitope, identified as an effective stimulatory adjuvant in many subunit DNA vaccines in our lab, has been utilized in this current investigation. The enhancement of humoral

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response against GRP, the increased avidity of antibodies and the efficacy on inhibition of the growth of GRP-dependent tumor RM-1 have been investigated.

2. Materials and methods

2.1. Tumor cell line and mice

The murine RM-1 prostate carcinoma cell line was cultured in growth medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C under humidified atmosphere of 95% air/5% CO_2. For all experiments, male, 5 weeks old, C57BL/6 mice were housed under specific pathogen-free conditions at Animal Center of Nanjing University (Nanjing, PR China). The implantation of subcutaneous tumors was carried out by injection of 2.5×10^5 RM-1 cells [9] for the prophylactic approach.

2.2. Construction of HSP65-GRP6 (HN) chimeric protein

The recombinant plasmid (pET28a-HN, Fig. 1A) was constructed according to the method described by Yi et al. [10] with some modifications. Briefly, inserting two copies of GRP_{18-27} gene into the plasmid pET28a-HSP65 downstream of the HSP65 three times to form tandemly repeated epitopes. The encoding sequence of two copies of GRP_{18-27} was synthesized by PCR with two oligonucleotides used as both primers and templates: G1 (5′-CGGGCTAGCGGCAACCACT GGGCGGTGGGGCACTTAATGGGCAACCACTGGGCGGTG-3′); G2 (5′-CCCAAGCTTA TC TAGACATTAAGTGCCCCACCGCCCAGTGGTTGCCCATTAAGTGCCC-3′).

2.3. Construction of plasmids

The plasmid pCR3.1 containing the VEGF183 signal peptide (VS, under the control of promoter CMV) and eight 5′-GACGTT-3′ CpG motifs (on its backbone) was used as eukaryotical expression vector. The gene encoding HSP65-GRP6 was obtained by PCR from plasmid pET28a-HN, using A1 (5′-CACCCATGGCCAAGACAATT-3′) and A2 (5′-ATCTAGACGCTAA GCTTGCG-3′) as forward and reverse primers, respectively. The gene encoding tetanus $toxoid_{830-844}$-PADRE (T) was amplified by PCR with the following four oligonucleotides: B1(5′-GCGTCTAGAGGTGGCGGTGGCTCCGGTGGCGGTGGCTCCGGATCTCAGTACATCAAGG-3′), B2(5′-GATCTCAGTACATCAAGGCTAACTCTAAGTTCATCGGCATTACCGAGCTGTC CCCTG-3′), B3(5′-CTTCAGGGTCCAAGCGGCCACGAACTTGGCAGAGCCAGGGGACAG CTCGGTAATGCC-3′), and B4(5′-ACTTGCTAGCGGATCCAGGGCCAGGGCCAGCAGCGGCCT TCAGGGTCCAAGCGGCC-3′) as both primers and templates. Both the plasmid vector and gene encoding HSP65-GRP6 were digested by NcoI and HimdIII, and then ligated. The resulting plasmid was digested again by NheI and treated with CIAP, then ligated with T gene digested by NheI and XabI to construct the plasmid pCR3.1-VS-HSP65-TP-GRP6 (pHTN) (Fig. 1C).

The method for inserting two copies of microbial $HSP70_{407-426}$ (M) gene (M2) into pHTN downstream of GRP6 was also similar to that described by Yi et al. [10]. The encoding sequence of M gene was synthesized by PCR with two oligonucleotides used as both primers and templates: C1(5′-ATAGCTAGCGCCAGATCTCCTGGCAGCCAGCCTTCCGTGCAGATCCAG GTCTACCAGGGCGAGCGC-3′) and C2(5′-GCGAAGCTTAGCGTCTAGATGAGG ATCCCTTGT TGTGAGCGGCAATCTCGCGCTCGCCCTGGTAGAC-3′). The resulting plasmid was pHTNM2 (Fig. 1C).

All constructed plasmids listed in Fig. 1C were verified by DNA sequencing. Plasmid DNA used for immunization was purified using Qiagen Plasmid Mega Kit (Qiagen, Germany) and suspended in sterile saline at a concentration of 0.5 μg/μL.

2.4. Expression and purification of the recombinant fusion protein

The *E. coli* BL21 (DE3) containing pET28a-HN, inoculated from a single bacterial colony, was growing for 10 h at 37 °C. The preculture was diluted 50-fold in LB medium containing kanomycin (50 μg/mL), and then growing at 37 °C. The recombinant engineering bacteria including HN fusion protein were induced at A550 of 1.3–1.5 by addition of lactose to a final concentration of 5 mM. After incubating for 6 h after induction, the bacterial were harvested. BL21(DE3) cells harboring HN were lysed with lysozyme and Dnase I. The supernatant fraction containing soluble fusion protein was precipitated with 10% saturated ammonium sulfate and redissolved in 10 mmol/L Tris–HCl (pH 8.0). After dialysis against 10 mmol/L Tris–HCl (pH 8.0), the solution was loaded on a DEAE-cellulose (Whatman) column equilibrated with 10 mmol/L Tris–HCl (equilibration buffer, pH 8.0) and was eluted with a linear gradient of 0–0.3 mol/L NaCl in the equilibration buffer. The peak fraction containing HN protein (determined by SDS-PAGE) was pooled, concentrated by pressure ultrafiltration, and applied to a column of Sephadex G-100 (Pharmacia, USA) previously equilibrated in 10 mmol/L Tris–HCl (pH 8.0), which was washed with 10 mmol/L Tris–HCl (pH 8.0). Fractions containing HN protein were pooled and dialyzed against water. The purified protein was stored at −20 °C after being lyophilized.

2.5. Immunization procedure and prostate carcinoma model

In order to analyze the antitumor effect of DNA prime and protein boost strategy, a vaccination protocol was designed as shown in Fig. 3. A total of eight C57BL/6 male syngeneic mice ($n = 8$) were used for each experimental group (one group for each of the five different vaccination treatment and PBS control). pHTN or PHTNM2 DNA vaccine (50 μg in a final volume of 100 μL PBS) was injected intramuscularly three times, on days −46, −25 and −11 before tumor cells challenge. To enhance the cell uptake of plasmid DNA, the quadriceps was injected with a total of 100 μL of 0.25% bupivacaine three days before each DNA administration. HN was applied intraperitoneally twice at 21 and 14 days before tumor cells challenge. Mice treated with HN without plasmid DNA priming received intraperitoneal injection of 10^6 CFU of BCG (Bacillus Calmette-Guerin; attenuated *Mycobacterium tuberculosis* var *bovis*) at 35 days before tumor cells challenge. At day 0, RM-1 cells (2.5×10^5) were injected subcutaneously into the left flank of mice. Tumors were measured in two dimensions with calipers every 2 days. The volume was calculated using the formula $V = 0.4ab^2$, with "*a*" as the larger diameter and "*b*" as the smaller diameter. Fifteen days after the tumor cells challenge, all mice were sacrificed for analysis of tumor weights.

In order to demonstrate an antibody-mediated mechanism of tumor destruction, GRP specific antisera collected from mice immunized pHTN or pHTNM2 51 days post-initial immunization were studied on RM-1 model. Briefly, C57BL/6 mice were s.c. implanted with 2.5×10^5 RM-1 tumor cells. Mice were randomly divided into three groups (five mice per group) when tumors became easily palpable (7 days post-tumor cell challenge). 200 μL of antisera (pHTN or pHTNM2) were i.v. injected into mice thrice per week for 2 successive weeks, whereas the controls received injection of PBS instead. The volumes were calculated at 9, 13, 17 and 21 days post-tumor cell challenge.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The amount of anti-GRP antibodies and anti-HSP65 antibodies in the immune sera was determined using ELISA as described by Yin et al. [11]. Briefly, 96-well flat-bottomed ELISA plates were coated with 100 μL/well of recombinant human VEGF121-