

# A novel vaccine targeting gastrin-releasing peptide: efficient inhibition of breast cancer growth *in vivo*

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## Abstract

Gastrin-releasing peptide (GRP), a bombesin-like peptide, is an autocrine growth factor that can stimulate the growth of various cancer cells. We developed a novel protein vaccine HSP65-(GRP-10)<sub>6</sub> (HG6) that consists of six copies of a 10-amino acid residue epitope of GRP C-terminal fragment carried by mycobacterial 65 kDa HSP65 and then immunized mice via subcutaneous injection. Strong humoral and cell-mediated immune responses were induced. High titer of anti-GRP antibodies was detected in immunized mice sera by ELISA and verified by Western blot analysis. Activity of CD4<sup>+</sup>T lymphocytes, especially high levels of interferon (INF)- $\gamma$ , were developed in mice immunized with HG6 when compared with HSP65 or PBS. We found that immunogene tumor therapy with a vaccine based on GRP was effective at both protective and therapeutic antitumor immunity in breast tumor models in mice. The purified GRP monoclonal antibody (McAb) was proved to be potential in inhibiting EMT-6 tumor cell proliferation *in vitro*. The attenuation induced by active immune responses on tumor-induced angiogenesis was observed with an intradermal tumor model in mice. Taken together, we demonstrate for the first time that immune responses that are elicited by a novel chimeric protein vaccine targeting GRP can suppress the proliferation of breast tumor cell EMT-6 in mice, and it may be of importance in the further exploration of the applications of other autocrine growth factor identified in human and other animal in cancer therapy.

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## Introduction

Gastrin-releasing peptide (GRP) is the primary member of the bombesin family of neuropeptides. Bombesin is originally isolated from the skin of the amphibian *Bombina* (Erspamer *et al.* 1972), while GRP is the peptide most pharmacologically homologous in mammals including humans (Benya *et al.* 1995). The GRP mediates its effects by binding to a specific member of the seven transmembrane spanning G protein-coupled receptor super families (Battey *et al.* 1991). Research has demonstrated that GRP and GRP receptor (GRPR) are expressed aberrantly in various cancer cells such as small cell lung, prostate, gastric, breast, pancreatic, and colorectal cancers (Carroll *et al.* 1999, Scott *et al.* 2004). The GRP has been presumed

to act as a clinically significant growth factor. Thus, the inhibition of GRP activity may be important to suppress the growth of cancer. Many kinds of GRPR antagonist, such as peptide antagonists RC-3095 and RC-3940-II (Miyazaki *et al.* 1998) as well as non-peptide antagonists PD176252 and PD168368 (Moody *et al.* 2000, 2003), have been developed to inhibit the growth of various cancers. Other works have shown that a mouse monoclonal antibody 2A11 against GRP is able to block proliferation of small cell lung cancer (SCLC) cell lines. The efficacy of the antibody has also been investigated in phase I and II trials with SCLC patients (Chaudhry *et al.* 1999).

When compared with the previous GRPR antagonists or monoclonal antibody, vaccines targeting GRP have

dual advantages: first, high titer of antibodies evoked by cancer vaccine can act as receptor antagonists to block GRPR activation; secondly, during vaccination, the activation of tumor-specific T lymphocytes can be induced. Thus, the induction of a strong and long-lasting immunity characterized by humoral and cell-mediated immune responses is one of the most important considerations for developing effective antitumor vaccines. But a key problem in developing subunit vaccines is that the immunogenicity of the vaccines is weak. Previous studies have demonstrated that mycobacterial 65 kDa heat shock proteins (HSP65) exhibit strong immunogenicity and contain strong T-cell epitopes presented by major histocompatibility II molecules, and accordingly they have been used as helper T-cell epitopes for delivering B-cell epitopes *in vivo* (Perraut et al. 1993). Thus, HSP65 can be used as suitable and safe carrier molecules for delivering B-cell epitopes to the immune system *in vivo* in the absence of adjuvants (Barrios et al. 1994). Another convenient feature of HSP65 as a carrier is its ability to enhance the molecular weight of incomplete antigen, like self-peptide GRP, and then effective immune response can be evoked. Besides, the low immunogenicity of self-peptides can also be overcome by immunization with immunogens containing multiple copies of the self-peptides in linear alignment. It has been shown that tandemly repeated epitopes are able to efficiently induce a strong immune response *in vivo* (Kim et al. 2005, Yi et al. 2006) and prime CD4<sup>+</sup>T cells *in vitro* (Barratt-Boyes et al. 1999).

In this study, we fused the strong helper T-cell epitope carrier molecule of HSP65 with the tandemly repeated epitopes of GRP and tested whether the fusion protein can induce strong humoral and cell-mediated immune responses after injection into mice. Moreover, we investigated whether recombinant protein has a protective effect as a vaccine before tumor cell challenge. In addition, we applied the same strategy for a therapeutic approach to assess the efficacy against established tumors. Furthermore, the inhibition induced by active immune responses on tumor-induced angiogenesis was evaluated with an intradermal tumor model in mice.

## Materials and methods

### Construction of recombinant plasmid containing the gene of HG6

The recombinant plasmid was constructed according to the method described by Yankai et al. (2006) with slight modifications. Briefly, two copies of the GRP-10

gene were inserted, marked as (GRP-10)<sub>2</sub>, into the plasmid pET28a-HSP65 behind the gene of HSP65 three times to form tandemly repeated epitopes. The encoding sequence of (GRP-10)<sub>2</sub> was synthesized by PCR with two oligonucleotides used as both primers and templates: P1 (5'CGGGCTAGCGGCAACCACTGGGCGGTGGGGCACTTAATGGGCAACCACTGGGCGGTG 3') as the forward primer; P2 (5'CCC AAGCTTATCTAGACATTAAGTGCCCCACCGC-CCAGTGGTTGCCCATTAAGTGCCC 3') as the forward primer. The final correct sequence was verified by Invitrogen.

### Expression and purification of the recombinant fusion protein

The *Escherichia coli* BL21 (DE3) containing pET28a-HSP65-(GRP-10)<sub>6</sub>, inoculated from a single bacterial colony, was grown for 10 h at 37 °C. The preculture was diluted 50-fold in LB medium containing kanamycin (50 µg/ml), and then grown at 37 °C. The recombinant engineering bacteria including HG6 was induced at A550 of 1.3–1.5 by addition of lactose to a final concentration of 5 mM. When harvested after 6 h induction, the bacteria were suspended in buffer of 50 mM Tris-HCl pH 8.0 and lysed with 40 µg/ml lysozyme in the presence of 1 U/ml DNaseI at 37 °C for 1 h. Centrifugation was carried out at 12 000 g for 20 min at 4 °C. The HG6 in the supernatant was precipitated with 10% saturated ammonium sulfate. The purified protein was stored at –20 °C after being lyophilized.

### Immunization procedure

Five-week-old BALB/c mice of three groups were immunized subcutaneously in the right flank with HG6, HSP65, or PBS (control; 50 µg per mouse). Sera were collected weekly for immunoassay after the initial immunization.

### ELISA analysis for GRP antibody

An ELISA was performed to detect the GRP antibody level as described elsewhere (Ghosh & Jackson 1999). Briefly, 96-well flat-bottomed ELISA plates (Costar, Kennebunk, ME, USA) were coated with 100 µl/well of recombinant green fluorescent protein (GFP)-(GRP-10) proteins (10 µg/well) and kept overnight at 4 °C. Plates were blocked with PBS containing 5% (w/v) bovine serum albumin (BSA; Sigma) and then incubated with 100 µl/well 1:100 dilution of serum collected from immunized animals. A secondary HRP-conjugated goat anti-mouse IgG (Sigma) was used for

the substrate reaction. The absorbance measured at a wavelength of 450 nm is proportional to the GRP-specific IgG titer within the sera. Each measurement was carried out in duplicate.

### Western blot analysis

Western blot analysis was performed as described (Gaofu *et al.* 2004). Briefly, recombinant proteins were electrophoresed on a 15% SDS-PAGE gel and then were transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% BSA, washed and probed with mice sera at 1:200. Blots were then washed and incubated with HRP-conjugated goat anti-mouse IgG (Sigma). After the membrane was washed again, reaction was developed using 0.05% (w/v) 3,3',5,5'-diaminobenzidine and 0.012% (v/v) H<sub>2</sub>O<sub>2</sub> for 15 min at 37 °C. Recombinant proteins GFP-(GRP-10) with the GFP as fusion partner and HSP65 were expressed and purified from *E. coli* (Gaofu *et al.* 2004).

### Preparation of GRP McAb and its inhibition of tumor cell proliferation *in vitro*

The GRP monoclonal antibodies (McAb) were prepared as per previous methods (Hendriksen & de Leeuw 1998). Briefly, BALB/c mice were immunized with recombinant protein HG6. Then spleen cells from the immunized mice were fused with SP2/0 myeloma cells to get hybridoma cells. Ascites from the mice vaccinated with hybridoma cells were collected and GRP monoclonal antibodies were purified through affinity chromatography.

The efficacy of GRP McAb in antitumor *in vitro* was tested by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye assay according to the method described by Bounous & Brown (1992) with slight modifications. Briefly, EMT-6 cells were cultured and transferred to 96-well cell culture plate with  $1 \times 10^4$  cells per well and then incubated with serially diluted GRP McAb. After 48 h of incubation, 20  $\mu$ l MTT were added to each well and plates were incubated for further 4 h. At the end of the incubation, the plates were read at 570 nm.

### Lymphocyte proliferative response

Mouse splenocytes were prepared as follows. Spleens were removed from the immunized mice and were homogenized in PBS (pH 7.4). The erythrocytes cell suspension was lysed with 0.75% Tris-NH<sub>4</sub>Cl (pH 7.4). After being washed three times with PBS, the splenocytes were resuspended at  $2 \times 10^6$  cells/ml

with the supplemented RPMI 1640 containing 10% fetal bovine serum, 14 mM HEPES, 50 mM 2-mercaptoethanol, 100  $\mu$ g/ml streptomycin, and 100 IU/ml penicillin. Splenocytes were plated in 96-well flat-bottom plates at 100  $\mu$ l per well ( $2 \times 10^5$  cells per well). Subsequently, 100  $\mu$ l per well of medium with HG6, HSP65 (10  $\mu$ g/ml), or PBS were added and mixed. Concanavalin A (1.25  $\mu$ g/ml; Sigma) was used as a positive control. Each splenocyte sample was plated in triplicate. The proliferative response was measured by MTT dye assay according to the method described previously. Briefly, after 72 h of incubation, 20  $\mu$ l MTT were added to each well and plates were incubated for a further 4 h. At the end of the incubation, the plates were read at 570 nm.

### INF- $\gamma$ release assays

T lymphocytes were prepared, cultured, and stimulated as described earlier (Julius *et al.* 1973). After incubation for 72 h, culture supernatants were harvested to test the presence of INF- $\gamma$ . Commercially available mouse INF- $\gamma$  ELISA kits (PharMingen, San Diego, CA, USA) were used according to the manufacturer's instructions.

### Quantification of angiogenesis *in vivo*

To visualize the induction of angiogenesis by a tumor *in vivo*, an intradermal tumor model was used. In this model, neovasculature observed predominantly at the tumor periphery can be quantified by vessel counting (Kreisle & Ershler 1988, Auerbach *et al.* 1991). Three groups of BALB/c mice (three mice each group) were immunized with PBS, HSP65, or HG6, respectively, for four times at biweekly intervals. At 2 weeks after the final vaccination, mice were anesthetized (inhalant 4% Halothane-vet) and 106 EMT-6 mouse breast tumor cells in 50  $\mu$ l PBS were implanted intradermally at two sites in the abdominal region. Two additional injections of PBS (50  $\mu$ l) were performed on each mouse as a control. All implant sites were marked with indelible ink to aid identification at the end of the experiment. After 7 days, a section of the abdominal wall skin encompassing all implant sites was removed and spread onto filter paper. Sections were examined by light microscopy ( $\times 10$  magnification) and the total number of blood vessels (major vessels and branching points) was determined within a 1 cm<sup>2</sup> area around each implant site. Vessel counts from implants of PBS were consistent irrespective of treatment; the mean value was therefore subtracted from control and vaccines immunized vessel counts to give a more accurate indication of tumor-induced blood vessel formation and to ascertain the effect of treatment.

## Statistical analysis

Data were shown as mean  $\pm$  s.e.m. Student's *t*-test was used in the analysis of data for statistical significance.

## Results

### Preparation of vaccine

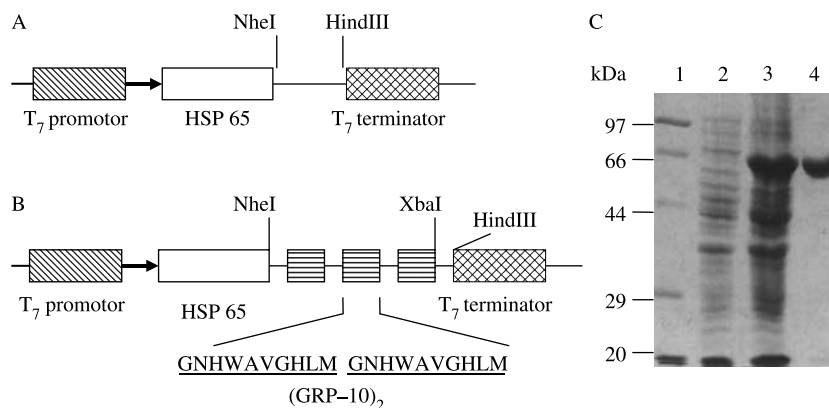
The native GRP is normally present in mammals, so it is not quite suitable for recombinant expression in bacterial cells. The two PCR primers used in the synthesis of the GRP gene were designed based on the genetic code rule in prokaryotes in order to express GRP gene in *E. coli* effectively. When two sites, which were digested by endonucleases NheI and XbaI respectively, as we knew they were isocaudamer, ligated again, the new site was recognized neither by NheI nor by XbaI. As a result, the plasmid pET28a-HSP65-(GRP-10)<sub>4</sub> and pET28a-HSP65-(GRP-10)<sub>6</sub> retained the site NheI only. With this strategy of isocaudamer technique, we construct the plasmid with six copies of GRP behind the gene of HSP65 (Fig. 1A and B).

The HG6 was expressed as soluble proteins in engineered strains. The amount of HG6 in engineered *E. coli* strains had increased to the highest level when cells were further cultured in LB medium for 6 h after lactose induction. Cells harboring HG6 were suspended in buffer of 20 mM Tris-HCl pH 8.0 and lysed with lysozyme in the present of DNaseI. The supernatant was collected after centrifugation. Most of HG6 in the supernatant could be precipitated with

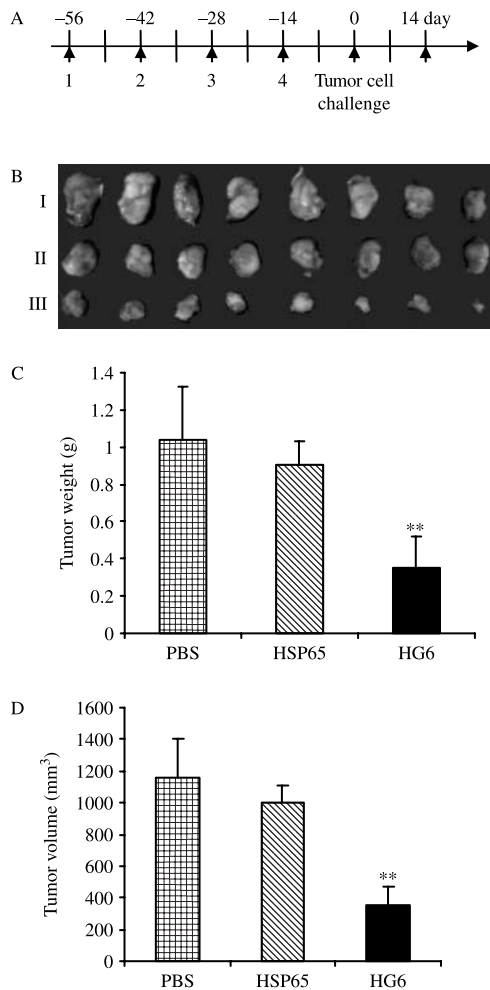
10% saturated ammonium sulfate. The precipitations were dissolved and dialyzed to PBS buffer to remove most of ammonium sulfate in solutions. The result of the purification process was exhibited on a 12% SDS-PAGE analysis (Fig. 1C).

### Induction of prophylactic antitumor immunity

In order to analyze the protective effect of this vaccine on breast cancer, a vaccination protocol was designed as shown in Fig. 2A. Always a total of eight BALB/c mice were used for each experimental group. Mice were immunized subcutaneously four times at biweekly intervals, on day -56, -42, -28, and -14, with the same doses (50  $\mu$ g per mouse) of HG6, HSP65, or PBS alone. At day 0, EMT-6 breast tumor cells ( $2 \times 10^5$ ) were injected subcutaneously into immunized mice. At day 14 after the cells challenged, all mice were killed and solid tumors were excised and weighed. As shown in Fig. 2B, tumors grew progressively in non-immunized mice (PBS alone) or in HSP65-immunized mice, but there was significant protection against tumor growth in HG6-immunized mice. In the group vaccinated with HG6, the mean mass of solid tumors was significantly less than the mean mass of solid tumors in the PBS ( $0.35 \pm 0.14$  vs  $1.037 \pm 0.28$  g;  $P=0.0004$ ) and HSP65 group ( $0.35 \pm 0.14$  vs  $0.907 \pm 0.103$  g;  $P=0.0001$ ). Vaccination with HG6 resulted in a three-fold reduction in tumor volume ( $360 \pm 120$  mm<sup>3</sup>) when compared with HSP65 group ( $1000 \pm 102$  mm<sup>3</sup>) and controls ( $1160 \pm 238$  mm<sup>3</sup>, PBS; Fig. 2D).



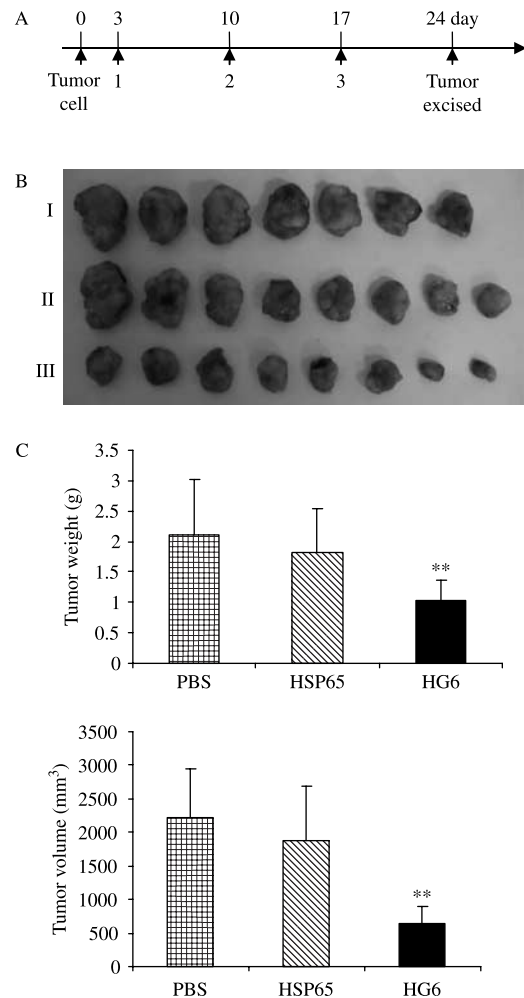
**Figure 1** Expression of HSP65-(GRP-10)<sub>6</sub>. (A) The HSP65 (represented by open box) gene was placed under the control of T<sub>7</sub> promoter (represented by bias box). (B) Six copies of GRP-10 (represented by linear box) were ligated to the HSP65 gene through the NheI. (C) SDS-PAGE analysis of purified HSP65-(GRP-10)<sub>6</sub>. The HSP65-(GRP-10)<sub>6</sub> expressed as soluble proteins in bacteria was purified by ammonium sulfate precipitation and then analyzed with 12% SDS-PAGE stained with Coomassie blue. Molecular weight marker (lane 1); total cell proteins of *E. coli* BL21 (lane 2); total cell proteins of *E. coli* BL21 harboring HSP65-(GRP-10)<sub>6</sub> induced by lactose (lane 3) and purified HSP65-(GRP-10)<sub>6</sub> (lane 4).



**Figure 2** Prophylactic vaccination of BALB/c mice with vaccines. (A) The immunization scheme indicates the days of vaccination by arrows. The PBS, HSP65, or HG6 (50 g per mouse) was injected subcutaneously at day -56, -42, -28, and -14. At day 0,  $2 \times 10^5$  EMT-6 cells were injected subcutaneously for tumor cell challenge. All mice were killed and tumors were excised 2 weeks later. (B) Solid tumors from three group mice were aligned and their photos were taken. The picture shows the tumors extracted from mice immunized with PBS (I,  $n=8$ ), HSP65 (II,  $n=8$ ), and HG6 (III,  $n=8$ ) from the top down. From this graph, tumors from mice immunized with HG6 were significantly smaller than that from HSP65-immunized mice and controls. (C) Tumor mass of mice treated using PBS (cross bar), HSP65 (bias bar), or HG6 (solid bar), respectively, is presented as mean  $\pm$  s.e.m. The graph demonstrates that the tumors from HG6-immunized mice were significantly less than those from the PBS (\*\* $P=0.0004$ ) and HSP65 groups ( $P=0.0001$ ). (D) The results of tumor volume are shown.

### Induction of therapeutic antitumor immunity

More relevant to the treatment of tumor by vaccines is the therapeutic potential vaccines. Therefore, a therapeutic vaccination protocol was designed as shown in Fig. 3A. At day 0, EMT-6 breast tumor cells



**Figure 3** Therapeutic vaccination of BALB/c mice with vaccines. (A) The immunization scheme indicates the days of vaccination by arrows. On day 0, tumor cells ( $2 \times 10^5$  EMT-6) were inoculated subcutaneously. Afterwards, on the days indicated, PBS, HSP65, or HG6 (50 g per mouse) were applied. On day 24, all mice were killed and tumors were excised. (B) Solid tumors from three group mice were aligned and their photos were taken. One mouse of the PBS group died on day 20. From this graph, tumors from mice immunized with HG6 (III) were significantly smaller than that from HSP65-immunized mice (II) and controls (I). (C) Tumor mass of mice treated using PBS (cross bar), HSP65 (bias bar), or HG6 (solid bar), respectively, is presented as mean  $\pm$  s.e.m. The graph demonstrates that the tumors from HG6-immunized mice were significantly less than those from the PBS (\*\* $P=0.0079$ ) and HSP65 groups ( $P=0.042$ ). (D) The tumor volumes from each group are shown consistent with indication of tumor mass.

( $2 \times 10^5$ ) were injected subcutaneously into three groups of mice. The therapeutic efficacy of vaccine HG6 was tested next in the established tumors. The mice were treated starting from day 3 after the injection of tumor cells, when the tumor was palpable. The mice were treated with HG6, HSP65, or PBS respectively in



the dose of 50 µg per mouse on day 3, 10, 17 as indicated (Fig. 3A). At day 7 after the third treatment, all mice were killed and solid tumors were excised and weighed. Unexpectedly, one mouse from the PBS group died on day 20. The tumor volumes of the mice treated with vaccine HG6 were strongly reduced whereas the mice treated with PBS or HSP65 showed tumors of larger size (Fig. 3B). In the group vaccinated with HG6, the mean mass of solid tumors was significantly less than the mean mass of solid tumors in the PBS ( $1.02 \pm 0.34$  vs  $2.115 \pm 0.9$  g;  $P=0.0079$ ) and HSP65 group ( $1.02 \pm 0.34$  vs  $1.832 \pm 0.7$  g;  $P=0.042$ ; Fig. 3C).

The mice immunized with these vaccines have been investigated particularly for potential toxicity. No adverse consequences were indicated in gross measures such as weight loss, ruffling of fur, behavior, and feeding. Furthermore, no pathologic changes in liver, lung, kidney, spleens, heart, pancreas, or intestines were found by microscopic examination.

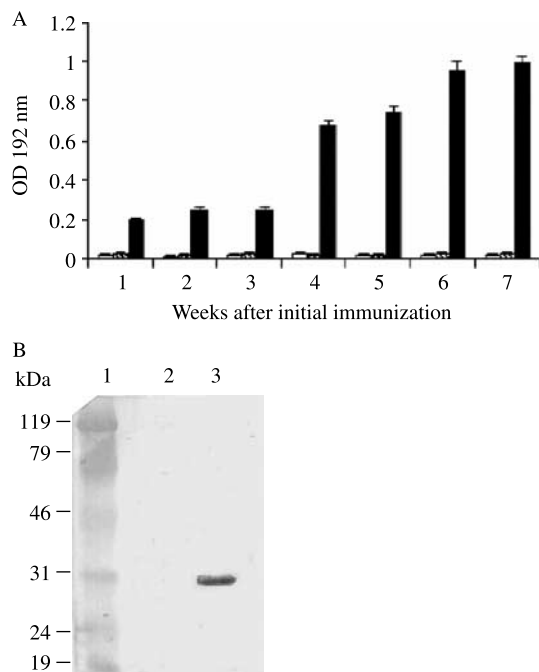
#### Characterization of GRP-specific IgG from immunized mice sera

In order to investigate whether the chimeric vaccine containing tandem repeats of GRP epitope could evoke strong humoral immune response, we compared the levels of GRP-specific IgG in the sera collected from mice immunized with HG6, HSP65, and PBS (as control) respectively by ELISA (Fig. 4A). The HG6 group showed a greatly increasing titer of GRP-specific IgG measured by ELISA as early as the first week following initial vaccination, while mice that were vaccinated with HSP65 alone or with PBS failed to elicit antibody formation. Later, anti-GRP antibodies could easily be detected by ELISA till the last week.

The fusion protein GFP-(GRP-10) and native GFP were processed for the Western blot using antisera from mice vaccinated with HG6 to demonstrate whether the antibodies produced in the immunized mice were specific for GRP. In this study, antibodies from immunized mice reacted with the GFP-(GRP-10) but not with the protein GFP (Fig. 3), suggesting that GRP-specific antibodies had been evoked by this vaccine.

#### Inhibition of tumor cells growth *in vitro* treated with GRP McAb

BALB/c mice were immunized with protein HG6 and then the spleen cells separated from the mice were fused into myeloma cells SP2/0 with the help of the fusogenic agent PEG to get hybridoma cells. Through the sandwich ELISA method, a cell strain of hybridoma cells were



**Figure 4** Characterization of GRP-specific antibodies from mice sera immunized with vaccines. (A) Anti-GRP antibody level in mice immunized by subcutaneous injection. The level of anti-GRP antibody was measured in mice ( $n=8$  per group) that received four biweekly subcutaneous injection of HG6, HSP65, or PBS. Serum samples were collected from each mouse each week after the initial injection and assayed by ELISA using GFP-(GRP-10) as antigen (PBS, empty bar; HSP65, bias bar; HG6, solid bar). (B) Western blot analysis of anti-GRP antibodies. Recombinant proteins GFP-(GRP-10), GFP were fractionated on 12% SDS-PAGE gel, transferred onto nitrocellulose membrane and hybridized with serum samples from mice immunized with HG6 by subcutaneous injection (lane1, pre-stained protein molecular weight marker; lane2, GFP; lane3, GFP-(GRP-10)).

selected, which can excrete antibody with high affinity against GRP. Then rats were vaccinated with hybridoma cells and its ascites was collected to be purified through saturated ammonium sulfate precipitation and affinity chromatography. The valency was determined through the sandwich ELISA method to be  $2 \times 10^6$ . The affinity constant between GRP McAb and GRP was calculated to be  $1.5 \times 10^9$ . The antibody was tested with immunoglobulin subtype kit and found to be IgG1 Type  $\kappa$  subtype.

To evaluate the inhibition effect of GRP McAb on EMT-6 cells *in vitro*, serially diluted GRP McAb (from  $1/1024$  µmol/l to 1 µmol/l) was added to tumor cells in a 96-well cell culture plate. The proliferation of EMT-6 cells *in vitro* was suppressed by GRP McAb as shown in Table 1. The effect was dose dependent. Treatment with a dose of  $3 \times 10^{-8}$  mol/l McAb had significant inhibition on tumor cells growth compared with that of the controls (IR% = 17.3%;  $P < 0.01$ ). With the dose of

**Table 1** Suppression on the proliferation of EMT-6 cell with different gastrin-releasing peptide (GRP) McAb level

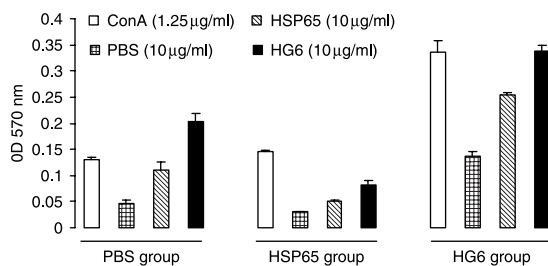
Dose ( $\mu\text{mol/l}$ )	$A_{570}$ ( $x \pm s$ , $n=3$ )	IR%	Dose ( $\mu\text{mol/l}$ )	$A_{570}$ ( $x \pm s$ , $n=3$ )	IR%
Control	$0.614 \pm 0.012$		1/32	$0.508 \pm 0.024^\dagger$	17.3
1/1024	$0.578 \pm 0.019$	5.9	1/16	$0.497 \pm 0.028^\dagger$	19.1
1/512	$0.567 \pm 0.014$	7.7	1/8	$0.476 \pm 0.017^\dagger$	22.5
1/256	$0.561 \pm 0.029$	8.6	1/4	$0.465 \pm 0.013^\dagger$	24.3
1/128	$0.528 \pm 0.011^*$	14.0	1/2	$0.402 \pm 0.021^\dagger$	34.5
1/64	$0.523 \pm 0.0006^*$	14.8	1	$0.324 \pm 0.021^\dagger$	47.2

\* $P < 0.05$  versus control;  $^\dagger P < 0.01$  versus control; IR%, inhibition rate (%).

McAb increasing to  $1 \times 10^{-6}$  mol/l, tumor cells were inhibited more significantly and the inhibition rate reached 47.2%. Upon further study, the phenomenon of cell apoptosis was observed when tumor cells were treated with a higher concentration of McAb.

### Cell-mediated immune responses

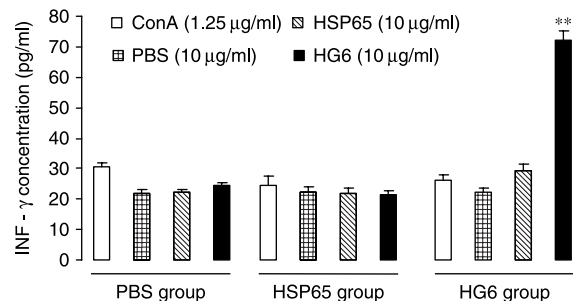
Although humoral immune responses were induced by vaccine HG6, it provided more efficient protective and therapeutic effect on mice against breast tumor, suggesting that the level of antibodies present in immunized animals do not correlate fully with antitumor results. Thus, we speculated that cell-mediated immunity also contributes to antitumor effect and this vaccine may induce stronger cellular immune responses. To test this hypothesis, another three groups of mice (four mice in each group) were immunized with PBS, HSP65, or HG6 as described earlier. Lymphocyte proliferative responses were analyzed 2 weeks after final immunization. As shown in Fig. 5, similar to the humoral immune responses, the



**Figure 5** Lymphocyte proliferative responses after *in vitro* stimulation with different stimulus. Each groups of mice ( $n=4$ ) were immunized subcutaneously with 100 g PBS, HSP65, or HG6, respectively, at 0, 2, and 4 weeks. At 2 weeks after third immunization, mice were killed and spleen lymphocytes from three groups were prepared separately. Lymphocytes of each group were stimulated with ConA, PBS, HSP65, or HG6 respectively. After 72 h stimulation, MTT was added and OD values were determined after a further 4 h inoculation. Each sample was assayed in triplicate. Treatment with HSP65 and HG6 proteins on lymphocytes from PBS or HSP65 group showed no stimulation effect. In contrast, lymphocytes from HG6 group exhibited strong proliferative responses to ConA, PBS, HSP65, and HG6 especially.

GRP-specific proliferative response was significantly higher ( $P < 0.05$ ) in mice immunized with HG6 than in mice immunized with HSP65 as well as the controls. Low levels of lymphocyte proliferative responses were observed in controls groups. Interestingly, the data presented that the response in lymphocyte from HSP65-immunized mice were even lower than that from PBS injected mice, suggesting that HSP65 have the function of down-regulation on immune system. Further researches on HSP65 were warranted.

To further confirm T-cell activation, production of INF- $\gamma$  in the supernatant of antigen-stimulated T lymphocyte from immunized mice's spleen was assessed. As shown in Fig. 6, the amount of INF- $\gamma$  measured in the supernatants of T lymphocyte from PBS-immunized mice stimulated with different antigen including ConA, PBS, HSP65, or HG6 exhibited no significant difference. Similar results were showed in T lymphocyte from HSP65 group mice. However, the release of INF- $\gamma$  measured in the supernatants of

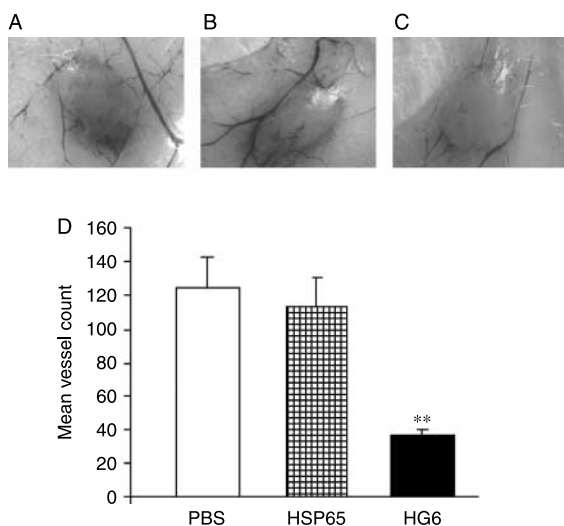


**Figure 6** Levels of production of INF- $\gamma$  from T lymphocyte in different group mice with stimulation with different stimulus. Each groups of mice ( $n=4$ ) were immunized subcutaneously with 100 g PBS, HSP65, or HG6, respectively, at 0, 2, and 4 weeks. At 2 weeks after third immunization, mice were killed and T lymphocytes from three groups were prepared separately according to 'Materials and methods'. T lymphocytes of each group were stimulated with ConA, PBS, HSP65, or HG6 respectively. After 72 h stimulation, supernatants of each group were harvested and analyzed by sandwich ELISA for INF- $\gamma$  production. Each sample was assayed in triplicate. The amount of INF- $\gamma$  measured in the supernatants of T lymphocyte from HG6-immunized mice was enhanced significantly with HG6 protein re-stimulation *in vitro* (\*\* $P < 0.01$  by *t*-test).

T lymphocyte from HG6-immunized mice stimulated with different antigen was significantly higher than the levels of INF- $\gamma$  measured in mice immunized with HSP65 and PBS ( $P < 0.01$ ). This result was consistent with that of lymphocyte proliferative responses. These INF- $\gamma$  release results indicate that HG6 had induced an enhanced Th1-type cell immune response.

### Intradermal tumor angiogenesis

To assess the effect of immune response on tumor angiogenesis induced by vaccine HG6, three groups of mice (three mice each group) were immunized with PBS, HSP65, or HG6, respectively, as described earlier. The EMT-6 tumor cells were implanted intradermally at two sites in the abdominal region, 14 days after final immunization. As shown in Fig. 7, tumor cells implanted intradermally were found to induce significant angiogenesis within a period of 7 days. The total number of blood vessels around each implant site from HG6-immunized mice was significantly lower than blood vessels from PBS ( $37 \pm 4$  vs  $125 \pm 18$ ;  $P < 0.001$ ) and HSP65-immunized mice ( $37 \pm 4$  vs  $113 \pm 17$ ;  $P < 0.001$ ).



**Figure 7** Effect of immune response on tumor-induced angiogenesis. To visualize the induction of angiogenesis by a tumor *in vivo*, EMT-6 tumor cells were implanted intradermally and new blood vessel formation was assessed 7 days later by light microscopy. The effect of immunization with (A) PBS, (B) HSP65, or (C) HG6 for the duration of assay is shown in representative images ( $\times 1$  objective magnification). The total number of blood vessels (major vessels and branching points) was determined within a precise 1-cm<sup>2</sup> area around each implant site (2 per mouse, 4 mice per group; refer to 'Materials and methods'). (D) Mean vessel count data indicate that HG6-induced immune response inhibited tumor-induced angiogenesis significantly when compared with PBS and HSP65 group (by 70.4% and 67.3% respectively;  $**P < 0.01$  by *t*-test).

### Discussion

In this current study, we developed a novel protein vaccine with the characteristics of six tandem repeats of a 10-amino acid residue epitope of GRP C-terminal fragment and fusion partner 65 kDa HSP65. The BALB/c mice were immunized with this vaccine and a high titer of GRP-specific antibodies was detected from the immunized mice sera. The immunized mice were injected subcutaneously with EMT-6 cells and then the inhibition of tumor growth was exhibited compared with the controls or the HSP65 group. To our knowledge, this is the first report of vaccine targeting GRP to inhibit the EMT-6 growth in mice compared with previous studies focusing on GRPR antagonists, monoclonal antibodies, or cytotoxic peptide conjugates.

The characteristics of this novel protein vaccine may contribute to its efficacy in the inhibition of tumor growth. First, this vaccine contains six tandem repeats of a 10-amino acid residue epitope of GRP. The induction of an immune response against self-peptides is problematic because of its low immunogenicity. Hsu *et al.* (2000) developed a new and efficient delivery system with the approach termed template-repeated polymerase chain reaction (TR-PCR) to induce an immune response against poorly immunogenic plasma peptides. They used multiple repeats of a small self-peptide (12 repeats of 10 amino acid residues of gonadotropin-releasing hormone) fused to the receptor binding domain (Ia) of *Pseudomonas* exotoxin A as an immunogen, which could evoke high-titer antibodies specific to the hormone in female rabbits. In this study, we take another approach to increase the copy number of self-peptide with the strategy of isocaudamer technique. With this efficient method, we can not only multiply a single kind of epitope but also tandemly link different kinds of epitopes to construct a multivalent vaccine. Our results showed that the introduction of six copies of a 10-amino acid residue sequence played a key role in enhancing the immunogenicity and antitumor effects of the fusion protein vaccine, which was more like the results of Kim *et al.* (2005) in that the low immunogenicity seemed to be overcome due to the tandem repeats. On the other hand, HSP65 may also play another important part in enhancing the immunogenicity of the vaccine in this study. According to the earlier literature, is able HSP65 to: (1) chaperone peptides, including antigenic peptides (Tamura *et al.* 1997, Srivastava *et al.* 1998); (2) interact with antigen presenting cells through a receptor (Arnold-Schild *et al.* 1999, Basu *et al.* 2001); (3) stimulate antigen-presenting cells to secrete inflammatory cytokines (Cho *et al.* 2000, Ausiello *et al.* 2006); (4) mediate maturation of dendritic cells,



making them a one-stop shop for the immune system (Cho *et al.* 2000, Ausiello *et al.* 2006); and (5) help HSP fusion protein elicit cytolytic T lymphocytes activity (Anthony *et al.* 1999). With such kinds of feature, HSP can assist the fusion protein to successfully induce a strong immune response including humoral and cell immunity.

The mechanisms underlying the ability of HG6 fusion protein to inhibit the proliferation of EMT-6 tumor cells *in vivo* have yet to be elucidated. First, it is clear that the humoral immune response has been elicited by the vaccination of this protein with high titer of GRP-specific antibodies detected. This phenomenon suggests that GRP-specific antibodies may neutralize the self-peptide GRP secreted by tumor cells and lower the concentration of GRP in tumor circumstances, for the activation of GRPR may be interrupted and the successive signal pathway can be blocked. Secondly, the antitumor result may be the synergistic effect of HSP65. Ausiello *et al.* (2006) have suggested that HSP65 could induce a strong monocyte-derived dendritic cells (MDDC) maturation. A clear T helper (Th) 1 immune response was also induced by HSP65 with the secretion of regulatory cytokines and enhancement of antigen presenting ability of mature MDDC. Our results show that the mice immunized with the native HSP65 also exhibit an extent of suppression on the tumor cells. The phenomenon may be explained by the fact that helper T cells induced by HSP65 can secrete a series of cytokines including INF- $\gamma$  and interleukin (IL)-2, which will stimulate the switching of natural killer cells (NK cells) to lymphokine activated killer cells (LAK cells) with enhanced nonspecific antitumor response. Thirdly, a putative effect of controlling tumor growth may come from the complement system. With the neutralization to the GRP by the GRP-specific antibodies, a large number of immune complexes will be produced, which may activate the complement cascade. The complement-mediated lysis of tumor cells can be enhanced by amplification of the amount of antibodies during the period of GRP secreted and released from the surface of tumor cell membranes.

It is known that CD4+T lymphocytes can steer and amplify immune responses through the secretion of cytokines and expression of surface molecules (Romagnani 1997, Schwartz 1997) and play a key role in antitumor immunity for establishing long-lasting and memory lymphocytes. Wei *et al.* (2001) have demonstrated that mice depleted of CD4+T lymphocytes by the injection of anti-CD4 McAb and vaccinated with *Xenopus* vascular endothelial growth factor (VEGF) were not protected from tumor challenge. In contrast, treatment with anti-CD8 or anti-NK McAb or control

IgG failed to abrogate the antitumor activity. Nawrath *et al.* (2001) have reported that CD4-depleted mice, vaccinated with DNA encoding gp100 and pps cells, did not benefit from vaccination. The survival rate of all of the mice challenged malignant melanoma declined rapidly. In the present study, we found that proliferative responses *in vitro* of lymphocytes from HG6-immunized mice were induced notably and production of INF- $\gamma$  from these T lymphocytes was enhanced significantly. These findings suggest that active CD4+T lymphocytes are absolutely essential for antitumor activity.

An important early event in the development of cancer is the induction of genes regulating angiogenesis, which is thought to be necessary for all macroscopic solid tumor growth. Several previous studies have demonstrated that GRP may contribute to the increased metastatic potential of cancers through the stimulation of proangiogenic gene expression. Lyuba Levine *et al.* (2003) have reported that GRP stimulation of GPR-R induced nuclear factor-kappa B (NF $\kappa$ B) activation and up-regulation of IL-8 gene expression in DU-145 and PC-3 cells, and increased VEGF gene expression in PC-3 cells. They also showed that GRP treatment of PC-3 cells induced I $\kappa$ B degradation, NF $\kappa$ B translocation to the cell nucleus, increased NF $\kappa$ B binding to its DNA consensus sequence, increased IL-8 and VEGF mRNA expression, and increased protein secretion. Kang *et al.* (2007) have showed that GRP is a tropic factor for highly vascular neuroblastomas with increased expression of angiogenic markers, PECAM-1 and VEGF, as well as phosphorylated (p)-Akt levels. Thus, GRP/GRPR is a potential target for inhibiting solid tumor growth by suppressing angiogenesis. It has been demonstrated that GRP or GRPR silencing significantly inhibited VEGF as well as p-Akt and p-mTOR expression *in vitro*, and inhibition of BBS/GRP with an antagonist, RC-3095, suppressed tumor progression and vascularization (Kang *et al.* 2007). Martinez *et al.* (2005) also reported that the specific GRP blocker 77 427 significantly reduced endothelial cell cord formation *in vitro* and angiogenesis *in vivo*. In our current study, we show that immune responses induced by vaccine HG6 significantly reduced angiogenesis and solid tumor proliferation when compared with controls in BALB/c model. It is suggested that high and GRP-specific antibodies evoked by HG6 might block the stimulation from GRP to GRPR and expression of proangiogenic gene, contributing to the inhibition of tumor growth *in vivo*.

In summary, we developed a novel protein vaccine consisting of six tandemly repeated GRP epitopes with

the mycobacterial HSP65 as a carrier and first showed efficient control of the growth of breast cancer. HG6 has the potential to be developed as a suitable vaccine against various cancers.

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