Strong humoral response elicited by a DNA vaccine targeting gastrin-releasing peptide with optimized adjuvants inhibits murine prostate carcinoma growth *in vivo*

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Abstract

Previous studies demonstrated that the elevated expression and receptor binding of gastrin-releasing peptide (GRP) in various types of cancer suggest that GRP might be a putative target for immunotherapy in neoplastic diseases. DNA vaccine for hormone/growth factor immune deprivation represents a feasible and attractive approach for cancer treatment; nevertheless, there is still a need to increase the potency of the DNA vaccine. Here, based on six copies of the B cell epitope GRP_{18–27} in a linear alignment as an immunogen, we designed several anti-GRP DNA vaccines containing different combinations of immunoadjuvants, such as HSP65, tetanus toxoid_{830–844} (T), pan HLA-DR-binding epitope (PADRE) (P), and mycobacterial HSP70_{407–426} (M), on a backbone of pCR3.1 plasmid vector with eight 5'-GACGTT-3' CpG motifs and the VEGF183 signal peptide (VS). The effects of these immunoadjuvants in enhancing GRP-specific humoral immune response were then evaluated by comparing the respective immunogenicity and antitumor effects. Immunization of mice with pCR3.1-VS-HSP65-TP-GRP6-M2 elicited much higher levels of specific anti-GRP antibodies and more effectively inhibited the growth of a GRP-dependent tumor RM-1 *in vivo*. Interestingly, plasmids encoding for 2HSP70_{407–426}, but not the one with 1 or 3HSP70_{407–426} showed stronger immune stimulatory potential as well as impressive antitumor activity, suggesting that 2HSP70_{407–426} is an efficient molecular adjuvant for developing self-epitope vaccines. The highly immunogenic, potent anti-tumorigenic and antiangiogenesis activities of the anti-GRP DNA vaccine offered a novel immunotherapeutic approach in the treatment of GRP-dependent tumors and their complications.

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Introduction

Over the last two decades, several lines of experimental evidence have suggested that the gastrin-releasing peptide (GRP) may act as a growth factor in many types of cancer. The GRP mediates its effects by binding to the GRP receptor (GRPR) which is expressed aberrantly in various cancer cells (Cornelio *et al.* 2007). In addition, recent data show that GRP is not only an autocrine mitogen, but also has paracrine and endocrine effects, and functions as a morphogen and a

pro-angiogenic agent (Xiangping *et al.* 2003). This elevated expressions and receptor-binding activity of GRP in various types of cancer suggest that GRP might be a viable target for immunotherapy in neoplastic diseases.

DNA vaccines have become an attractive approach for generating antigen-specific immunotherapy. The naked plasmid DNA is safe, has low immunogenicity, and can be repeatedly administered. DNA vaccines can be easily prepared on a large scale with high purity and are highly stable relative to proteins and other biological polymers (Donnelly *et al.* 1997). However,

a key issue in developing subunit DNA vaccines is their relatively weak immunogenicity. Consequently, alternative strategies have been considered and tested experimentally to avoid this significant problem. This led to the identification of effective DNA vaccine delivery systems and the optimization of the expression of the encoded vaccine antigens (Greenland & Letvin 2007), DNA-elicited immune responses can be enhanced and modulated by the use of plasmid-based molecular adjuvants, for example, targeting antigens to the endoplasmic reticulum for rapid intracellular degradation (Hung *et al.* 2001), directing antigens to antigen presenting cells (APCs) by fusion to ligands for APC receptors (Cusi *et al.* 2004), fusing antigens to chemokines (Biragyn *et al.* 1999) or to pathogen sequences (King *et al.* 1998), coinjecting cytokines (Calarota & Weiner 2004), costimulatory molecules (Calarota & Weiner 2004) and co-administration with CpG oligonucleotides (Klinman *et al.* 1997). However, DNA vaccines with these adjuvants have not been proven to induce impressive protection against infection of several pathogens or neoplastic diseases, particularly in non-human primates. Thus, additional research for novel and potent adjuvants for DNA vaccine is needed.

In the current study, anti-GRP DNA vaccines have been designed to induce specific deprivation of GRP by a strong humoral immune response. To break the already established tolerance of this self-peptide *in vivo*, six copies of the B cell epitope GRP_{18–27} (Papac *et al.* 1994) in a linear alignment (GRP6) have been inserted into an eukaryotic expression vector (pCR3.1) as an immunogen. Furthermore, tandem repeated GRP_{18–27} was fused to different immunoadjuvants to optimize its immunogenicity, including mycobacterial heat shock protein 65 kDa (HSP65; Perraut *et al.* 1993), tetanus toxoid fragment 830–844 (Kumar *et al.* 1992), pan HLA-DR-binding epitope (Alexander *et al.* 2000), and mycobacterial HSP70 fragment 407–426 (Wang *et al.* 2005). The efficacy of the anti-GRP DNA vaccines were tested on the RM-1 murine prostate carcinoma model, the growth of which can be regulated by GRP and anti-GRP antibodies (Abs) *in vitro*. Finally, the inhibition of tumor-induced angiogenesis induced by active immune responses was evaluated in an intradermal tumor model *in vivo*.

Materials and methods

Tumor cell line and mice

The murine RM-1 prostate carcinoma cell line was obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences,

Beijing, China. Tumor cells were cultured in growth medium containing 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% CO₂. For all experiments, male C57BL6 mice, 5 weeks old, obtained from the Chinese Academy of Medical Sciences, Beijing, China were used. Animals were housed in pathogen-free conditions during the studies, and their care was in accord with institutional guidelines. All procedures in animal experiments were approved by the Institutional Animal Ethical Committee of China Pharmaceutical University.

Construction of DNA vaccines

Construction of plasmid

pCR3.1-VS-HSP65-TP-GRP6

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 the eukaryotic expression vector. The gene encoding HSP65-GRP6 was obtained by PCR from plasmid pET28a-HG6 (Guojun *et al.* 2008), using A1 (5'-CACCCATGGCCAAGACAATT-3') and A2 (5'-ATCTAGACGCTAAGCTTGCG-3') as forward and reverse primers, respectively. The gene encoding tetanus toxoid_{830–844}-PADRE (TP) was amplified by PCR with B1 (5'-GCGTCTAGAGGTGGCGGTGGC-TCCGGTGGCGGTGGCTCCGGATCTCAGTACATCAAGTCAAGG-3'), B2 (5'-GATCTCAGTACATCAAGGCTAACTCTAAGTTCATCGGCATTACCGAGCTGTCCCTG-3'), B3 (5'-CTTCAGGGTCCAAGCGGCCACGAAGTGGCAGAGCCAGGGGACAGCTCGGTAATGCC-3'), B4 (5'-ACTTGCTAGCGGATCCAGGGCCAGGGCCAGCAGCGGCCTTCAGGTCCAAGCGGCC-3') as both primers and templates. Both the plasmid vector and gene encoding HSP65-GRP6 were digested by NcoI and HindIII, and then ligated. The newly formed plasmid was named pCR3.1-VS-HSP65-GRP6 and digested again by NheI and treated with calf intestinal alkaline phosphatase (CIAP), then ligated with TP gene digested by NheI and XbaI to construct the plasmid pCR3.1-VS-HSP65-TP-GRP6.

Construction of plasmid containing tandem repeats (from 1 to 3) of HSP70_{407–426} gene (M)

These three plasmids were constructed according to the method described by Yankai *et al.* (2006) with slight modifications. The encoding sequence of M gene was synthesized by PCR with two

oligonucleotides used as both primers and templates: C1 (5'-ATAGCTAGCGCCAGATCTCCTGGCAGC-CAGCCTTCCGTGCAGATCCAGGTCTACCAGG-GCGAGCGC-3'), C2 (5'-GCGAAGCTTAGCGTCTA-GATGAGG ATCCCTTGTGTGAGCGGCAATCT-CGCGCTCGCCCTGGTAGAC-3'). M gene was inserted into the plasmid pCR3.1-VS-HSP65-TP-GRP6 behind GRP6 gene.

Construction of plasmid pCR3.1-VS-HSP65-TP-M2

The gene encoding two copies of M (M2) was amplified by PCR from plasmid pCR3.1-VS-HSP65-TP-GRP6-M2 using D1 (5'-TTAGCTAGCGCCAGATCTCCTGG-CAGC-3') and D2 (5'-TTAAAGCTTAGCGTCTA-GATGAGGATC-3') as forward and reverse primers respectively. pCR3.1-VS-HSP65-TP-M2 was constructed by digestion of pCR3.1-VS-HSP65-TP-GRP6-M2 with NheI and HindIII, and then ligated with M2 digested by the same restriction enzymes. This plasmid served as a vaccine control.

All constructed DNA vaccines and controls listed in Fig. 1 were verified by DNA sequencing.

DNA immunization procedure

Plasmid DNA used for immunization was purified using Qiagen Plasmid Mega Kit (Qiagen) and suspended in sterile saline at a concentration of

0.5 µg/µl. A total of eight C57BL6 male syngeneic mice ($n=8$) were used for each experimental group (one group for each of the six plasmid DNA and saline control). Mice were anesthetized via i.m. injections in the anterior thigh with 100 µl of 0.25% bupivacaine hydrochloride solution. Seventy-two hours later, mice received i.m. injections of 100 µl each of the plasmid DNA vaccines (0.5 µg/µl) or saline. Booster injections were given every other week (weeks 3 and 5) using the same protocol. Sera were taken at various time points for later analyses of antibody titers.

Detection of antibodies by ELISA

The amount of anti-GRP Abs and anti-HSP65 Abs in the immune sera was determined using ELISA as described by Yankai *et al.* (2006). Briefly, 96-well flat-bottomed ELISA plates were coated with 2 µg/well of GRP_{1–27} peptide (Sigma) or 2 µg/well of recombinant HSP65 (Xu *et al.* 2008). Plates were washed and blocked, and individual sera from immunized and control mice were added to the wells at the indicated dilutions. This was followed by incubation in HRP-conjugated goat anti-mouse IgG (Sigma). The reaction was completed using 3,3',5,5'-tetramethylbenzidine and absorbances were measured at a wavelength of 450 nm. Endpoint titers were defined as the dilution where the optical density (OD₄₅₀) exceeded the cut-off value. Cut-off was calculated as the mean OD₄₅₀ + three times the s.d., from sera from control plasmid vaccinated mice at a dilution of 1:200. Each sample in the assay was performed in duplicate.

To determine the specific isotypes, individual sera from mice were diluted 1:200 and tested in duplicate. For detection of mouse IgG1 and IgG2a isotypes, we used anti-mouse Ig subclass-specific, HRP-conjugated secondary Abs (Sigma).

Specificity of anti-GRP and anti-HSP65 antibodies

The method for Dot Blots was similar to that described by Perschinka *et al.* (2003) and Guojun *et al.* (2008). Briefly, a 2 µg/dot GRP_{1–27} was air-dried on a nitrocellulose membrane (Millipore, Bedford, MA, USA). For detection of the specificity of anti-HSP65 Abs, purified HSP65 recombinant proteins were separated on a 12% SDS-PAGE gel, then transferred onto nitrocellulose membranes. The membranes were blocked, washed and probed with 1:200 dilution of pooled immune sera collected from mice immunized with various DNA vaccines. Blots were then washed and incubated with HRP-conjugated goat anti-mouse

Figure 1 Schematic diagram of the constructed anti-GRP DNA vaccines. Take pCR3.1-VS-HSP65-TP-GRP6-M2 as an example. The VS gene (VS,) was placed under the control of promoter CMV ( ) followed sequentially by the genes encoding for HSP65 ( ) tetanus toxoid_{830–844} (T,) PADRE (P,) six tandem repeats of hGRP_{18–27} (GRP6,) and two copies of mycobacterial HSP70_{407–426} (M2,)

IgG (Sigma). The reaction was developed by using 0.05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.012% (v/v) H₂O₂ for 15 min at 37 °C.

Determination of relative avidity of anti-GRP antibodies

The method for avidity assays was similar to that described by Pullen *et al.* (1986). Briefly, ELISA plates were coated with 2 µg/well of GRP_{1–27} peptide. Plates were blocked and then incubated with 100 µl/well 1:200 diluted sera from pCR3.1-VS-HSP65-TP-GRP6 immunized mice and 100 µl/well 1:12 800 diluted sera from pCR3.1-VS-HSP65-TP-GRP6-M2 immunized mice (sera were collected 2 weeks after the last booster injection). After incubation, wells were washed and incubated with 100 µl of serial dilutions of 0–3 M sodium thiocyanate (NaSCN, Sigma), a chaotropic compound that interferes with the antigen–antibody reaction, in PBS. The wells were incubated for 15 min at room temperature before washing. The remainder of the assay was done similar to the ELISA analysis for anti-GRP Abs.

T cell proliferation assay

Fourteen days after the last nasal immunization, mice were killed to obtain splenocytes. Proliferation of purified T cells to various antigens was detected following the procedure previously described (Jin *et al.* 2008). Briefly, 100 µl T cell cultures (5 × 10⁶ cells/ml) were incubated for 72 h in the presence of 10 µg/ml HSP65, tetanus toxoid_{830–844}, PADRE, and mHSP70_{407–426} respectively (peptides were synthesized by using an automated multiple peptide synthesizer following the company's protocols for *N*-α-fluorenylmethoxycarbonyl synthesis). ConA (1.25 µg/ml) was used as a positive control. Each sample was plated in triplicate. T cell responses were detected by the methyl thiazolyl tetrazolium (MTT) method.

Affinity chromatography isolation of anti-GRP antibodies

Recombinant GFP–GRP_{18–27} proteins (Guojun *et al.* 2008) was coupled to NHS-activated sepharose 4FF (Pharmacia) according to the manufacturer's recommendation. Pooled anti-GRP sera from mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 were inactivated and precipitated twice with 50%-saturated and 33%-saturated (NH₄)₂SO₄ respectively. Then the precipitated IgGs, dialyzed against PBS overnight, were added to the column. After 30 min

incubation, 20 mmol/l Gly-HCl buffer (pH 2.5) was added to wash antibodies. The eluant was immediately neutralized with 1 mol/l Tris-HCl (pH 8.0), then dialyzed against PBS. The concentration of anti-GRP Abs was determined with the Coomassie Plus protein assay reagent kit (Pierce, Rockford, IL).

Cell proliferation assay

The effects of the GRP_{1–27} full peptide, immune sera or purified anti-GRP Abs on the proliferation of RM-1 cells were measured using the MTT assay (Guojun *et al.* 2008). Briefly, RM-1 cells plated on 96-well plates at 1 × 10⁴ cells per well were incubated with serial dilutions of the GRP_{1–27} peptide, pooled immune sera from each immunized group or purified anti-GRP Abs for 48 h followed by 20 µl of MTT for 4 h. At the end of the incubation, absorbances were read at 570 nm. All assays were performed in triplicate.

RM-1 prostate carcinoma model

In order to analyze the antitumor effect of anti-GRP DNA vaccines, a vaccination protocol was designed as shown in Fig. 2A. Mice (eight per group) were immunized three times every 2 weeks followed by s.c. injections of 1 × 10⁶ RM-1 cells into the left flank. After 14 days, the animals were killed, and solid tumors were excised and weighed.

Quantification of angiogenesis *in vivo*

As shown in Fig. 2A, a vaccination protocol was designed for each experimental group (four mice per group). 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). At day 0, mice were anesthetized (inhalant 4% Halothane-vet), and 2.5 × 10⁶ RM-1 viable cells in 50 µl of PBS were implanted intradermally at two sites in the abdominal region. Two additional injections of PBS (50 µl) 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. When the intradermal tumor grows up to 5 mm in diameter, a section of the abdominal wall skin encompassing all implant sites was removed and spread onto filter paper. Sections were examined by light microscopy (×10 magnification), and the total number of blood vessels (major vessels and branching points) was determined within a 1 cm² area around each implant site. Vessel counts from implants

Figure 2 Effects on RM-1 tumor model of anti-GRP DNA vaccine. (A) The immunization scheme indicates the days of vaccination by arrows. Mice were immunized with anti-GRP DNA vaccines day -42 , -28 , and -14 . At day 0, 1×10^6 RM-1 prostate tumor cells were injected subcutaneously for tumor cell challenge. Fourteen days later, all mice were killed and tumors were excised and weighted. (B) The RM-1 prostate tumors from immunized mice. The picture shows the tumors extracted from mice immunized with saline (a, control $n=8$), pCR3.1-VS-HSP65-TP-M2 (b, control $n=8$), pCR3.1-VS-HSP65-TP-GRP6 (c, $n=8$), pCR3.1-VS-HSP65-TP-GRP6-M1 (d, $n=7$), pCR3.1-VS-HSP65-TP-GRP6-M2 (e, control $n=8$).

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.

Histological evaluation of the intradermal tumors

Intradermal tumors (five tumors per group) were fixed in 4% paraformaldehyde, paraffin embedded, cut into 5 μ m sections. Periodic acid–Schiff (PAS) staining with hematoxylin counterstaining was then performed to the tumor sections.

Statistical analysis

Data were collected and subjected to statistical analyses using Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Induction of anti-GRP and anti-HSP65 antibodies in immunized mice

To investigate whether DNA vaccines (Fig. 1) containing 6 tandem repeats of GRP_{18–27} could evoke a strong humoral immune response, we compared the

levels of GRP-specific IgG in the sera collected from immunized mice by ELISA (Fig. 3). Sera samples were collected at 3, 5, and 7 weeks after initial immunization. All GRP-based DNA vaccines, as compared with the control vaccine (pCR3.1-VS-HSP65-TP-M2) or saline control, greatly increased titers of specific anti-GRP Abs 5 weeks post-inoculation and the antibody levels remained high up to 7 weeks post-inoculation. The average anti-GRP Abs titers in mice immunized with the plasmid containing two copies of the M (endpoint dilution: 51 200) were the highest 7 weeks post-immunization as compared with other GRP-based vaccines (endpoint dilution: from 800 to 3200). These data clearly demonstrated that two copies of HSP70_{407–426} gene were highly potent in augmenting humoral response and immunogenicity of the anti-GRP vaccine.

Interestingly, the antibody response to GRP (endpoint dilution: 51 200) was superior to the carrier protein HSP65 (endpoint dilution: 6400) when plasmids containing two copies of the M (Fig. 4A) were present. It appears that the reduced humoral responses to the carrier protein may allow a greater portion of the immune response to be directed to the therapeutically relevant portion of the vaccine.

Figure 3 Detection of anti-GRP antibodies. Each group of mice ($n=8$) was immunized i.m. with 50 μ g of plasmids at 1, 3, and 5 weeks. Individual serum samples were collected at 3 weeks (A), 5 weeks (B), and 7 weeks (C). The sera were tested for the presence of anti-GRP Abs in ELISA. Data represent mean $\pm$ S.E.M. Mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 produced the highest titers of anti-GRP Abs than any other groups. (D) The specificity of anti-GRP Abs elicited in immunized mice was established by immunoblots using GRP₁₋₂₇ as antigen. Sera from mice immunized with all GRP-based DNA vaccines reacted with GRP₁₋₂₇ (dots 3–7), while sera from vaccine control group or saline group did not react with GRP₁₋₂₇ (dots 1–2).

Anti-GRP and anti-HSP65 antibodies analysis by western blot

The specificity of antibodies elicited in immunized mice was established by immunoblots using GRP₁₋₂₇ and recombinant HSP65 protein as antigens. Antibodies from mice immunized with all GRP-based DNA vaccines reacted with GRP₁₋₂₇ (dots 3–7, Fig. 3D), while sera from vaccine control group or saline group did not react with GRP₁₋₂₇ (dots 1–2, Fig. 3D). Specific anti-HSP65 Abs were also detected by immunoblots using purified HSP65 as antigen (Fig. 4B). These findings suggested that the antibodies in the immune sera specifically recognized the GRP and HSP65 antigens.

Relative avidity of anti-GRP antibodies

To further investigate the relationship between the antibody titers and the biological effects of the GRP vaccines, avidity assays were performed on diluted antisera with equalized anti-GRP Abs titers from mice ($n=8$, week 7) immunized with

pCR3.1-VS-HSP65-TP-GRP6 or pCR3.1-VS-HSP65-TP-GRP6-M2. The relative avidities of antibodies were observed based on the concentration of NaSCN required to depress the value of OD₄₅₀ to half its initial value. The mean relative avidity of antibodies in the group vaccinated with pCR3.1-VS-HSP65-TP-GRP6-M2 was significantly higher than that from the group immunized with pCR3.1-VS-HSP65-TP-GRP6 (1.09 ± 0.14 vs 0.64 ± 0.11 M, $P < 0.01$; Fig. 5C). All these data clearly demonstrated that two copies of HSP70₄₀₇₋₄₂₆ gene were highly potent in augmenting the humoral response and immunogenicity of the anti-GRP vaccine.

HSP70₄₀₇₋₄₂₆-specific T cell responses induced Th1-polarized immune responses

To investigate which T cell epitopes are responsible for the generation of Th cells, T cell proliferation to various T cell epitopes was tested (Fig. 5A). Although T cell proliferation was noticeably induced by the stimulation of ConA in saline, pCR3.1-VS-HSP65-TP-GRP6 and pCR3.1-VS-HSP65-TP-GRP6-M2

Figure 4 Detection of anti-HSP65 antibodies. Each group of mice ($n=8$) was immunized i.m. with 50 μ g of plasmids at 1, 3 and 5 weeks. Individual serum samples were collected at 7 weeks (A). The sera were tested for the presence of anti-HSP65 Abs in ELISA. Data represent mean $\pm$ S.E.M. Mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 produced the lowest titers of anti-HSP65 Abs than any of the other anti-GRP DNA vaccine immunized groups. (B) The specificity of anti-HSP65 Abs elicited in pCR3.1-VS-HSP65-TP-GRP6-M2 immunized mice was established by immunoblots using HSP65 as antigen. (a) Proteins on nitrocellulose membrane stained by ponceau after being transferred from 12% SDS-PAGE gel. (b) Result of western blotting (lane1, protein molecular weight marker; lane2, HSP65). Sera from mice immunized with other plasmids showed the similar results (data not shown).

treated groups, HSP65, tetanus toxoid_{830–844}, and PADRE did not induce T cell proliferation. Importantly, T cells isolated from mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 induced strong T cell proliferation after stimulation with HSP70_{407–426}, indicating HSP70_{407–426} was responsible for the generation of Th cells and GRP-specific Abs.

When functioning *in vivo* as an adjuvant, the C-terminal portion of mHSP70 (aa 359–610) is well known for the induction of Th1 polarized immune responses (Wang *et al.* 2002). In addition, mHSP70_{407–426} was identified as a major epitope

stimulating Th1-polarizing cytokines (Wang *et al.* 2005). In this study, we measured the production of IgG1 (Th2 response) and IgG2a (Th1 response) anti-GRP Abs in our experiments to indirectly demonstrate the relative contributions of Th2 versus Th1 responses (Agadjanyan *et al.* 2005). As expected, the anti-GRP Abs induced by pCR3.1-VS-HSP65-TP-GRP6-M2 were almost exclusively of IgG2a

Figure 5 HSP70_{407–426}-specific T cell responses induced a Th1-polarized immune response. Mice ($n=4$) were immunized with pCR3.1-VS-HSP65-TP-GRP6-M2, pCR3.1-VS-HSP65-TP-GRP6 and PBS. Two weeks after the last immunization, the spleens were removed and the T cell proliferative responses were assayed *in vitro* after 72 h of stimulation with 10 μ g/ml mHSP65, tetanus toxoid_{830–844}, PADRE, and mHSP70_{407–426} or 1.25 μ g/ml T cell mitogen ConA. Data were expressed as mean $\pm$ S.E.M. $**P<0.01$ compared with medium treated T cells in the same group. (B) IgG subclass determination. Sera from mice ($n=8$, week 7) immunized with pCR3.1-VS-HSP65-TP-GRP6-M2, pCR3.1-VS-HSP65-TP-GRP6 were tested on GRP_{1–27}-coated wells and detected with peroxidase conjugated monoclonal anti-mouse IgG1 and IgG2a Abs. Data were expressed as mean $\pm$ S.E.M. (C) Diluted antisera with equalized anti-GRP Abs titers from mice ($n=8$, week 7) immunized with pCR3.1-VS-HSP65-TP-GRP6 or pCR3.1-VS-HSP65-TP-GRP6-M2 were examined for their binding avidity to GRP in a modified ELISA. The results were expressed as the concentration of sodium thiocyanate (NaSCN) that was required to disrupt antibodies binding to GRP. Anti-GRP Abs from sera of mice immunized with HSP70_{407–426}-containing plasmids had significantly higher relative avidity as compared with those without. $**P<0.01$.

subclass, which is indicative of Th1 response (Fig. 5B). Furthermore, we determined the IgG2a/IgG1 ratios for anti-GRP Abs. The IgG2a/IgG1 ratio for pCR3.1-VS-HSP65-TP-GRP6-M2 immunized mice (8.5) was 15 times greater than that for pCR3.1-VS-HSP65-TP-GRP6 (0.56). These data suggest that the introduction of two copies of HSP70₄₀₇₋₄₂₆ as a T-helper epitope induced a highly Th1-polarized immune responses in mice.

GRP₁₋₂₇ peptide stimulated the proliferation of RM-1 cells *in vitro*

To determine the effect of GRP on murine prostate carcinoma, RM-1 cells plated in 96-well plates were incubated with full GRP peptide (GRP₁₋₂₇) in concentrations of 10⁻⁵–10⁻¹¹ mol/l. The number of RM-1 cells increased in a dose-dependent fashion in direct relation to the concentration of the full-length peptide GRP₁₋₂₇ (Table 1). A significant mitogenic effect, as compared with control, was observed starting initially at 10⁻⁹ mol/l (SR%=17.6%; *P*<0.05) and increased at higher doses with a stimulation rate of 57.9% at 10⁻⁵ mol/l.

Anti-GRP Abs inhibited the growth of RM-1 cells *in vitro*

To determine the inhibitory effect of anti-GRP Abs on RM-1 cells *in vitro*, cultured RM-1 cells were incubated with antibodies against GRP (affinity purified) 50 to 0.87 µg/ml. The growth of RM-1 cells was suppressed by anti-GRP Abs in a dose-dependent manner (Table 2). Significant inhibitory effects as compared with control were observed initially at 1.56 µg/ml (inhibition rate % (IR%)=19.7%; *P*<0.05) and further increased at higher doses with an IR of 43.3% at 50 µg/ml. Moreover, similar results were also obtained *in vitro* showing inhibition of RM-1 cell proliferation in a dose-dependent manner by the antisera from pCR3.1-VS-HSP65-TP-GRP6-M2 immunized mice (Table 3). With the dilutions at 1:10 and 1:2, the antisera showed IRs similar to that

obtained from purified anti-GRP Abs at 1.56 and 12.5 µg/ml, respectively. Antisera from other groups of mice did not significantly inhibit the proliferation of RM-1 cells, possibly due to the low titers of anti-GRP Abs generated in these groups of mice.

Effect on RM-1 prostate carcinoma model

To determine whether vaccination with pCR3.1-VS-HSP65-TP-GRP6-M2 could protect mice against RM-1 prostate carcinoma and whether the introduction of two copies of the HSP70₄₀₇₋₄₂₆ gene in this DNA vaccine would also enhance the anti-tumor effects along with remarkably increased levels of specific antibodies, we applied the RM-1 prostate carcinoma model in this study. The plasmids pCR3.1-VS-HSP65-GRP6 and pCR3.1-VS-HSP65-TP-GRP6-M3 were excluded from this study because the former elicited an anti-GRP Abs level comparative to that elicited by pCR3.1-VS-HSP65-TP-GRP6, and the latter elicited an anti-GRP Abs level comparative to that elicited by pCR3.1-VS-HSP65-TP-GRP6-M1. The size and weights of tumors removed from all immunized mice on day 14 after tumor cell challenge of the five experimental groups are shown in Fig. 2B and Table 4 respectively. The tumor weights distinctly indicated that tumor regression was more pronounced in the animals immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 compared with that in the mice immunized with saline (*P*=0.004), pCR3.1-VS-HSP65-TP-M2 (*P*=0.0003), pCR3.1-VS-HSP65-TP-GRP6 (*P*=0.003), or pCR3.1-VS-HSP65-TP-GRP6-M1 (*P*=0.002). However, tumor weight in mice immunized with pCR3.1-VS-HSP65-TP-M2 (*P*=0.985), pCR3.1-VS-HSP65-TP-GRP6 (*P*=0.238), or pCR3.1-VS-HSP65-TP-GRP6-M1 (*P*=0.579) was not significantly lower than that of the saline control. As the results show, pCR3.1-VS-HSP65-TP-GRP6-M2 could strongly elicit anti-tumor immunity against the growth of RM-1 mainly benefiting from the introduction of two repeats of M fragments after the GRP6 fragment.

Table 1 GRP₁₋₂₇ peptides stimulate the proliferation of cultured RM-1 cells

Dose (mol/l)	A ₅₇₀ (x±s, n=3)	SR%	Dose (mol/l)	A ₅₇₀ (x±s, n=3)	SR%
Control	0.693±0.097		10 ⁻⁸	0.886±0.009 [†]	27.8
10 ⁻¹¹	0.767±0.159	10.7	10 ⁻⁷	0.972±0.065 [†]	40.2
10 ⁻¹⁰	0.788±0.043	13.7	10 ⁻⁶	1.003±0.084 [†]	44.7
10 ⁻⁹	0.816±0.134*	17.6	10 ⁻⁵	1.095±0.117 [†]	57.9

**P*<0.05 versus control; [†]*P*<0.01 versus control; SR%, stimulation rate (%).

Table 2 Suppression on the proliferation of RM-1 cells with different gastrin-releasing peptide (GRP) Abs level

Dose ($\mu\text{g/ml}$)	A_{570} ($\bar{x} \pm s$, $n=3$)	IR%	Dose ($\mu\text{g/ml}$)	A_{570} ($\bar{x} \pm s$, $n=3$)	IR%
Control	1.236 ± 0.117	—	6.25	$0.913 \pm 0.017^{\dagger}$	26.1
0.78	1.080 ± 0.049	12.6	12.5	$0.865 \pm 0.025^{\dagger}$	30.1
1.56	$0.993 \pm 0.054^*$	19.7	25	$0.804 \pm 0.056^{\dagger}$	35.0
3.13	$0.942 \pm 0.069^*$	23.8	50	$0.701 \pm 0.031^{\dagger}$	43.3

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

Intradermal tumor angiogenesis

To assess the effect of the immune response on tumor-associated angiogenesis induced by anti-GRP DNA vaccine pCR3.1-VS-HSP65-TP-GRP6-M2, RM-1 tumor cells were implanted intradermally at two sites in the abdominal region. Three groups of mice were immunized with saline, vaccine control pCR3.1-VS-HSP65-TP-M2, or anti-GRP DNA vaccine pCR3.1-VS-HSP65-TP-GRP6-M2 respectively, as described earlier. As shown in Fig. 6, tumor cells implanted intradermally were found to induce significant angiogenesis when the intradermal tumor grows up to 5 mm in diameter. The total number of blood vessels around each implant site from mice immunized with anti-GRP DNA vaccine pCR3.1-VS-HSP65-TP-GRP6-M2 was significantly lower than blood vessels from saline (21 ± 7 vs 56 ± 9 ; $P < 0.001$) and mice immunized with vaccine control pCR3.1-VS-HSP65-TP-M2 (21 ± 7 vs 60 ± 9 ; $P < 0.001$).

Histological examination of tumor sample sections by PAS staining

Sections of intradermal tumors were stained with PAS base staining and viewed on the microscope. As shown in Fig. 7, tumor sections from the vaccine control group or saline group have been detected with the

presence of PAS-positive loops which are known to be a marker of aggressive tumors. In contrast, these PAS-positive loops were highly diminished in the tumor sections from pCR3.1-VS-HSP65-TP-GRP6-M2 immunized group or even absent in some sections.

Discussion

Over the past 20 years, abundant evidence has been collected to suggest that GRP and its receptors play an important role in the development of a variety of cancers (Patel *et al.* 2006). These observations have inspired researchers to find a suitable approach to treat cancers by taking advantage of blocking the GRP to GRPR signal pathway. 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 (Chaudhry *et al.* 1999). In addition, such cytotoxic peptide conjugates as doxorubicin–bombesin conjugates (Nagy *et al.* 1997, Szepeshazi *et al.* 2003) and camptothecin–bombesin conjugates (Moody *et al.* 2004) have also shown impressive antitumor activity. In the current investigation, the strategy of GRP

Table 3 Suppression on the proliferation of RM-1 cells with immune sera

Group	A_{570}^a ($\bar{x} \pm s$, $n=3$)	IR%	A_{570}^b ($\bar{x} \pm s$, $n=3$)	IR%
Control	1.174 ± 0.058	—	1.166 ± 0.055	—
Saline	1.187 ± 0.041	−1.2	1.162 ± 0.069	0.4
pCR3.1-VS-HSP65-TP-M2	1.170 ± 0.046	−0.3	1.151 ± 0.087	1.3
pCR3.1-VS-HSP65-GRP6	1.160 ± 0.035	0.6	1.118 ± 0.083	4.1
pCR3.1-VS-HSP65-TP-GRP6	1.133 ± 0.067	2.9	1.048 ± 0.087	10.1
pCR3.1-VS-HSP65-TP-GRP6-M1	1.167 ± 0.046	−0.1	1.108 ± 0.084	5.0
pCR3.1-VS-HSP65-TP-GRP6-M2	$0.918 \pm 0.062^*$	21.3	$0.795 \pm 0.054^*$	31.8
pCR3.1-VS-HSP65-TP-GRP6-M3	1.152 ± 0.053	1.2	1.105 ± 0.086	5.8

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

^a10% of sera in culture medium (10% of PBS in control culture medium).

^b50% of sera in culture medium (50% of PBS in control culture medium).

Table 4 Average tumor weight of tumors from immunized mice

Group	Tumor weight/g	Inhibitory rate/%
Saline	2.025 ± 0.766	–
pCR3.1-VS-HSP65-TP-M2	2.018 ± 0.492	0.31
pCR3.1-VS-HSP65-TP-GRP6	1.652 ± 0.388	18.41
pCR3.1-VS-HSP65-TP-GRP6-M	1.833 ± 0.486	9.47
pCR3.1-VS-HSP65-TP-GRP6-M2	0.748 ± 0.440*	63.06

* $P < 0.01$ versus saline.

deprivation by anti-GRP DNA vaccines has been applied and proved to be an appealing approach for cancer treatment. This vaccine-based therapy has substantial advantages over these previously described cancer chemotherapies: it is less toxic and allows long-term treatments while significantly reducing the cost involved in cancer treatment.

The mechanisms underlying the ability of the anti-GRP DNA vaccine to inhibit the proliferation of RM-1 cells *in vivo* have yet to be elucidated. It is clear that the humoral immune response has been elicited by vaccination of DNA vaccine with a high titer of GRP-specific Abs detected. This phenomenon suggests that GRP-specific Abs neutralize the self-peptide GRP and lower the concentration of GRP around the tumor, the activation of GRPR may therefore be interrupted and the successive signalling pathways can be blocked. Very recently, Garcia *et al.* (2008) reported the humoral immune response of patients enrolled in a randomized phase II clinical trial using epidermal growth factor (EGF) vaccination blocked the binding of EGF to its receptor (EGFR), which slowed down tumor cell proliferation and correlated with survival in vaccinated non-small-cell lung cancer (NSCLC) patients. The strategy of exploiting anti-EGF autoantibodies to block the EGF/EGFR autocrine loop reduces the growth rate of EGF-dependent tumors, which can also support the function of anti-GRP autoantibodies in reducing the growth rate of GRP-dependent tumors. Both GRP and EGF deprivation therapy can be classified into therapeutic interventions aiming to deprive cancer cells of their required hormone or growth factor, as reviewed in Gonzalez & Lage (2007). Clinical trials have been initiated using immunization with human chorionic gonadotrophin, gastrin, LHRH/GnRH, and EGF. Preliminary data already show that both the antibody titers and the decrease in the hormone level are related to survival. These vaccines are not meant to destroy the tumor by inducing immune effector mechanisms, but to re-set the cell proliferation rate below tumor cell death rate, resulting in inhibition of further tumor cell growth.

Although the target sequence chosen in this study is a B cell epitope, the potential T cell epitope in this region of GRP might also exist. With this hypothesis, the T cell-mediated immune responses induced by anti-GRP vaccines have been tested *in vitro* as described elsewhere, for example, Jin *et al.* (2008). Based on our unpublished results, splenocytes isolated from mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 induced strong T cell proliferation after stimulation with ConA, but not with chemo-synthesized peptide GRP_{18–27}. In addition, the supernatants of splenocyte culture were used for detection of interferon- γ (IFN- γ) and interleukin-10 (IL-10) by ELISPOT technique. As expected, splenocytes from immunized mice did not generate IFN- γ or IL-10 cytokines after stimulation with peptide GRP_{18–27}. All these results definitely showed that a T cell-mediated immune response targeting GRP was not induced after immunization with anti-GRP DNA vaccine. The target sequence chosen in this study is merely a B cell epitope.

Figure 6 Effects of anti-GRP vaccine on the angiogenesis of intradermal tumors. Light microscopical picture of RM-1 tumor cells implanted intradermally in the anterior abdominal wall and the development of new blood vessels. Tumor-associated angiogenesis (2/mouse, 4 mice/group) in mice injected with saline (A) and pCR3.1-VS-HSP65-TP-M2 control vaccine (B) appeared to be significantly higher than in mice immunized with the anti-GRP vaccine pCR3.1-VS-HSP65-TP-GRP6-M2 (C). Representative images were taken at 10 $\times$ objective. (D) The total number of blood vessels (major vessels and branching points) was determined within the precise 1 cm² area around each implant site. *** $P < 0.001$.

Figure 7 Light microscopic view of PAS-hematoxylin-stained tissue sections of intradermal tumors. Tumor sections from both saline group (A) and vaccine control group pCR3.1-VS-HSP65-TP-M2 (B) have been detected with the presence of PAS-positive loops within which red blood cells were identified. These PAS-positive loops were highly diminished in the tumor sections from pCR3.1-VS-HSP65-TP-GRP6-M2 immunized group (C). Arrows demonstrate PAS-positive loops with red blood cells.

GRP is a self-peptide, and appears to be very effective at establishing immunological tolerance. There are hardly any reports of circulating autoantibodies to hormone/growth factors being detected in

humans. Thus, the production of sufficient and sustained levels of GRP-specific antibody should require a special strategy to vaccine design, especially for subunit DNA vaccines with relatively weak immunogenicity. In the current DNA vaccine construction, human GRP_{18–27} was chosen as the targeting B cell epitope based on reports that the 7-amino-acid C-terminal sequence of Bombesin-like peptides (BLPs) is required for immunogenicity and bioactivity (Papac *et al.* 1994). In order to optimize its immunogenicity, the following strategies have been exploited: 1) GRP_{18–27} epitope was tandemly repeated six times to enhance the humoral immune response. Our previous study has established that increased immunogenicity as well as anti-tumor effects of immunogens containing tandemly repeated B cell epitopes is associated with the increased copy number of the target sequence (Yankai *et al.* 2006); 2) HSP65 served as a suitable and safe carrier molecule for delivering B cell epitopes to the immune system; 3) eight 5'-GACGTT-3' CpG motifs which can activate antigen presenting cells through Toll-like receptors (Shah *et al.* 2003) have been constructed into the plasmid vector as an adjuvant; and 4) synthetic peptides representing helper T-lymphocyte epitopes such as those encoded by tetanus toxoid fragment 830–844, pan HLA-DR-binding epitope, or mHSP70 fragment 407–426, have been incorporated as immuno-adjuvants. Our results showed that two copies of mHSP70_{407–426} epitope downstream of GRP6 not only significantly boosted the anti-GRP Abs in sera, but also markedly enhanced the relative avidity of anti-GRP Abs.

Studies have already shown the linkage of antigens to both mHSP70 and the C-terminal portion of mHSP70 (359–610) to represent a potential approach to increase the potency of DNA vaccines (Chen *et al.* 2000, Li *et al.* 2006). We have now introduced the peptide HSP70_{407–426}, identified as a major epitope stimulating tumor necrosis factor- α , IL-12, and CCL-5 in monocytes and dendritic cells in mHSP70 (Wang *et al.* 2005), into anti-GRP DNA vaccines as immunoadjuvants. Our data also showed a highly Th1-polarized immune responses in mice when HSP70_{407–426} was used as an immunoadjuvant, which might be more beneficial in cancer treatment. Interestingly, plasmids encoding for 2HSP70_{407–426}, but not the one for 3HSP70_{407–426} showed stronger immune stimulatory potential as well as impressive antitumor activity. The reasons for this phenomenon could be that: 1) although mer-epitope effects can induce stronger immunogenicity of the epitope, as the number of the epitope copies increases, the

immunogenicity of this epitope becomes stronger, and this effect could then meet a crucial point in which the number is still increasing while the immunogenicity decreases (based on unpublished data from our lab). Thus, it is not the more repeated copies of epitope, but rather the moderate one which can induce the strongest immune responses; or 2) HSP70_{407–426} epitope in these plasmids is the T-helper epitope, and the leading role should be played by the GRP epitope. Too many repeated copies of HSP70_{407–426} will decrease the immune responses to GRP. The exact mechanism under this phenomenon is now under detailed investigation in our lab. However, the current results have shown that 2HSP70_{407–426} is an efficient molecular adjuvant for developing self-epitope vaccines.

GRP has been reported to stimulate pro-angiogenic gene expression as well as the expression of various angiogenic markers including platelet-endothelial cell adhesion molecule (PECAM-1), vascular endothelial growth factor (VEGF) and phosphorylated-Akt levels (Kang et al. 2007). Moreover, GRP or GRPR silencing has been reported to significantly suppress tumor progression and vascularization (Cornelio et al. 2007). In this study, the ability of anti-angiogenesis by anti-GRP DNA vaccine has been investigated in an intradermal tumor model. Our results demonstrate the immune responses induced by pCR3.1-VS-HSP65-TP-GRP6-M2 significantly reduced the tumor-associated angiogenesis and vascularization of RM-1 solid tumors. In addition, histological examination of intradermal tumors showed the PAS-positive loops were attenuated by GRP-immune arrest, indicating that intratumor microhemorrhages and tumor cell nutrition (which can be represented by the presence of PAS-positive loops (Lee et al. 2002, Clarijs et al. 2003)) were highly diminished. The anti-angiogenesis effects of plasmid pCR3.1-VS-HSP65-TP-GRP6-M2 may further hamper tumor progression, contributing to the inhibition of tumor growth *in vivo*.

In conclusion, we constructed a novel anti-GRP DNA vaccine with a high immunogenicity by the introduction of a new efficient molecular adjuvant 2HSP70_{407–426}. The highly immunogenic and potent anti-tumorigenic activities of the anti-GRP DNA vaccine offer a novel immunotherapeutic approach in the treatment of the GRP-dependent tumors and their complications.

Declaration of interest

There is no conflict of interest that would prejudice the impartiality of this scientific work.

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