Specific Antibodies Elicited by a Novel DNA Vaccine Targeting Gastrin-Releasing Peptide Inhibit Murine Melanoma Growth In Vivo

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The elevated expression and receptor binding of gastrin-releasing peptide (GRP) in various types of cancer, especially in malignant melanoma of the skin, suggest that GRP might be a putative target for immunotherapy in neoplastic diseases. We have therefore constructed a novel DNA vaccine coding for six tandem repeats of a fragment of GRP from amino acids 18 to 27 (GRP6) flanked by helper T-cell epitopes for increased immunogenicity, including HSP65, a tetanus toxoid fragment from amino acids 830 to 844 (T), pan-HLA-DR-binding epitope (PADRE) (P), and two repeats of a mycobacterial HSP70 fragment from amino acids 407 to 426 (M). The anti-GRP DNA vaccine (pCR3.1-VS-HSP65-TP-GRP6-M2) was constructed on a backbone of a pCR3.1 plasmid vector with eight 5'-GACGTT-3' CpG motifs and the VEGF183 signal peptide (VS). Intramuscular (IM) injections of anti-GRP vaccine in mice stimulated the production of high titers of specific antibodies against GRP and suppressed the growth of subcutaneous tumors of B16-F10 melanoma cells. Parallel results were obtained in vitro, showing inhibition of B16-F10 cell proliferation by GRP antisera. IM injections of the DNA vaccine also significantly attenuated tumor-induced angiogenesis associated with intradermal tumors of B16-F10 cells. In addition, lung invasion of intravenously injected cells was highly diminished, suggesting potent antimetastatic activity of the DNA vaccine. These findings support the highly immunogenic and potent antitumorogenic activity of specific anti-GRP antibodies elicited by the anti-GRP DNA vaccine.

In recent years, gastrin-releasing peptide (GRP) has been shown to be a potent mitogen for a variety of tumors (23). GRP plays an important role in human cancers exerting autocrine, paracrine, or endocrine growth factor effects (34). The GRP receptor (GRPR) is expressed aberrantly in various cancer cells (23), and GRP binding appears to activate multiple cellular signaling pathways, resulting in cellular proliferation and tumor formation (2, 8). Moreover, bombesin-like peptide (BLP) family members are involved in many steps of tumor progression, including angiogenesis (9, 14, 20) and distant metastasis (19, 22), resulting in increased aggressiveness and poorer prognosis of tumors.

Various GRPR antagonists, anti-GRP antibodies, and cytotoxic immunocomplexes have exhibited impressive antitumor activity both in vitro and in vivo against human and murine tumors (3). DNA vaccines targeting GRP represent another promising approach. However, a key issue in developing subunit DNA vaccines is their relatively weak immunogenicity. The effectiveness of subunit vaccines could be increased by delivering them with adjuvants. Previous studies have demonstrated that the mycobacterial 65-kDa heat shock protein (HSP65) exhibits strong immunogenicity and contains strong T-cell epitopes presented by major histocompatibility complex class II molecules; accordingly, it has been used as a helper T-cell epitope for delivering B-cell epitopes in vivo (24). The low immunogenicity of self-peptides can also be overcome by immunization with immunogens containing multiple copies of the self-peptides in linear alignment (36). In addition, unmethylated bacterial DNA oligonucleotides (CpG motifs) (16) and synthetic peptides representing helper T-lymphocyte epitopes, such as those encoded by a tetanus toxoid fragment from amino acids 830 to 844 (tetanus toxoid 830–844) (18), pan-HLA-DR-binding epitope (PADRE) (1), or mycobacterial HSP70 fragment 407 to 426 (HSP70407–426) (32), can be incorporated as immunoadjuvants in vaccines to augment immunogenicity and promote biostable antibody response.

In this study, we constructed anti-GRP DNA vaccines incorporating various immunoadjuvants and tested whether they could induce strong humoral responses in immunized mice. The efficacy of the anti-GRP DNA vaccines against tumor-associated angiogenesis or distant metastases was evaluated with various tumor models utilizing the well-characterized mouse melanoma B16-F10 cell line.

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In vivo tumor studies. Mice were immunized four times every 2 weeks followed by subcutaneous injections of 1 × 10^6 B16-F10 cells into the left flank. After 14 days, animals were sacrificed, and solid tumors were excised and weighed.

In vivo angiogenesis assay. To investigate the effects of anti-GRP DNA vaccines on tumor angiogenesis in vivo, an intradermal tumor model using B16-F10 cells was used. Tumor-associated angiogenesis was quantified by vessel counting methods described previously (17). Briefly, mice immunized four times every 2 weeks were injected intradermally with 1.0 × 10^6 B16-F10 cells in 50 μl of PBS at two sites in the anterior abdominal wall. When the tumors reached ~4 mm in diameter, the flap of the abdominal wall skin containing the injected cells was removed. Sections were examined by light microscopy at low magnification (>10), and the total number of blood vessels (major vessels and branch points) was determined within a 1-cm² area around each implant site.

In vivo assay for tumor metastasis. Mice immunized four times every 2 weeks were injected intravenously with 1.0 × 10^6 B16-F10 cells into the tail veins. After 21 days, mice were sacrificed, and each body organ was examined grossly for the presence of metastatic tumor nodules.

Statistical analysis. Data were collected and subjected to statistical analyses using Student’s t test. Differences were considered significant at a P value of <0.05.

RESULTS

DNA vaccines elicited production of specific antibodies against GRP. To investigate whether DNA vaccines containing six tandem repeats of GRP 18–27 could evoke a strong humoral immune response, we compared the levels of GRP-specific immunoglobulin G (IgG) in the sera collected from immunized mice by ELISA (Fig. 1B and Table 1). Sera samples were collected 1 week, 3 weeks, 5 weeks, 7 weeks, and 9 weeks after the initial immunization. All GRP-based DNA vaccines, compared with the control vaccine (pCR3.1-VS-HSP65-TP-M2) or the saline control, greatly increased titers of specific anti-GRP antibodies 5 weeks postinoculation, and the antibody levels remained high for up to 9 weeks postinoculation. Among the GRP-based vaccines, the anti-GRP antibody titers in mice immunized with the plasmid containing two copies of the mycobacterial HSP70 0403–0426 gene (M) were highest at 9 weeks postimmunization.

The specificity of antibodies elicited in immunized mice was established with immunobLOTS using the rhVEGF121-GRP 18–27 fusion protein as the antigen (Fig. 1C). Both the rhVEGF121-GRP 18–27 fusion protein and rhVEGF121 are capable of forming homodimers through the disulfide bonds between monomers (11). Antibodies from mice immunized with pCR3.1-VS-HSP65-TP-GRP-M2 reacted with the rhVEGF121-GRP 18–27 fusion protein (lanes 2 and 3) but not with the rhVEGF121 proteins (lanes 4 and 5). Under nonreducing conditions, two reactive bands consistent with the sizes of the dimer and monomer of the rhVEGF121-GRP 18–27 were observed; under reducing conditions, a single reactive band (lane 3) compatible with the size of the rhVEGF121-GRP 18–27 monomer (lane 2) was observed. These findings suggest that the antibodies in the immune sera specifically recognized the GRP antigen.

To further investigate the relationship between the antibody titers and the biological effects of the GRP vaccines, avidity assays were performed with antisera from mice (n = 8, week 9) immunized with pCR3.1-VS-HSP65-TP-GRP6 or pCR3.1-VS-HSP65-TP-GRP-M2. The relative avidities of antibodies were observed based on the concentrations of NaSCN required to depress the OD at 450 nm 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 (1.18/0.15 versus 0.66/0.14 M, \( P < 0.0001 \)) (Fig. 1D).

The GRP1–27 peptide stimulated the proliferation of B16-F10 cells in vitro. To determine the effect of GRP on malignant melanoma cells, B16-F10 cells plated on 96-well plates were incubated with the full GRP peptide (GRP1–27) in concentrations of \( 10^{-5} \) mol/liter to \( 10^{-11} \) mol/liter. The number of the GRP1–27 peptide stimulated the proliferation of B16-F10 cells in vitro.

**Table 1.** Titers of anti-GRP antibodies determined by ELISA

| Group | Antibody titer (log2) |
|-------|----------------------|
| pCR3.1-VS-HSP65-GRP6 | 10.56 ± 0.81 |
| pCR3.1-VS-HSP65-TP-GRP6 | 10.68 ± 1.97 |
| pCR3.1-VS-HSP65-TP-GRP6-M1 | 10.37 ± 0.76 |
| pCR3.1-VS-HSP65-TP-GRP6-M2 | 14.84 ± 0.99 |
| pCR3.1-VS-HSP65-TP-GRP6-M3 | 10.49 ± 0.61 |

\( ^a \) Sera for detection of antibody titers were collected at week 9 after the initial immunization. Values are means ± standard deviations; there were eight mice in each experimental group.

\( ^b \) \( P < 0.01 \) by Student’s \( t \) test.
B16-F10 cells increased in a dose-dependent fashion in direct relation to the concentration of the full-length GRP 1–27 peptide (Fig. 2A). Significant mitogenic effects, compared with those for the control, were observed initially starting at 10⁻⁸ mol/liter (stimulation rate, 19.5%; P < 0.05) and further increased at higher doses, with a stimulation rate of 41.7% at 10⁻⁵ mol/liter.

Sera from immunized mice inhibited the growth of B16-F10 cells in vitro. To evaluate the inhibition effect of sera from immunized mice on B16-F10 cells in vitro, cultured cells were incubated with medium-diluted PBS, preimmunized serum, sera (n = 8) from mice immunized with pCR3.1-VS-HSP65-TP-M2, or sera (n = 8) from mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2. The growth of B16-F10 cells was significantly suppressed by GRP antisera in a dose-dependent manner (Fig. 2B).

The anti-GRP vaccine suppressed the growth of subcutaneous B16-F10 cells in vitro. To evaluate the inhibition effect of sera from immunized mice on B16-F10 cells in vitro, cultured cells were incubated with medium-diluted PBS, preimmunized serum, sera (n = 8) from mice immunized with pCR3.1-VS-HSP65-TP-M2, or sera (n = 8) from mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2. The growth of B16-F10 cells was significantly suppressed by GRP antisera in a dose-dependent manner (Fig. 2B).

The anti-GRP vaccine suppressed the growth of subcutaneous B16-F10 tumors. B16-F10 cells injected subcutaneously formed large solid tumors in nonimmunized mice (saline) or in mice injected with a non-GRP-containing plasmid (pCR3.1-VS-HSP65-TP-M2). The tumor sizes decreased progressively in mice immunized with the anti-GRP vaccine (pCR3.1-VS-HSP65-TP-GRP6-M2) (Fig. 2C). The average weight of solid tumors in mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 was significantly lower than that for mice in the saline group (0.517 ± 0.203 g versus 1.671 ± 0.579 g; P < 0.0001) or for mice injected with the control vaccine (0.517 ± 0.203 g versus 1.475 ± 0.835 g; P < 0.0001) (Fig. 2D).

The anti-GRP vaccine inhibited angiogenesis associated with intradermal B16-F10 tumors. 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, B16-F10 tumor cells were implanted intradermally at two sites in the abdominal region. It took approximately 7 days for the cells to form ~4-mm intradermal tumors in the two control groups; however, the growth of intradermal tumors in pCR3.1-VS-HSP65-TP-GRP6-M2-immunized group was slightly delayed and required almost 11 days to form ~4-mm tumors. As shown in Fig. 3A, tumor cells implanted intradermally were associated with significant angiogenesis. The total number of blood vessels around each implant site from pCR3.1-VS-HSP65-TP-GRP6-M2-immunized mice was significantly lower than that from the saline group (22 ± 4 versus 72 ± 14; P < 0.01) or from non-GRP-containing plasmid-immunized mice (22 ± 4 versus 63 ± 19; P < 0.01) (Fig. 3B). There were no significant differences between the two control groups (P > 0.5).

The anti-GRP vaccine inhibited lung metastasis of intravenous B16-F10 tumors. To further test the efficacy of the anti-GRP vaccine, the extent of lung metastasis by intravenously
administered tumor cells in the tail vein of immunized mice was evaluated. Metastatic tumor nodules were often detected in the lungs 21 days after injection of tumor cells (Fig. 4A). The average weight of lungs of mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 was significantly lower than that of the saline group (0.215 ± 0.020 g versus 0.301 ± 0.068 g; \( P < 0.05 \)) or the pCR3.1-VS-HSP65-TP-M2 control group (0.215 ± 0.020 g versus 0.289 ± 0.087 g; \( P < 0.05 \)) (Fig. 4B), which indicates that fewer metastases were formed in the lungs of the anti-GRP DNA vaccine-immunized group. In addition, the average number of metastatic nodules in mice immunized with pCR3.1-VS-HSP65-TP-GRP6-M2 was significantly less than that in mice that received saline (37.2 ± 9.4 versus 88.0 ± 22.6; \( P < 0.001 \)) or in mice in the pCR3.1-VS-HSP65-TP-M2 control group (37.2 ± 9.4 versus 79.3 ± 16.8; \( P < 0.001 \)) (Fig. 4C). There were no significant differences between results for the two control groups (\( P > 0.5 \)).

**DISCUSSION**

The main obstacle in developing subunit cancer vaccines targeting self-proteins is the low immunogenicity of their epitopes. Our previous study has established that the increased immunogenicity as well as antitumor effects of immunogens containing tandemly repeated B- or T-cell epitopes is associated with the increased copy number of the target sequence (35). These results could be due to the enhanced recognition of the tandemly repeated self-epitope by Th1 cells, resulting in efficient inhibition of Th1 cells and a consequent increase in recruitment of Th2 cells (10). In this study, human GRP_{18-27} was chosen as the target epitope (21). The GRP epitope was tandemly repeated six times in order to enhance the humoral immune response. To further enhance the immunogenicity of the DNA vaccine, HSP65, tetanus toxoid 830–844, PADRE, and mycobacterial HSP70_{407–426} were used as fusion partners. Our results showed that the DNA vaccine pCR3.1-VS-HSP65-TP-GRP6-M2, containing two tandem repeats of the mHSP70_{407–426} epitope (M2), generated the highest titers of anti-GRP antibodies among the DNA vaccines.

HSP70_{407–426}, located in the 70-kDa microbial protein HSP70, has been identified as a major epitope stimulating tumor necrosis factor alpha, interleukin-12, and CCL-5 in monocytes and dendritic cells, comparable to those stimulated by native HSP70 (32). Interestingly, the plasmid coding for two copies of HSP70_{407–426} showed a stronger immune-stimulatory potential than the one with three copies of HSP70_{407–426}. It could be speculated that the reasons for this phenomenon are as follows. (i) Although mer-epitope effects can induce stronger immunogenicity of the epitope, as the number of epitope copies increases, the immunogenicity of the epitope becomes stronger, and then this effect could reach a crucial point at which the number is still increasing while the immunogenicity decreases (based on unpublished data from our lab). Thus, it is not the immunogen with more copies of the epitopes but rather the moderate one which can induce the strongest immune responses. (ii) The HSP70_{407–426} epitope in these plasmids is the T-helper epitope, and the leading role should be played by GRP epitope. Too many repeated copies of HSP70_{407–426} will decrease the immune responses to GRP. The exact mechanism underlying this phenomenon is now under detailed investigation in our lab.

The growth of B16-F10 cells has been shown to be regulated by both GRP and monoclonal antibodies in vitro. Our data demonstrate that the anti-GRP DNA vaccine was highly efficacious against B16-F10 tumors in vivo. High titers of GRP-specific antibodies could neutralize elevated levels of the GRP.

![FIG. 3. Effects of the anti-GRP vaccine on the growth and angiogenesis of intradermal tumors. (A) Light microscope picture of B16-F10 tumor cells implanted intradermally in the anterior abdominal wall and the development of new blood vessels. Tumor-associated angiogenesis in mice injected with saline (a and b) and the pCR3.1-VS-HSP65-TP-M2 control vaccine (c and d) appeared to be significantly greater than in mice immunized with the pCR3.1-VS-HSP65-TP-GRP6-M2 anti-GRP vaccine (e and f). Representative images were taken with an objective that was ×10 (a, c, and e) or higher (b, d, and f). (B) The total number of blood vessels was determined within the precise 1-cm² area around each implant site. **, \( P < 0.01 \).](http://cvi.asm.org/)

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and VEGF, and to increase phosphorylated Akt levels (14). To our knowledge, our study is the first to demonstrate that immune responses induced by the pCR3.1-VS-HSP65-TP-GRP6-M2 anti-GRP vaccine significantly reduced tumor-associated angiogenesis and vascularization of solid tumors.

The metastatic spread of cancer cells to different organs represents the major cause of death in cancer patients. BLPS have been shown to stimulate invasion and/or migration of many tumor cell lines (3, 23). BLPS mediate tumor cell invasion and metastasis by stimulating the expression and activation of several integrins, PP125FAK tyrosine phosphorylation, and MMP-9 (4, 5, 19, 28, 33). Bombesin significantly increased the incidence of peritoneal metastasis from gastric cancers and intestinal adenocarcinomas (30, 31). Very recently, Qiao et al. have demonstrated that GRPR silencing suppressed the metastatic potential of neuroblastoma (26). In the current study, we showed for the first time that anti-GRP vaccine-activated GRP immune responses suppressed the lung metastasis of intravenously injected tumor cells. The inhibition of tumor invasion to the lungs could be attributed to the following: (i) inhibition of the proliferation of B16-F10 cells invading the lungs and suppression of tumor-associated angiogenesis (29); (ii) downregulation of PKC (27), resulting in antimetastatic effects (37); (iii) upregulation of wild-type p53 (13), leading to the induction of apoptosis of B16-F10, which has very low levels of endogenous p53 (12); or (iv) antigenic epitopes in degenerating B16-F10 cells engulfed by immune-activated antigen-presenting cells could be presented to cytotoxic T lymphocytes, leading to a potent immune response against the remaining tumor cells (15).

In conclusion, we have demonstrated for the first time that immune responses which are elicited by a novel anti-GRP DNA vaccine suppress the proliferation and growth of melanoma tumors in mice. The antiangiogenesis and antimetastatic activities of this DNA vaccine suggest a novel approach against various cancers, especially malignant melanoma.

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