Effective Particle-mediated Vaccination against Mouse Melanoma by Coadministration of Plasmid DNA Encoding Gp100 and Granulocyte-Macrophage Colony-Stimulating Factor

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INTRODUCTION

Certain human cancers have been found to express TAAs that can be recognized immunologically. One such antigen is the gp100 glycoprotein, which is expressed on most human melanoma cells (1) and can also be recognized by immune cells including melanoma-derived tumor-infiltrating lymphocytes (2). Moreover, it has been reported that the adoptive transfer of autologous, gp100-reactive, tumor-infiltrating lymphocytes into melanoma patients, along with IL-2 treatment, has been associated with tumor regression in some patients (3). Therefore, gp100 can be considered as a candidate TAA molecule for the induction of an efficacious immune response in melanoma patients. Indeed, initial studies have reported that immunization with gp100-derived peptides can induce a measurable antitumor immune response in some cancer patients (4).

Genetic immunization using genes that code for TAAs is evolving as a strategy for cancer therapy. This vaccination strategy is formulated on the hypothesis that a sustained local expression of transgenic TAAs may confer a greater immunogenic presentation of tumor antigens, and this will enhance in vivo sensitization and activation of T cells that are capable of recognizing the TAA-associated peptides on the tumor cell surface. Indeed, several studies have demonstrated the T cell-dependent efficacy of this genetic TAA immunization strategy in murine models (5, 6). The efficacy of genetic vaccines in these preclinical murine studies has led to several clinical trials using genetic vaccination with TAA genes, including trials of MART-1 and gp100 DNA for melanoma patients. A preclinical study using genetic vaccination with recombinant adenovirus vector expressing gp100 has documented protection against murine melanoma (7). However, the limitations of virus-mediated delivery systems (8) and the recent concerns on biosafety of the current adenovirus vectors for gene therapy trials (9) have fueled attempts to develop alternative, nonviral means of gene delivery into somatic cells in vivo.

The PMGT technology (10, 11) makes use of a burst of

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helium to accelerate DNA-coated microscopic gold particles into target cells. This results in transgene expression levels that are often comparable with and sometimes superior to those achieved by other DNA delivery systems (10). Potential advantages of the PMGT approach (12, 13) include its ability to: (a) physically confer gene delivery into cells by nonviral means; (b) perform a transfection in several seconds; (c) transfect resting, nondividing cells, irrespective of cell lineage; (d) alter or modify levels of transgene expression over time via a multiple gene delivery regimen; and (e) simultaneously deliver multiple candidate therapeutic genes into individual cells. The latter feature might be beneficial for skin DNA vaccination strategies when a TAA gene is used in combination with other immunostimulatory reagents, such as cytokine genes. Vaccine strategies using tumor cells transfected with cytokine genes, including IL-2, IL-4, IL-12, IFN-γ, and GM-CSF, have been effective in mediating either T cell-dependent or inflammatory responses that lead to tumor regression (14–18). A separate strategy involving vaccination using TAA peptides has shown that enhanced antitumor immunity can be obtained by systemic coadministration of cytokines, such as IL-2 (19), IL-12 (20), or GM-CSF (21). However, systemic immune activation with infusions of recombinant cytokines, such as IL-2 or IL-12, might have unwanted toxicities or immunological effects. Localizing the cytokine expression to the vaccine site may enhance immunization without the undesired systemic effects.

We report here that co-delivery of hugp100 and murine GM-CSF DNA expression plasmids resulted in a greater vaccination effect than the gp100 DNA vaccine alone. Vaccination of naïve mice with the gp100 + GM-CSF plasmid combination protected them from subsequent tumor challenge. Moreover, treatment of tumor-bearing mice with this plasmid combination suppressed growth of established tumors.

**MATERIALS AND METHODS**

**Plasmids.** The hugp100 expression plasmid, pWRG1644, was constructed by ligating an EcoRI/Xhol DNA fragment from pCRII-gp100 (pCRII-gp100 was kindly provided by Dr. Nicholas Restifo at the National Cancer Institute, Bethesda, MD) into the mammalian expression vector pWRG7077 (provided by PowderJect Vaccines, Inc., Madison, WI). pWRG1644 is a pUC19-based plasmid and contains a kanamycin resistance gene from Tn903, the human cytomegalovirus immediate early enhancer/promoter and intron A, the coding sequence for hugp100, and the bovine growth hormone polyadenylation sequence. pWRG1644 was propagated in *Escherichia coli* DH5α. Supercoiled plasmid DNA was purified on Qiagen columns according to the manufacturer's instructions. The expression plasmid pWRG3226 containing the coding sequence for mGM-CSF was described previously (22).

**Mice.** C57BL/6 mice were obtained from Harlan-Sprague-Dawley (Madison, WI) or from Taconic (Germantown, NY). Female mice between 8 and 12 weeks of age were used in this study. Housing, care, and use of mice were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH publication 86-23, NIH, Bethesda, MD, 1985).

**Tumor Cells and Transfectants.** The B16F10 (B16) melanoma cell line, syngeneic in C57BL/6 mice, was originally obtained from Dr. William Ershler (University of Wisconsin Institute of Aging, University of Wisconsin-Madison). Tumor cell cultures were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and gentamicin at 50 μg/ml. For B16 cell transfection, the cDNA construct of pNASS/CMV-hugp100 was used. It was generated by inserting the hugp100 DNA fragment, excised from the pWRG1644 vector, into the pNASS/CMV-neo vector (23). B16 cells were transfected *in vitro* using the PowderJect gene delivery system (PowderJect Vaccines) as described previously (24), followed by selection in the presence of G418 and cloning by limiting dilution. Clones expressing high levels of gp100 (B16-gp100) were identified by flow cytometry, and one of the clones was used in this study. Control B16-neo cells were generated by transfecting B16 cells with the pNASS/CMV-neo vector without the hugp100 cDNA insert.

**PCR.** C57/BL6 mice were vaccinated using the PowderJect gene delivery system. One transfection contained 625 ng of DNA of either vector alone, or vector encoding hugp100, or vector encoding gp100 mixed at a 1:1 ratio with vector encoding mGM-CSF. Twenty-four h later, skin samples were excised from the transfected area, and total RNA was extracted using the guanidinium method as described previously (25).

All RNA samples were quantified spectrophotometrically and tested for DNA contamination by performing a PCR using 40 ng of RNA as template and the specific primers as described in this section. RNA samples that showed signs of DNA contamination were subjected to DNase treatment according to standard protocols. Single-stranded cDNA was generated from RNA with oligo-dT priming and AMV-RT according to the manufacturer’s instructions (Promega Corp., Madison, WI). The resulting cDNA was amplified using primers obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Primers were designed to specifically amplify hugp100 (upper, 5’-TAT-TGAAAAGTGGCAGATCC-3’; lower, GCTGTTCCTCAC-CAATGGACAAG-3’). The conditions for the PCR reaction were: initial denaturation step 4 min at 94°C; 35 cycles of denaturation (94°C for 45 s), annealing (55°C for 45 s), and extension (72°C for 1.5 min), and a final extension at 72°C for 7 min. The PCR products were then evaluated by 1.4% agarose gel electrophoresis in ethidium bromide and visualized by UV light.

**Flow Cytometry.** Because gp100 is a transmembrane glycoprotein, parental (B16-wild) and hugp100-transfected (B16-gp100) B16 cells were first permeabilized in ORTHO PermaFix solution (Ortho Diagnostics Systems, Raritan, NJ) for 1 h. Then, the cells were incubated with anti-gp100 mAb, HMB-45 (26), obtained from Coulter-Immunotech (Westbrook, ME), for 40 min at 4°C. As a negative control, mouse IgG (Sigma Chemical Co., St. Louis, MO) was used. After washing, the cells were stained with FITC-conjugated goat-antimouse IgG (Becton Dickinson and Co., San Jose, CA) for 40 min at 4°C. Stained cells were analyzed using a FACScan cytofluorometer (Becton Dickinson), and data were collected for 10,000 events/sample.

**Tumor Models.** Mice were shaved in the abdominal area and injected i.d. with 5 × 10⁴ or 1 × 10⁵ B16 cells in 50 μl of PBS. Tumor growth was monitored two to three times/week by measuring two perpendicular tumor diameters using calipers.
Because of humane considerations, mice were sacrificed when their tumor reached a diameter of 15 mm. For in vivo experiments, five mice/group were routinely used; in some experiments, eight to nine mice/group were used. Each experiment was repeated at least once.

**In Vivo Gene Transfer.** We used a helium-pulse Dermal PowderJect-XR device (PowderJect Vaccines) as described previously (27). Briefly, plasmid DNA was precipitated onto gold particles (2 μm in size; Degussa, South Plainfield, NJ) at a loading rate of 2.5 μg of DNA/mg of gold, mixed with an equal amount of gold, and coated onto the inner surface of Tefzel tubing. Each half-inch segment of tubing conferred the delivery of 0.5 mg of gold and 625 ng of plasmid DNA/transfection. When two plasmids were used in combination, each plasmid was precipitated onto separate gold particles, which were then mixed together to deliver 625 ng of each plasmid DNA per 0.5 mg gold/transfection. In some experiments, the gp100 cDNA expression plasmid was diluted 10- or 100-fold before precipitating onto gold particles. For DNA vaccination, mouse skin was shaved and transfected in vivo with the cDNA vectors with a 400 psi helium gas pulse. DNA vaccine was given to mice either 7 days before tumor challenge or on days 7, 10, 13, and 17 after tumor cell implantation.

**T-Cell Depletion in Vivo.** Anti-CD4 mAb and anti-CD8 mAb were produced from ascites of nude mice injected i.p. with GK1.5 and 2.43 hybridomas, respectively (both obtained from American Type Culture Collection). The mAbs, enriched for IgG by ammonium sulfate precipitation, were mixed at a dose of 98% of T cells in the spleen or peripheral blood for 4 days after anti-CD4 and anti-CD8 mAb administration (28).

**Immunohistochemistry.** C57BL/6 mice were injected i.d. with B16-gp100, B16-wild melanoma cells, and SP-1 sarcoma cells. Tumors were removed when the tumor diameter reached 6–8 mm and were fixed in 4% paraformaldehyde in PBS overnight. The tumors were then washed in 30% sucrose in PBS three times and cryoprotected in 30% sucrose solution overnight. They were embedded in OCT and cut in 7-μm sections. Cryostat sections were placed onto preclean slides (Fisher Scientific, Fair Lawn, NJ) and dried at room temperature overnight. Cryostat sections were rehydrated in TBS for 20 min and incubated for 30 min in TBS containing 0.3% hydrogen peroxide (J. T. Baker Chemical Co., Phillipsburg, NJ) and 1% Triton X-100 (Sigma) to eliminate endogenous peroxidase activity. The sections were then washed in TBS for 20 min and blocked in 3% BSA (ICN Biomedicals, Inc. Aurora, OH) in TBS for 30 min. The sections were then incubated with HMB-45/HRP antibody (Dako, Carpinteria, CA) at room temperature for 1 h. For control staining, the sections were incubated without HMB-45/HRP or with goat antirabbit IgG/HRP (1:200 dilution; Southern Biotechnology Associates, Inc., Birmingham, AL). After being washed twice with TBS for 10 min, the sections were incubated with 3,3′-diaminobenzidine (Sigma). The sections were washed in TBS for 20 min, counterstained with hematoxylin (Fisher Scientific), dehydrated, and mounted in a mounting medium (Premount; Fisher Scientific).

**Fluorescence Intensity**

*Fig. 1* Expression of hugp100 in B16-gp100 melanoma cells. Mouse B16 melanoma cells (B16-wild) or the cell clone derived from B16 cells after particle-mediated transfection with hugp100 DNA and selection in G418-containing medium (B16-gp100) were used for flow cytometry. The cells were first permeabilized, then incubated with HMB-45 mAb reactive with human gp100, and stained with FITC-conjugated goat-antimouse IgG (open curve). The control staining was performed with mouse IgG isotype control, followed by FITC-conjugated goat-antimouse IgG (dark curve). Mean fluorescent intensity (MFI) values are shown.

**Statistical Analysis.** A two-tailed Student’s *t* test was used to determine the significance of differences between experimental and relevant control values.

**RESULTS**

**gp100 Expression in Vitro and in Vivo in B16 Tumors and in Skin after Particle-mediated Transfection with a DNA Plasmid Expressing hugp100.** Before evaluating antitumor efficacy of hugp100 DNA vaccination, it was necessary to determine whether cell transfection with the hugp100 DNA expression plasmid could result in production of transgenic gp100 protein. B16 cells were transfected with hugp100 DNA, selected in *vitro* for G418-resistant clones (B16-gp100), and then analyzed for gp100 expression using flow cytometry. Wild-type B16 cells (B16-wild) were used as control. The B16-wild cells demonstrated consistent, although weak, staining over background with HMB-45 mAb (Fig. 1). These data suggest that HMB-45 mAb recognizes an epitope that is shared between human and mouse gp100, because these proteins have 79.7% amino acid sequence homology (29). Furthermore, Fig. 1 shows that B16-gp100 cells bound more HMB-45 mAb than control mouse B16 melanoma cells (mean fluorescence intensity, 29.0 versus 7.4, compared with 2.9 for mouse IgG isotype control), suggesting that the B16-gp100 clone expresses hugp100. A nonmelanoma murine cell line, 4T1 adenocarcinoma, showed no binding with HMB-45 mAb over mouse IgG isotype control.
(mean fluorescence intensity, 10.3 versus 6.7), documenting the specificity of the HMB-45 mAb (data not shown). When injected into mice, B16-gp100 cells formed tumors that continued to bind HMB-45 mAb far more strongly than similar tumors obtained with the nontransfected B16-wild tumor cells, as shown by immunohistological analysis (Fig. 2).

The expression of the hugp100 transgene in B16-gp100 cells was also confirmed using RT-PCR analysis. Several previously published primer sets for the hugp100 sequence proved unsuitable for the current study because they amplified the mouse gp100 gene expressed by murine B16-wild cells. We therefore designed a new set of primers that is capable of specifically amplifying only the human sequence. RT-PCR tests using the human-specific primer set confirmed such specificity. Only B16-gp100 cells (Fig. 3a, Lane 2), but not B16-wild cells (Fig. 3a, Lane 1), were found to generate the hugp100 PCR amplification product.

The vaccine strategy reported here involves direct in vivo transfection of mouse epidermis with hugp100 DNA by PMGT. To confirm the transcriptional expression of hugp100 in DNA-transfected mouse skin, PCR testing was performed on epidermal specimens from C57/BL6 mice that were treated with the gold beads coated with either empty vector DNA (control), hugp100 DNA, or a mixture of hugp100 and mGM-CSF DNA. The mice were given one transfection on each side of the abdomen, using 625 ng (gp100) or 1250 ng (gp100 + GM-CSF) of total DNA/transfection. Twenty-four h later, skin samples were excised from the transfected area, and total RNA was extracted. The clean RNA samples were reverse transcribed and amplified by PCR to detect transcripts for the housekeeping gene G3PDH or for the hugp100 gene. Lane 3 in Fig. 3a shows that no hugp100 was detected in the empty vector-transfected skin, indicating that the primers for the hugp100 sequence are specific and no

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Fig. 2 Detection of hugp100 antigen in B16-gp100 tumor by immunohistochemistry. Frozen sections of established B16-gp100 tumors (A and B), B16-wild tumors (C), and SP-1 sarcomas (D) were incubated with the human gp100-specific HMB-45 mAb conjugated to HRP (B–D). The sections were then developed with 3,3’-diaminobenzidine and counterstained with hematoxylin. Only B16-gp100 tumor sections were hugp100 positive (B). As a negative control, B16-gp100 tumors were incubated only with diaminobenzidine (A). As an additional control, B16-gp100 tumor sections were incubated with goat-antirabbit IgG/HRP followed by diaminobenzidine, which resulted in a low level of background staining similar to that seen in A (data not shown).
Genetic Vaccination with Gp100 Plus GM-CSF
cotransfected with hugp100 DNA and mGM-CSF DNA. From skin transfected with hugp100 DNA; Lane 5 shows the positive amplification product of the hugp100 DNA- transfected skin. Lane 3, RNA from control B16-wild cell line; Lane 2, RNA from the B16-gp100 stably transfected cell line; Lane 3, RNA from the skin from a control mouse transfected with empty vector; Lane 4, RNA from skin transfected with hugp100 DNA; Lane 5, RNA from skin cotransfected with hugp100 DNA and mGM-CSF DNA.

mouse-derived products are interfering with the PCR reaction. A clear band in Lane 4 shows the positive amplification product of the hugp100 DNA- transfected skin. Lane 5 confirms the expression of hugp100 when transfected along with mGM-CSF DNA, indicating that mGM-CSF DNA did not interfere with the expression of hugp100 in the skin. Transcription efficiency for all of the samples was confirmed by amplifying the housekeeping gene G3PDH. a, Lane 1, RNA from control B16-wild cell line; Lane 2, RNA from the B16-gp100 stably transfected cell line; Lane 3, RNA from the skin from a control mouse transfected with empty vector; Lane 4, RNA from skin transfected with hugp100 DNA; Lane 5, RNA from skin cotransfected with hugp100 DNA and mGM-CSF DNA.

Protection against B16-gp100 Tumor after gp100 DNA Vaccination. The skin of mice was transfected at four abdominal sites with hugp100-encoding plasmid DNA, alone or in combination with mGM-CSF-encoding plasmid DNA. This resulted in a total delivery of 2.5 μg of each DNA. Seven days later, vaccinated or naive mice were challenged i.d. in the middle of the abdomen with 5 × 10^4 of either B16-wild cells or B16-gp100 cells, and tumors were serially measured. No tumor protection was observed against wild-type B16 tumor (Fig. 4, left), or B16 cells transfected with empty vector (data not shown) after either gp100 or gp100 + GM-CSF gene vaccination. In contrast, gp100 gene vaccination resulted in substantial protection against B16-gp100 tumors, with 40% of the mice remaining tumor free for at least 2 months. Importantly, co-delivery of mGM-CSF DNA with hugp100 DNA resulted in complete tumor protection in all five vaccinated mice (Fig. 3, right). This ability of mGM-CSF gene co-delivery to enhance the antitumor effect of hugp100 DNA vaccination was consistently reproducible in all 12 subsequent experiments performed (not shown), although the degree of protection varied. In separate experiments, mice were challenged 21 days after the gp100 + GM-CSF DNA vaccination, and enhanced antitumor efficacy was consistently observed when compared with vaccination with gp100 DNA alone.

Dose Effect of gp100 DNA Gene Vaccination. Because an enhancing effect of mGM-CSF DNA on hugp100 gene vaccination was observed, we evaluated whether a reduction of hugp100 DNA dose in a combined DNA vaccine could still result in tumor protection. The vaccination was designed as follows. The initial hugp100 cDNA dose (625 ng/transfection; one transfection/mouse) was decreased 10- and 100-fold, whereas the GM-CSF DNA dose was kept constant (625 ng/transfection). Control mice received a vaccination with the empty vector alone or in combination with mGM-CSF DNA. Seven days after vaccination, mice were challenged with 5 × 10^4 B16-gp100 cells, and tumor growth was measured. The results in Fig. 5 show that, in combination with the mGM-CSF gene, a 10-fold reduced dose of hugp100 plasmid DNA (62.5 ng) still induced an appreciable level of protection (two of five mice were tumor free), which was less than that induced by 625 ng of gp100 DNA + GM-CSF DNA (three of five mice were tumor free), but still much higher than that obtained by using 625 ng of hugp100 DNA alone (no tumor-free mice). A smaller dose of gp100 DNA (62.5 ng) in combination with GM-CSF DNA did not noticeably affect tumor growth when compared with vaccination with vector DNA plus GM-CSF DNA. Fig. 5 also shows that the protection of gp100 + GM-CSF DNA
vaccine against B16-gp100 tumors, although substantial, was not strong enough to completely eradicate the tumor in all vaccinated mice, in that some mice eventually developed the tumors 20–28 days after the tumor challenge.

Role of T Cells in Tumor Protection in gp100\(^1\)GM-CSF DNA-Vaccinated Mice. Antibody depletion studies were used to determine whether T cells were responsible for the protection from B16-gp100 tumors induced by gp100\(^1\)GM-CSF gene vaccination. Mice were immunized in the skin with the gp100\(^1\)GM-CSF combined DNA vaccine 7 days before challenge with B16-gp100 tumor cells and injected i.p. with a mixture of anti-CD4 and anti-CD8 mAbs 1 day before and 4 days after the tumor challenge. Control mice received rat IgG. Fig. 6 shows that in vivo depletion of T cells abrogated the protection induced by the DNA vaccine, indicating that this antitumor protection is T cell mediated.

RT-PCR Analysis of Recurrent Tumors in Vaccinated Mice. Although hugp100 gene vaccination, especially in combination with mGM-CSF DNA, resulted in tumor protection, delayed tumor growth was observed in some mice several weeks after tumor challenge (Fig. 5, bottom left and bottom middle). An explanation for these relapsed tumors could be that B16-gp100 tumor cells injected into vaccinated mice have lost expression of the hugp100 transgene and, therefore, became resistant to the hugp100-specific T-cell immune response. To address this possibility, we analyzed the expression of hugp100 RNA in three tumors that developed in vaccinated mice at different time points after a first or second tumor challenge. These three tumors developed in distinct vaccinated mice 8 days after the challenge, 4 weeks after the challenge, or 4 weeks after a second tumor challenge. RT-PCR analysis results presented in Fig. 7 show that these three tumors continued to make hugp100 transcripts. Of all three tumors evaluated, none have lost hugp100 transcription, as determined by RT-PCR.

Therapy of Established Tumors with the gp100\(^1\)GM-CSF DNA Vaccine. The experiments described above showed that hugp100 gene vaccination of naïve mice, especially in combination with mGM-CSF DNA, can result in the induction of a T cell-mediated immune response that is capable of protecting mice from a subsequent tumor challenge. To evaluate this vaccination strategy in a model that is more analogous to the clinical setting, the therapeutic effect of gp100\(^1\)GM-CSF gene therapy was tested in mice bearing established tumors. Mice were injected i.d. in the middle of the abdomen with \(5 \times 10^4\) B16-gp100 cells. Seven days later, when the tumors reached \(\sim 4\) mm in diameter, a combined gp100\(^1\)GM-CSF DNA vaccine was administered to two sides of the abdomen. This treatment was repeated on days 10, 13, and 17 after tumor cell implantation. The results in Fig. 8A show the suppression of tumor growth in treated mice when compared with control mice (\(P < 0.05\) starting from day 17 of tumor growth).

The effect of gp100\(^1\)GM-CSF gene therapy against established tumors was further confirmed by evaluating the survival of mice. Survival time for the animals shown in Fig. 8A
was calculated based on the day after the tumor establishment that mice required sacrifice because their tumor diameter reached 15 mm. The unvaccinated tumor-bearing mice and mice treated with the empty vector
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GM-CSF DNA showed mean survival of 26.11 ± 0.96 and 25.5 ± 1.42 days, respectively, whereas gp100
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GM-CSF DNA-treated mice "survived" for 37.5 ± 2.72 days (P < 0.005). The experiment depicted in Fig. 8 was repeated two times, with the vaccine treatment started on day 4 or on day 7 after tumor cell implantation, and similar results were obtained.

\section*{Vaccination with hugp100 + GM-CSF DNA Induces Antitumor Effect against a Wild-Type B16 Tumor.}

The primary purpose of these studies was to measure the influence of GM-CSF DNA administration on the immunity induced by hugp100 DNA vaccination, as measured by protection from challenge with B16-gp100 cells. Because the hugp100 molecule is cross-reactive with the murine gp100 molecule, we also tested whether this hugp100 + GM-CSF DNA vaccination regimen induced any antitumor effect against the wild-type murine B16 melanoma, which expresses the murine gp100 molecule. Although a single vaccination was not protective against the challenge with the wild-type tumor cells (Fig. 4, left), three vaccinations with gp100 + GM-CSF DNA induced a statistically significant (P < 0.025) inhibition of growth of wild-type B16 tumor as compared with control mice (Fig. 8B).

\section*{DISCUSSION}

Immune responses against TAA have been induced by several cancer vaccine strategies, including vaccination using...
tumor cells, TAA as proteins or peptides, or genes encoding for TAs. Genetic vaccination against cancer may have clinical potential as a simple and effective way of inducing a protective antitumor immune response. The experimental results presented in this study show that particle-mediated vaccination of mice with the hugp100 DNA expression plasmid, especially in combination with a mGM-CSF DNA vector, resulted in effective protection against the hugp100-expressing murine B16 melanoma. As little as 62.5 ng of human gp100 plasmid DNA was found to be sufficient to achieve a substantial level of tumor protection when combined with the GM-CSF gene expression plasmid.

Immunostimulatory peptides of hugp100 have been recently synthesized and modified to confer more immunogenicity (29–32). This need for peptide modification is largely based on the findings that hugp100 induced much more effective immune and antitumor responses than murine gp100 in a mouse melanoma model (32–34). These modified peptides were found to induce an antitumor immune response in mice (7) and in cancer patients (3, 21). An initial clinical study has indicated that 42% of melanoma patients demonstrated objective antitumor responses as a result of gp100 peptide vaccination followed by a course of treatment with recombinant IL-2 protein (35). Thus, vaccination against the gp100 antigen seems to be a promising approach for melanoma treatment.

An alternative method to achieve an immune response against gp100 is to use genetic immunization with gp100 DNA. DNA immunization could potentially be more effective than peptide immunization, as has been indicated recently in a comparative experimental study using β-galactosidase and ovalbumin as model tumor antigens (36). Genetic vaccination with i.m. injection of naked hugp100 plasmid DNA, but not murine gp100 DNA, has been shown to result in the generation of CTLs and protection against hugp100-expressing B16 tumors (33). The results presented here confirm these findings using PMGT and further demonstrate that augmented antitumor efficacy is obtained when GM-CSF DNA is coadministered. Furthermore, the results show that comparable antitumor effects can be achieved by PMGT using 2.5 μg of the hugp100 DNA (Fig. 4), corresponding to only 5% of the hugp100 DNA (50 μg) that is used for the i.m. injection of naked DNA (33). This difference may be important for future clinical applications of genetic vaccines. In addition, the effective dose of hugp100 DNA can be further reduced to as little as 62.5 ng by coadministration with the gene encoding for mGM-CSF (Fig. 5). An adjuvant effect of GM-CSF protein (21) or DNA (14, 24, 37, 38) for experimental cancer vaccination has been reported previously. Our findings that GM-CSF augments gp100 DNA vaccination, however, seem to contradict the results of Hawkins et al. (34), who reported that recombinant mouse GM-CSF given in combination with hugp100 DNA did not provide any additional antitumor effect. A possible explanation may be that transgenic GM-CSF secreted at the site of tumor vaccine is much more potent than exogenous GM-CSF, as we have shown previously (22). The mechanism of the adjuvant effect of GM-CSF may be attributed to its ability to induce and activate antigen-presenting cells, such as dendritic cells and macrophages, and thereby potentiate a specific immune response elicited by tumor vaccines. At least in some tumor systems, GM-CSF was found to be the most potent adjuvant as compared with other tested cytokine genes (14). In agreement with those findings, our preliminary results indicate that GM-CSF DNA is more potent in enhancing the protective effect of gp100 DNA vaccination than IL-4 or B7.1 DNA expression vectors (data not shown).

A theoretical disadvantage of a vaccination approach based on using a specific TAA peptide or plasmid DNA encoding a TAA is the possibility of tumor escape from the immune control by developing "antigen loss" or antigen-negative tumor variants (39). In some patients, gp100 peptide vaccination was associated with gp100 antigen loss (40). In a recent clinical study using gp100 peptide vaccination, it was observed that a patient had initially responded to the vaccination by tumor shrinkage, but later the tumor relapsed. This relapsed tumor was shown to have lost the expression of gp100 and was resistant to gp100-specific CTLs (41). The results of this study on experimental gp100 gene vaccination also provide the evidence that some tumors may escape from the T cell-mediated immune control. However, we found that the relapsing tumors, being insidious for several weeks in vaccinated mice, have continued to express hugp100 transcripts. Although this observation does not exclude the loss of hugp100 on the protein level, it is possible, instead, that when the immune response to hugp100 weakens below a certain threshold, dormant tumor cells from the challenge inoculum begin to grow. Future experiments are warranted to determine the mechanism of tumor escape in these gp100-vaccinated mice.

Collectively, these results demonstrate that particle-mediated gene vaccination with hugp100 and mGM-CSF DNA in combination provides an effective approach for inducing immunologically mediated antitumor responses against murine B16 melanoma expressing hugp100 and weak but measurable antitumor effects against wild-type B16 melanoma. The preclinical model described in this study also allows the testing of different cytokine genes or other immunomodulatory genes for serving as a potential adjuvant in combination with the hugp100 DNA. Results from this study suggest that the combination of gp100 and GM-CSF DNA warrants testing as a genetic vaccine for clinical evaluation in patients with melanoma.

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