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Cloning and Characterization of the Genes Encoding the Murine Homologues of the Human Melanoma Antigens MART1 and gp100

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Abstract

The recent identification of genes encoding melanoma-associated antigens has opened new possibilities for the development of cancer vaccines designed to cause the rejection of established tumors. To develop a syngeneic animal model for evaluating antigen-specific vaccines in cancer therapy, the murine homologues of the human melanoma antigens MART1 and gp 100, which were specifically recognized by tumor-infiltrating lymphocytes from patients with melanoma, were cloned and sequenced from a murine B16 melanoma cDNA library. The open reading frames of murine MART1 and gp 100 encode proteins of 113- and 626-amino acids with 68.8 and 77% identity to the respective human proteins. Comparison of the DNA sequences of the murine MART1 genes, derived from normal melanocytes, the immortalized nontumorgenic melanocyte line Melan-a and the B16 melanoma, showed all to be identical. Northern and Western blot analyses confirmed that both genes encoded products that were melanocyte lineage proteins. Mice immunized with murine MART1 or gp 100 using recombinant vaccinia virus failed to produce any detectable T-cell responses or protective immunity against B16 melanoma. In contrast, immunization of mice with human gp 100 using recombinant adenoviruses elicited T cells specific for hgp100, but these T cells also cross reacted with B16 tumor in vitro and induced significant but weak protection against B16 challenge. Immunization with human and mouse gp100 together [adenovirus type 2 (Ad2)-hep100 plus recombinant vaccinia virus (rVV)-mgp100], or immunization with human gp100 (Ad2-hgp100) and boosting with heterologous vector (rVV-hgp100 or rVV-mgp100) or homologous vector (Ad2-hgp100), did not significantly enhance the protective response against B16 melanoma. These results may suggest that immunization with heterologous tumor antigen, rather than self, may be more effective as an immunotherapeutic reagent in designing antigen-specific cancer vaccines.

Keywords

Cancer vaccine; In vivo model; B16 melanoma; Adenovirus; Vaccinia virus

Active immunotherapy using antigen-specific cancer vaccines is being developed as an experimental method for the treatment of patients with metastatic melanoma (1-5). Recenlty, a variety of melanoma-associated antigens (MAAs) have been identified using lymphocytes from patients with melanoma (6-12). MART1 and gp100 are melanocyte differentiation antigens specifically recognized by HLA-A2 restricted tumor-infiltrating lymphocytes (TILs) derived from patients with melanoma, and seem to be involved in tumor regression (6,7). Phase

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I clinical trials using immunodominant peptides from thes antigens and recombinant adenoviruses encoding human MART1 or gp100 for the treatment of patients with melanoma have begun in the Surgery Branch, National Cancer Institute (1,13,14). However, a major obstacle to the development of effective cancer vaccines with these melanocyte differentiation antigens is the paucity of animal models, in which the target tumor antigens are normal, nonmutated differentation antigens. In this communication, we describe our attempts to determine whether melanocyte differentiation antigens can serve as immunotherapy targets in the B16 murine melanoma in syngeneic C57BL/6 mice. Therefore, we have cloned and characterized the genes encoding the murine homologues of the human melanoma antigens, MART1 and gp100, and evaluated whether these self-antigens can serve as immunotherapy targets for the development of experimental antigen-specific vaccine strategies for cancer therapy.

MATERIALS AND METHODS

Cell Cultures and Animals

The Murine B16 melanoma has been maintained in our laboratory for >15 years. After transduction with a retrovirus encoding the murine B7.1 gene, B7.1-expressing B16 cells were selected with the neomycin analogue G418 and were designated B16.B7. Hepa-1 and its subclone, K78-1.16, are murine hepatoma lines; MC-38 is a 1,2-dimethylhydrazine-induced murine colon adenocarcinoma; MCA-205 is a murine fibrosarcoma; EL4 is a T-cell lymphoma; 3LL is a lung carcinoma. All tumor lines already described are of H-2^b origin. CT26 is a murine colon carcinoma derived from BALB/c mice; K1735 is a nonpigmented melanoma line derived by ultraviolet irradiation of C3H mice, whereas, M3 is a murine melanoma syngeneic with DBA/2 (H-2^d) mice. Both K1735 and M3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD.U.S.A.). Another C57B/J (H-2^b) derived melanoma JB/MS and an immortalized melanocyte line Melan-a were provided by Dr. Vincent Hearing (National Cancer Institute, Bethesda, MD, U.S.A.).

Human melanoma cell line SK-23, human embryonic kidney line 293, monkey embryonic kidney line Cos7, and breast carcinoma line MDA-231 were purchased from ATTC. All tumor lines were grown and maintained in RPMI 1640 medium containing 10% fetal calf scrum, except MDA-231 and 293 cells, which used Dulbecco's Modified Eagle Medium as basal medium. HLA-A2-restricted TIL 1200, TIL 771, and TIL 1520 specifically recognized gp100, and TIL 1235 recognized MART1, whereas TIL 620 recognized both gp100 and MART1. These TILs were generated by culturing fresh tumor cell suspensions with 6,000 IU/ml interleukin-2 (IL-2) for 30-70 days as previously described (6,7,14,15). Female C57BL/6 mice, 6-12 weeks old, were obtained from the Frederick Cancer Research and Development Center, National Institutes of Health (Frederick, MD,U.S.A.) and Charles River Laboratories (Raleigh,NC, U.S.A.).

cDNA Library Screening and Sequence Analysis

A cDNA library was constructed from poly (A)RNA from the B16 murine melanoma cell line using the Lambda ZAP expressing vector cloning system (Stratagene, La Jolla, CA, U.S.A.). Briefly, cDNA was synthesized and the fractionated cDNA was ligated in to the EcoR1 predigested ZAP Express vector arms, then followed by in vitro packaging with Gigapack II gold packaging extract. The cDNA libraries (a total of 6×10^6 palques) were screened with [α32P]ATP-labeled human MART1 or human gp100 cDNA probes. MART1 or gp100-positive pBK-cytomegalovirus phagemids were purified, excised, and further characterized by DNA sequencing.

To test whether the cloned cDNAs encoding murine MART1 or gp100 were nonmutated, total RNA was isolated from the Melan-a melanocyte line and from melancoytes that were obtained from the skin of normal C57BL/6 mice. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using murine MART1 or gp100 specific primers. The amplified products were cloned into the pCRIII expression vector (Invitrogen, San Diego, CA, U.S.A.). DAN sequencing of the cloned genes was performedusing the Sequenase quick-denature plasmid sequencing kit (United States Biochemical Corp., Cleveland, OH, U.S.A.).

Northern Blot Analysis

Total RNA from tumor cell lines was isolated using RNAzol (Tel-Test, Friendswood, TX, U.S.A.). Total RNA from mouse normal tissues was purchased from Clontech (Palo Alto, CA, U.S.A.). Fifteen micro-grams of RNA was subjected to electrophoresis in a 0.6% formaldehyde-agarose gel. After transfer to anylon membranc, the membranes were hybridized with cither a mouse MART1, or mouse gp100-specific probe, then wahsed twice according to the QuikHyb procedure (Stratagene) before autoradiography.

Western Blot Analysis

Immunoblotting procedures were carried out using standard protocols. Briefly, all tumor cell lines were washed twice with ice-cold phosphate-buffered salineand then lysed with NP-40 lysis buffer (20m*M* Tris HCl, pH 8.0; 1% NP-40; 0.9% NaCl; 5 m*M* EDTA; 0.23 U/ml aprotinin; 2 m*M* phenylmethylsulfonyl fluoride; 1 μ*M* pepstatin A; 1 μ*M* leupeptin; 50 μg/ml RNAse and 50 μg/ml DNAase I). The protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockville, MD, U.S.A.). Equivalent amounts of protein (15 μg) from eachtumor line were separated by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (4–15% gradient) and then were transferred onto a nylon membrane. The membranes were probed either with αPEP13, a rabbit polyclonal antibody that recognized a 15-amino-acid peptide from the carboxyl terminus of mouse Pme117 (a gift from Dr. Vincent Hearing),or with M2-9E3, a mouse mAb that recognized human MART1 but also cross reacted with its murine homologue. Subsequent washing and visualization of antibody binding was carried out with Enhanced Chemiluminescence (Amersham Corp., Arlington Heights, IL, U.S.A.) according to the manufacturer's instructions.

Construction of Recombinant Vaccinia Viruses Encoding Murine MART1 and gp100

To construct plasmids containing the murine MART1 (mMART1) or gp100 (mgp100), cDNAs (Sal I/Xho I predigested fragments) were inserted into the Sal I digested parental plasmid pSC65, in which the transgene was under the transcriptional control of the strong synthetic early/late promoter $S_{F/L}$. After homologous recombination in BSC-1 cells, three rounds of plaque purification under BrdU selection were carried out in huTK$^-$ cells, and the recombinant vaccinia viral vectors (rVV-mMART1 and rVV-mgp100) were amplified and purified on sucrose cushions. Expression of mMART1 and mgp100 in infected cells was confirmed by immunostaining and RT-PCR analyses. The recombinant vaccinia and fowlpox viruses encoding human MART1 or gp100 (named as rVV-hMART1, rVV-hgp100, rFPV-hMART1, and rFPV-hgp100, respectively)were generated in a similar fashion and provided by Therion Biologics (Cambridge, MA, U.S.A.).The control vaccinia and fowlpox viruses containing LacZ (rVV-LacZ and rFPV-LacZ, respectively) were described previously (16).

In Vivo Protection Studies

C57BL/6 mice were immunized intravenously with 10^9 plaque-forming units (PFU) of recombinant adenoviruses encoding either human MART1, or human gp100 or LacZ gene. Fourteen days later, immunized mice were boosted with various recombinant adenoviruses (10^9 PFU) or vaccinia viruses (2×10^7 PFU) encoding human MART1/gp100 or the mouse

homologues. Twenty-one days later, mice were challenged intravenously with 2×10^5 B16 cells. Mice were tagged, randomized, and killed on day 14. Lung metastatic nodules were enumerated in a blinded, coded fashion.When metastases exceeded 250, they were deemed too numerous to count and were arbitrarily assigned a value of 250. Statistical evaluation of the data was performed using the nonparametric two-tailed Kruskal-Wallis test.

Generation of gp100-Reactive T Cells

Splenocytes from two mice from each of the vaccinated groups already described were harvested before the remaining mice were challenged with B16 tumor cells. These splenocytes were stimulated in vitro with either irradiated (3×10^4 cGy B16.B16.B7, or with irradiated K78-1.16 cells infected for 24–48 h at a multiplicity of infection of 100 with adenoviruses encoding human MART1/gp100 or control LacZ. K78-1.16 was selected as the H-2^b tumor line for which adenovirus type 2 (Ad2) has the greatest trapism. The 3×10^6 splecnocytes were added to 2×10^5 tumor cells (stimulators) in 2 ml of complete medium/well in 24-wellplates plus a final concentration of 30 IU IL-2/ml. The T-cell lines were tested for reactivity by measuring the secretion of murine interferon-γ(IFNγ) after coculture with MC-38 cells infected with rVV encoding different genes including human MART1/gp100, their murine homologues, and control LacZ.

Cytokine Release Assay

Lymphokine release assays were performed to detect T-cell reactivity with the MART1 and gp100 antigens. To test whether the murine MART1 and gp100 genes isolated from the B16 cDNA library were functional, the cloned mMART1 or mgp100 genes were transiently cotransfected into Cos-7 cells along with the HLA-A2 gene using lipofectamine(Life Technologies, Gaithersburg, MD, U.S.A.). After 24-h incubation, cells were washed, and the ability of the transfected Cos-7 cells to trigger IFNγ release from TILs was assessed. An equal number(10^5) of responder and stimulator cells were mixed,incubated for 20 h, and then supernatants were collected. Secretion of human IFNγ was detected using enzyme-linked immunosorbent assay kits purchased from Endogen. Murine IFNγ secretion was determined using biotinylated anti-mouse IFNγ monoclonal antibody (mAb) (Endogen) as previously described (13).

RESULTS

Comparisons of Nucleotide and Amino AcidSequences of Mouse MART1 and gp100

MART1 and gp100 cDNA clones, isolated from the murine B16 melanoma, normal skin melanocytes, and the immortalized melanocyte line Melan-a, were sequenced and compared. The nucleotide sequence of mouse MART1 cDNA from B16 and its predicted amino acid (AA) sequence is shown in Fig. 1. The full-length open reading frame of mMART1 consists of 342 bp encoding a protein of 113 AA with a predicted molecular weight of ~13. The mMART1 sequence contains two small deletions resulting in a protein that is 5AA shorter than the human MART1 protein. Alignment of human and mouse MART1 AA sequences showed 68.6% identity. The mouse MART1 gene cloned from B16 did not show any variation from the MART1 genes colned from normal melanocytes and Melan-a.

The mouse gp100 gene cloned from the B16 cDNA library consists of 1,878 bp encoding a protein with 626 AA bearing 75.5% identity to human gp100. The mgp100 gene cloned from B16 melanoma was almost identical to the published sequences of the murine Pme117 gene cloned from melanocytes from normal C57BL/6 mice and silver mice (17,18). The nucleotide and amino acid differences among mgp100 from B16, Pme117, and the silver mouse are summarized in Table 1. The most striking difference between mgp100 and murine Pme117 is

deletion of codon 2 and an insertion of A, G, and C at codons 582, 603, and 610,respectively, which result in AA changes as shown in Table 1.

Expression of Murine MART1 and gp100

Northern blot analyses of a variety of murine cell lines including pigmented and nonpigmented melanomas, melanocyte and nonmelanoma cancer cell lines, and normal tissues were performed to evaluate the expression pattern of mMART1 and magp100. As shown in Fig. 2, among normal tissues tested, only testis contained the gp100 mRNA transcript (Fig. 2B). Among cell lines tested to date, both murine MART1 and gpl00 were expressed only in pigmented melanomas and theimmortalized melanocyte line Melan-a, but were not expressed in the K1735 amelanotic melanoma and in nonmelanoma tumor lines examined. This result was confirmed at the protein level by Western blot analyses (Fig. 3) and by fluorescence activated cell sorter analysis (data not shown). Thus, like human MART1 and gp100, the expression pattern of murine homologues appeared to be restricted to melanocyte lineage cells.

The observed molecular weight of murine MART1 is ~16, bigger than we predicted, probably because of glycosylation (Fig. 3A). The murine gp100 protein is about 85 kDa by immunoblotting analysis (Fig. 3B), and was larger than the predicted size from the AA sequence as previously reported by other laboratories (19). It has been demonstrated that the silver mutation is the result of a single base insertion that alters the predicted carboxyl terminus sequence normally recognized by αPEP13 mAb, and consequently would not be recognized in immunoblotting. As shown in Fig. 3B, the 85-kDa band was detected in both melanocytes (Melan-a) and all pigmented melanoma lines tested (B16, M3 and JB/MS) as well. These results, combined with our sequence data, suggest that the murine gp100 from tumor cell is anormal protein not identical to the silver.

Cross-Reactivity Between Human Melanoma Antigens MART1 and gp100 and Their Murine Homologues Recognized by Human MART1/gp100 Specific TILSs

$MART1_{27-35}$ is the highly immunogenic dominant epitope of human MART1 (14), whereas five different epitopes have been identified in human gp100 (15). These epitopes were recognized by different MART1 or gp100-specific, HLA-A2-restricted TILs. Comparing the AA sequences of human and mouse MART1 and gp100, THE G9-209 epitope recognized by TIL 1520 was the only epitope among the six epitopes identified that was identical in mice and humans (Table 2). To determine whether the murine MART1 and gp100 cDNAs isolated from the B16 cDNA library encoded functional proteins, the cytokine release assays were performed using human TIL. Specific secretion of human IFNγ was observed when the cloned mMART1 gene was transiently contrans-fected into Cos7 cells along with HLA-A2, and these cells were with human MART1-specific TIL 1235(Table 3), even though one amino acid substitution (Tto I at position 8) was found in the murine sequence compared with the human epitope. Similarly, specific recognition of murine gp100 by TIL 1520 (reactive with G9-209) occurred when Cos7 cells were contrans-fected with HLA-A2 and mgp100 (Table 4). Expression of the HLA-A2, human and mouse MART1/gp100 were confirmed by FACS analysis (data not shown). Thus, expression of murine MART1 and gp100 cDNA in nonpigmented Cos 7 cells results in immunoreactivity with selected human MART1 or gp100-specific TILs.

Protective Anti-Melanoma Immune Response in Mice Immunized with Human gp100, but Not Murine gp100

In previous studies, we have demonstrated that immunization of mice with recombinant adenovirus encoding human gp100 (Ad2-hgp100) protected syngeneic mice from B16 challenge (13). In vivo protection assays were performed to investigate whether mouse MART1 or gp100, the “self-proteins,” were able to generate protective immunity against B16 challenge. As shown in Table 5, no protection against B16 melanoma was observed in mice

immunized with recombinat vaccinia viruses encoding mgp100 or mMART1. In contrast, immunization with Ad2-hgp100 significantly reduced the number of lung metastases compared with control mice [65 versus 224 ($p = 0.007$) and 66 versus 163 ($p = 0.001$) in experiments 1 and 2, respectively]. Mice immunized with recombinant vaccinia virus encoding human gp100, however, did not elicit a protective effect against B16. Neither homologous boosting (with Ad2-hgp100) nor heterologous boosting (with rVV-hgp100 or rVV-mgp100) significantly reduced the number of lung metastases when compared with a single Ad2-hgp100 immunization (Tabel 5, experiment 2).

In Vitro Generation of Human gp100 Specific TCells that Cross React with Murine B16 Melanoma

Studies were undertaken to determine which immunization strategy was most effective in eliciting T-cell responses to the murine homologues of the human melanoma antigens. As shown in Table 6, immunization of mice with Ad2-hgp 100 and stimulation in vitro with B16.B7 or K78-1.16 cells expressing hgp100 elicited T cells that recognized murine melanoma B16 as well as murine MC-38 cells infected with rVV-hgp100, little, if any release of murine IFNγ was seen with MC-38 cells infected with rVV-mgp100, but not uninfected MC-38 cells, or cells infected with rVV expressing LacZ, or mouse or human MART1. These same T cells also recognized MC-38 cells infected with rFPV expressing hgp100 but not LacZ or hMART1. No other immunization stategies were able to elicit clear specific T-Cell response to murine gp100. Murine gp100 reactivity in mice immunized with rVV-mgp100 could not be evaluated against target cells infected with rVV-map100 because of confounding antivaccinia reactivity. All attempts to generate murine MART1 reactive T cells were also unsuccessful.

DISCUSSION

Recent studies identifying MAAs or melanocyte differentiation antigens on melanomas have generated renewed enthusiasm for the development of cancer vaccines. Evidence has suggested that the melanocyte differentiation antigens MART1 and gp100 may be tumor rejection antigens in vivo (1,6,7). We have thus isolated genes encoding the murine homologues of the human MART1 and gp100 antigens and have studied their possible role as tumor antigens using the B16 melanoma in C57BL/6 mice.

This is the first report of the DNA sequence of the gene encoding murine MART1 and the tissue distribution of the encoded protein. Previous studies established that the murine Pme117 gene (similar to gp100) isolated from normal melanocytes mapped near the si (silver) locus on mouse chromosome 10 and encoded a melanosomal matrix protein with an unknown enzymatic function (19). The mouse silver mutation was caused by a single G insertion at codon 603 in the putative cytoplasmic tail of Pme117, which altered the last 24 AA sequence at the C-terminus(18). Thus, it was important to determine whether the gp100 expressed by B16 was a mutated or nonmutated self-antigen. Studies presented in this communication demonstrate that murine gp100 is indeed a melanocyte lineage protein and that the product encoded by mgp100 cDNA isolated from B16 tumor cells is not a protein with the silver mutation. Even though serveral nucleotide differences and insertions were observed when comparing the gp100 from B16 to the published DNA sequence of murine Pme117 derived from normal melanocytes, it is not not yet clear whether these differences result from alternative splicing of the same gene or from polymorphism. Nevertheless, the human $MART1_{27–35}$ and gp100 G9-209 epitopes, processed and presented from their murine homologues, could be recognized by selected human TILs. Thus, these epitopes may provide a useful in vivo model to study peptide-based vaccines against melanoma in HLA-A2 transgenic mice.

Because human MART1 and gp100 are human melanoma antigens recognized by human T cells, we next evaluated whether the murine homologues of these proteins might serve as T-

Cell antigens on the B16 melanoma. Murine MART1 was a melanocyte lineage protein as demonstrated by DNA sequence, Northern blot, and Western blot analyses. However ,all attempts to generate murine MART1 specific CTL in vitro, and to generate in vivo protective immunity against the B16 melanoma in mMART1 immunized mice under our experimental conditions were unsuccessful. As shown in Table 6, human gp100-reactive T cells generated from Ad2-hgp100 immunized mice did recognize B16 and B16.B7 melanoma, but only weak reactivity was seen to murine gp100. In addition, attempts to generate murine gp100 reactive T cells from mice immunized with rVV expressing murine gp100 (rVV-mgp100) were unsuccessful. Because the anti-B16 reactivity seen was disproportionately greater than any anti-mgp100 activity seen, it is possible that B16 reactivity exists to targets not related to mgp100. Moreover, normal mgp100 alone was clearly insufficient to elicit an autoreactive T-cell response using the immunizations described.

Our studies demonstrated that immunization with Ad2-hgp100, containing the human homologue of mgp100, significantly reduced the number of lung metastases compared with control mice as previously reported (13). However, mice immunized with rVV encoding human gp100 (rVV-hgp100), or murine gp100 (rVV-mgp100) did not elicit a protective effect against B16. Among recombinant adenoviruses examined, Ad2-hgp100, but not the Ad2-hMART1 nor Ad2-LacZ, reproducibly elicited anti-B16 tumor effects (13). We cannot rule out the possibility that the adenoviral vector itself presents unknown or nonspecific T-helper epitopes in vivo that augment the anti-tumor effects of gp100 vaccination as an explanation for the efficacy of Ad2-hgp100 but not rVV-hgp100. The generation of potent protective immunity against melanoma may require additional interactions of tumor-associated antigens and other “helper epiopes” presented by adenoviral vectors, but not the rVV vectors. Vaccinia viruses are replicating viruses, and the timing of transgene expression, the expression of multiple viral proteins, and the replication processes of rVV may explain the different results obtained, compared with nonreplicating rAd2 vectors. All of these factors could affect antigen presentation, and lead to the inability of rVV to generate protective immunity in vivo in this model.

The mechanisms by which Ad2-hgp100 immunization mediated protective immunity against B16 challenge in mice are not yet understood. The concept of molecular “mimicry,” in which heterologous proteins can induce strong T-cell responses against cryptic self-determinants, is similar to the concept of molecular mimicry of viral peptides in the initiation of autoimmune disorders (20-23). Previous studies using cytochrome *c* (cyt *c*) as a model autoantigen (24,25) have demonstrated that mice failed to elicit cyt-*c*-specific T cells in response to immunization with either human or mouse cyt *c* alone; howerver, sequential immunization with foregin cyt *c* followed by self (mouse) cyt *c*, or immunization with human and mouse cyt *c* together, was able to break T-cell tolerance and generated T cells that could be stimulated by either human or mouse cyt *c*. Similarly, in a murine systemic lupus erythematosus model, neither T- nor B-cell responses could be detected after immunization with native self-snRNPs (small nuclear ribonucleoprotein). T cells elicited by self-peptides did not respond to stimulation with native snRNPs, suggesting that the peptides were cryptic and were not processed from the native protein for presentation by antigen-presenting cells (26,27). Influenced by the cross-priming theory from autoimmune diseases, we tested vaccine strategies immunizing mice with self (mgp100) or human “mimic” (hgp100) alone, or human and mouse gp100 together, or sequentially. No immunization approach using these strategies generated a better antitumor response than using single vaccination with recombinant adenovirus encoding hgp100, the homologue of mouse gp100. It seems that foregin molecule mimicry (hgp100) probably provided the stimulus required for breaking T-cell tolerance to self-mgp100 in syngeneic mice, although further studies with recombinant adenovirus expressing mgp100 (which is not currerntly available) would be necessary to support this conclusion.

In summary, the present study provides an animal model of molecular minicry of the melanoma antigen gp100 for the induction of antigen-specific T-cell response in vitro and a protective immune response against B16 tumor in vivo. This phenomenon deserves further study in other murine and human tumor models for clinical design of more potent cancer vaccines.

REFERENCES

1. Rosenberg SA. The development of new cancer therapies based on the molecular identification of cancer regression anti-gens. Cancer J 1995;1:89–101.
2. Nanda NK, Sercarz EE. Induction of anti-self-immunity to cure cancer. Cell 1995;82:13–7. [PubMed: 7606778]
3. PardollDMTumour antigens: a new look for the 1990s. [News; comment].Nature1994369357 [PubMed: 8018207]
4. Boon, T.; Coulie, P.; Marchand, M.; Weynants, P.; Wolfel, T.; Brichard, V. Genes coding for tumor rejection antigens: perspectives for specific immunotherapy. In: Devita, VT.; Hellman, S.; Rosenberg, SA., editors. Important Advances in Oncology. J. B. Lippincott; Philadelphia: 1994. p. 58-69.
5. HoughtonANCancer antigens: immune recognition of self and altered self. [Comment].J Exp Med199418014 [PubMed: 8006576]
6. Kawakami Y, Eliyahu S, Delgado CH, et al. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc Natl Acad Sci USA 1994;91:6458–62. [PubMed: 8022805]
7. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci USA 1994;91:3515–9. [PubMed: 8170938]
8. CouliePGBrichardVVan PelAA new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. [see Comment].J Exp Med19941803542 [PubMed: 8006593]
9. Brichard V, Van Pel A, Wolfel T, et al. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 metanomas. J. Exp Med 1993;178:489–95. [PubMed: 8340755]
10. Robbins PF, el-Gamil M, Li YF, et al. Cloning of a new gene encoding an antigen recognized by melanoma-specific HLA-A24-restricted tumor-infiltrating lymphocytes. J Immunol 1995;154:5944–50. [PubMed: 7751637]
11. Topalian SL, Rivoltini L, Mancini M, et al. Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc Natl Acad Sci USA 1994;91:9461–5. [PubMed: 7937789]
12. WangRFRobbinsPFKawakamiYKangXQRosenbergSAIdentification of a gene encoding a melanoma tumor antigen recognized by HLA-A31-restricted tumor-infiltrating lymphocytes. [published erratum appears in J Exp Med 1995 Mar 1;181(3):1261].J Exp Med1995181799804 [PubMed: 7836932]
13. Zhai Y, Yang JC, Kawakami Y, et al. Antigen-specific tumor vaccines: development and characterization of recombinant adenoviruses encoding MART1 or gp100 for cancer therapy. J Immunol 1996;156:700–10. [PubMed: 8543823]
14. Kawakami Y, Eliyahu S, Sakaguchi K, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J Exp Med 1994;180:347–52. [PubMed: 7516411]
15. Kawakami Y, Eliyahu S, Jennings C, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J. Immunol 1995;154:3961–8. [PubMed: 7706734]
16. Wang M, Bronte V, Chen Pw, et al. Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. J Immunol 1995;154:4685–92. [PubMed: 7722321]
17. Kwon BS, Kim KK, Halaban R, Pickard RT. Characterization of mouse Pmel 17 gene and silver locus. Pigment Cell Res 1994;7:394–7. [PubMed: 7761347]

18. Kwon BS, Halaban R, Ponnazhagan S, et al. Mouse silver mutation is caused by a single base insertion in the putative cytoplasmic domain of Pmel 17. Nucleic Acids Res 1995;23:154–8. [PubMed: 7870580]
19. Kobayashi T, Urabe K, Orlow SJ, et al. The Pmel 17/silver locus protein: characterization and investigation of its melanogenic function. J Biol Chem 1994;269:29198–205. [PubMed: 7961886]
20. Mamula MJ. Lupus autoimmunity: from peptides to particles. Immunol Rev 1995;144:301–14. [PubMed: 7590818]
21. Mamula MJ, Craft J. The expression of self antigenic determinants: implications for tolerance and autoimmunity. Curr Opin Immunol 1994;6:882–6. [PubMed: 7536009]
22. Theofilopoulos AN. The basis of autoimmunity, Part I. Mechanisms of aberrant self-recognition. Immunol Today 1995;16:90–8. [PubMed: 7888073]
23. Theofilopoulos AN. The basis of autoimmunity: Part II. Genetic predisposition. Immunol Today 1995;16:150–9. [PubMed: 7718089]
24. Mamula MJ, Lin RH, Janeway CA Jr. Hardin JA. Breaking T cell tolerance with foreign and self co-immunogens: a study of autoimmune B and T cell epitopes of cytochrome C. J Immunol 1992;149:789–95. [PubMed: 1321851]
25. Mamula MJ. The inability to process a self-peptide allows autoreactive T cells to escape tolerance. J Exp Med 1993;177:567–71. [PubMed: 8381158]
26. Mamula MJ, Fatenejad S, Craft J. B cells process and present lupus autoantigens that initiate autoimmune T cell responses. J Immunol 1994;152:1453–61. [PubMed: 8301145]
27. Bockenstedt LK, Gee RJ, Mamula MJ. Self-peptides in the initiation of lupus autoimmunity. J Immunol 1995;154:3516–24. [PubMed: 7534800]

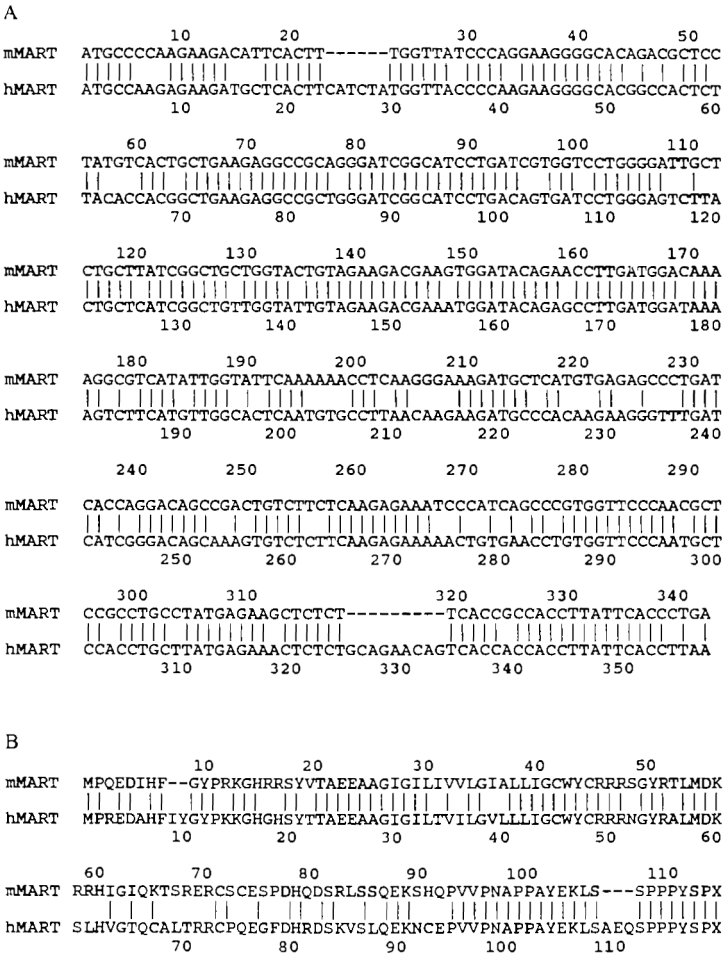

FIG. 1.

Comparison nucleotide sequences between mouse and human MART1 cDNA **(A)** and their predicted amino acid sequences **(B)**.

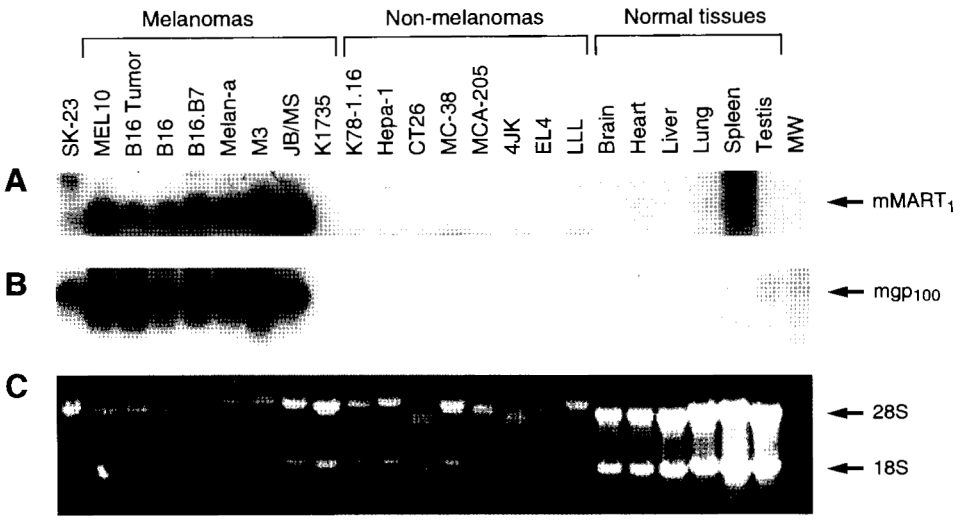

FIG. 2.

Northern blot analyses of the expression of mouse MART1 and gp100 mRNA. Fifteen micrograms of total RNAs from different murine melanoma tumor lines, nonmelanoma lumor lines, and normal tissues were loaded in each lane. Mouse MART1 mRNA **(A)** and gp100 mRNA **(B)** were detected using the full-length murine MART1 and gp100 cDNA as the probes, respectively. Ethidium bromide staining of RNA as shown in **(C)** was used as a control. The positions of mMART1 RNA, mgp100 RNA, AND 28S and 18S RNA were marked.

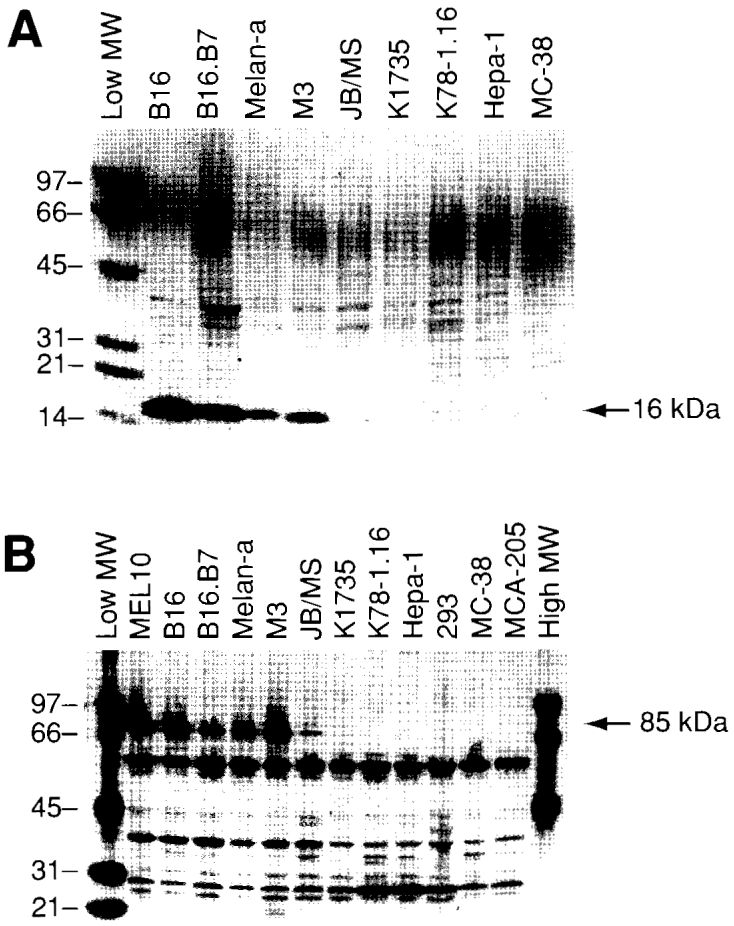

FIG. 3.

Western blot analyses of mouse MART1 and gp100 proteins. Equal quantities of protein (10 μg) from each cell line were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting with MART1-specific monoclonal anibody (mAb)2D12 **(A)** or gp100-specific mAb α PEP13 **(B)**. The 16-kDa mouse MART1 and the 85-kDa mouse gp100 proteins are marked. The position and size of molecular weight markers are indicated.

TABLE 1

Differences between B16 gpl00 and published murine Pmel17 sequences

		Codon								
		2	**98**	**170**	**175**	**373**	**396**	**444**	**471**	**531**
Nucleotides	B16	-	CCG	CTG	GGC	GAT	ACC	GTG	TTC	ACA
	mPmel 17	GTG	CCG	TCG	CGC	GAT	ACC	GTG	TTc	ACA
	Silver	GTG	CCA	CTG	GGC	AAT	ACT	GTA	TTC	ACT
Amino acids	B16	-	P	L	G	D	T	V	F	T
	mPmel 17	V	P	S	G	D	T	V	F	T
	Silver	V	P	L	G	N	T	V	S	T

	Insertion and substitutions at codons:		
	582	**603**	**610**
B16	ATA CAT AGG	CAC TGG CTG	GTC TTC CGC
mPmel17	ATA C—T AGG	CAC T—G CTG	GTC TT— CGC
Silver	ATA C—T AGG	CAC TAG CTG	GTC TT— CGC

	Result in the following amino acid changes:					
	582	**603**	**610**			
B16	l I HRHR L KKQ	GSVSOMPHGS	THWLRLPPVF	RARGLGENSP	LL S GQQVX	
mPmel17	- - LGID-RSR	AQFPKC*-MV	ALTAAPASGL	RARGLGENSP	LL S GQQVX	
Silver	--MGID-RSR	AQFPKC*-MV	ALTSCACLRS	SRPRPWKQPA	PQWTAGLI IL	KAPWISWGX

TABLE 2

Comparison of amino acid sequences of HLA-A2 restricted epitopes between human (h) and mouse (m) MART1 or gplO0 (identical amino acids are indicated by dashes)

Gene	Epitope		Sequence	Recognition by
MART1	$MART_{27-35}$	(h)	AAGIGILTV	TIL1235
		(m)	-------I-	
gp100	G9-154	(h)	KTWGQYWQV	TIL1200
		(m)	----K----	
	G9-209	(h)	ITDQVPFSV	TIL1520
		(m)	---------	
	G9-280	(h)	LEPGPVTA	TIL771
		(m)	--S-S---	
	G10-457	(h)	LLDGTATLRL	TIL1200
		(m)	--D-D-IM-	
	G10-476	(h)	VLYRYGSFSV	TIL660
		(m)	---------L	

TABLE 3

Recognition of mouse MART1 by human MART1-reactive tumor-infiltrating lymphocytes (TILs)

Stimulator	Transfcted gene(s)	HLA-A2	IFNγ secretion (pg/ml/10^5 cells)[a]		
			TIL620[b]	TIL1200	TIL1235
Cos7	none	-	0	0	0
Cos7	HLA-A2	+	12	73	46
Cos7	hMART1	-	0	99	28
Cos7	mMART1	-	0	62	43
Cos7	hMART1 + HLA-A2	+	112	90	460
Cos7	mMART1 + HLA-A2	+	168	83	552
SK-23[c]		+	1,702	1,712	1,663
B16		-	0	10	54

[a] Human interferon-γ (IFNγ) concentration was determined by enzyme-linked immunosorbent assay after TILs were coincubated for 24 h with stimulators with or without transiently transfected genes indicated.

[b] TIL620 and TIL235 are HLA-A2-restricted cytotoxic T-lymphocytes that recognize the MART1 antigen, whereas TIL1200 recognizcs human gp100 specifically.

[c] SK-23 and B16 are melanoma tumor lines used as positive and negative controls, respectively.

TABLE 4

Recognition of mouse gp100 by human gp100-reactuve tumor-infiltrating lymphocytes (TILs)

Cells transfected genes		Human IFNγ secretion (U/ml/10^5 cells)[a]		
	HLA-A2	TIL771	TIL1200	TIL1520
Cos7 Nonc	-	0	0	0
Cos7 HLA-A2[b]	+	0	0	1
Cos7 hgp100	-	0	0	0
Cos7 mgp100	-	0	0	0
Cos7 hgp100 + HLA-A2	+	4	179	146
Cos7 mgp100 + HLA-A2	+	0	0	236
SK-23[c]	+	37	274	314
397mcl	-	0	1	0
B16	-	0	0	0

[a] Human interferon-γ (IFNγ) concentration was measured after TILS were coincubatcd for 24 h with Cos7 cells that were transiently transfected with or without gene(s) indicated.

[b] TIL771 recognizes G9-280, TIL1200 recognizes G9-154 and G10-457, and TIL1520 recognizes G9-209 epitope specifically.

[c] SK-23, 397mel and B16 are melanoma tumor lines used as positive and negative controls, respectively.

TABLE 5

Protection of C57BL/6 mice against challenge with B16 melanoma using different immunization strategies

Group	Immunization[a]	Boost	No. of lung metastases		p value compared with group 1 (no. 6)
			Avg.	Metastases/mouse	
Exp. 1					
1	None	None	224	250 × 4,173,168	
2	rVV-hgp100	None	162	220,228,205,104,53	0.13
3	rVV-mgp100	None	250	250 × 6	0.98
4	rVV-mMART1	None	199	248,205,199,195,181,160	0.25
5	rVV-LavZ	None	193	250,243,223,144,103	0.36
6	Ad2-hgp100	None	65	78,74,66,65,44	0.001
Exp. 2					
1	None	None	163	240,225,207,153,148,121,116,90	
2	rVV-hpg100	None	164	219,214,211,207,198,168,168,135,120	0.5
3	rVV-mgp100	None	212	250 × 3,200,182,180,169	0.07
4	rVV-mMART1	None	ND		
5	rVV-LacZ	None	186	250 × 3,230,190,183,156,130,37	0.23
6	Ad2-hgp100	None	66	102,77,61,49,41	0.007
7	Ad2-hgp100	Ad2-hgp100	133	250,203,195,158,136,104,78,51,16	0.41 (0.08)
8	Ad2-hgp100	rVV-hgp100	52	172,98,49,39,34,10,8,3	0.007 (0.19)
9	Ad2-hgp100	rVV-mgp100	92	132,123,105,97,91,79,64,41	0.002 (0.14)
10	Ad2-hgp100 + rVV-mgp100	None	79	120,105,100,96,96,71,25,22	0.007 (0.51)
11	Ad2-hMART1	Ad2-hMART1	169	218,201,194,188,173,160,153,149,146,104	0.79
12	Ad2-hMART1	rVV-mMART1	215	250 × 3,222,214,203,190,139	0.08
13	Ad2-LacZ	None	143	180,172,156,152,147,145,137,133,112,97	0.56
14	Ad2-LacZ	Ad2-LacZ	179	250 × 2,198,198,183,170,168,164,106,105	0.50
15	Ad2-LacZ	rVV-LacZ	157	208,174,165,157,149,139,131,129	0.87

[a] C57B1/6 mice were immunized intravenously with either 10^9 or 2×10^7 plaque-forming units of either recombinant adenovirus type 2 (Ad2) or recombinanat vaccinia virus (rVV) encoding gene as indicated. Eighteen days later, these mice were boosted with same amount of recombinant virus indicated. These mice were then injected intravenously with 2×10^5 B16 cells 21 days after boosting. On day 14 after tumor challenge, lungs were harvested, and the number of pulmonary metastases were enumerated in a coded and blinded fashion.

TABLE 6

Specific interferon-γ (IFNγ) secretion by murine T-cell lines induced by adenovirus type 2-human gp100 (Ad2-hgp100) immunization[a]

			IFNγ secretion (× 10^{-3} pg/10^5 cells)											
			MC-38 cells infected with[b]											
			rVV encoding						rFPV encoding					
Immunization	**Boost**	**In vitro stimulation**	**None**	**LacZ**	**hMART1**	**mMART1**	**hgp100**	**mgp100**	**LacZ**	**hMART**	**hgp100**	**B16**	**B16.B7**	**E22[c]**	**EL4**
Ad2-hgp100	None	B16.B7	1	1	1	1	34	6	1	1	21	77	74	0	0
		K78-Adz-hgp100	1	1	1	1	42	8	1	1	75	65	74	1	2
None	rVV-mgp100	B16.B7	2	73	75	75	72	71	2	2	2	74	74	2	1
		K78-Ad2-hgp100	1	70	75	77	61	73	3	3	29	62	72	2	0
Ad2-hgp100	rVV-mgp100	Bl6.B7	1	75	56	81	65	71	1	2	5	69	70	1	0
		K78-Ad2-hgp100	1	72	75	75	59	73	4	3	77	38	61	1	0
Ad2-hMART1	rVV-MART1	Bl6.B7	1	67	41	45	56	66	1	1	1	70	76	1	1
		K78-Ad2-hMART1	2	74	74	78	65	72	3	3	3	67	73	5	1
None	None	B16.B7	0	0	0	0	1	0	0	1	1	15	19	3	1
Ad2-LacZ	rVV-LacZ	K78-Ad2-LacZ	0	80	67	72	83	78	77	0	0	5	6	80	1

[a] C57BL/6 mice were immunized intravenously with 10^9 plaque-forming units (PFU) of recombinant Ad2 (rAd2) as indicated. Two weeks later. mice were boosted with 5×10^7 PFU of recombinant vaccinia virus (rVVs) as indicated. Twenty-one days after boosting, splenocytes were harvested and stimulated in vitro either with irradiated Bl6-B7 cells or with irradiated K78-1.16 cells infected with Ad2-hgp100 or Ad2-LacZ for 2 weeks. These T cells were then plated in 96-well plates with tumor stimulators at a 1:1 ratio (10^5 cells each) for 24 h. Supernatants were harvested and murine IFNγ concentrations were determined by enzyme-linked immunosorbent assay.

[b] MC-38 cells were infected with 10 multiplicities of infection of different rVVs or rFPVs as stimulators right before being mixed with T cells.

[c] E22 and EL4 are H-2b tumors transfected with or without β-gal, respectively.