Genetic Immunization for the Melanoma Antigen MART-1/Melan-A Using Recombinant Adenovirus-transduced Murine Dendritic Cells


Abstract

Dendritic cells (DCs) are professional antigen-presenting cells that process and present antigenic peptides and are capable of generating potent T-cell immunity. A murine tumor model was developed to evaluate methods of genetic immunization to the human MART-1/Melan-A (MART-1) melanoma antigen. A poorly immunogenic murine fibrosarcoma line (NFSA) was stably transfected with the MART-1 gene. This transfected tumor (NFSA[MART1]) grows progressively in C3H/Ham/Sod (H-2b) mice. Partial protection against a challenge with NFSA[MART1] could be achieved with i.m. injections of a MART-1 expression plasmid or with systemic administration of an adenovirus vector expressing MART-1. However, superior protection was achieved when granulocyte-macrophage colony-stimulating factor/Interleukin-4-differentiated murine DCs transduced with an adenovirus vector expressing MART-1 were used for immunization. Both partial and complete protection could be achieved with i.v. administration of MART-1-engineered DCs. Splenocytes from immunized mice contained MHC class I-restricted CTLs specific for MART-1. This preclinical model of genetic immunization supports a therapeutic strategy for human melanoma.

Introduction

Several groups have described the existence of melanoma antigens and suggested a potential role in tumor immunity (1, 2). The MART-1 antigen is one of the best characterized and is expressed by most melanomas (2, 3). Peptides encoded by this gene can be recognized by melanoma-specific CD8-positive CTLs in the context of MHC molecules. DCs are the most potent APCs known, and it is now possible to isolate, expand, and study their function (4, 5). Recently, investigators have demonstrated that specific antitumor responses can be achieved by pulsing tumor peptide antigens onto DCs (6, 7). These tumor antigens may be MHC class I synthetic peptides (8) or autologous acid-eluted tumor peptides (7). The opportunity for DCs to express the entire tumor antigen polypeptide has a number of potential advantages to peptide-based strategies. These include the expression of multiple and/or undefined peptide epitopes, possibly in the context of both MHC class I and II molecules, and with any MHC allele. We have previously demonstrated that the most efficient way to express foreign genes in human DCs is with recombinant Ad vectors (9). In this study, we used a recombinant Ad vector to express the MART-1 gene into murine DCs, and we demonstrate that these engineered DCs can generate MART-1-specific protective immunity in vivo.

Materials and Methods

Mice and Cell Lines. Female C3H (H-2b) mice, 5–8 weeks old, were bred and kept in the Animal Facility of the Department of Radiation Oncology at the University of California Los Angeles. All animal studies were conducted in accordance with the Animal Care Policy of the University of California Los Angeles Chancellor’s Animal Research Committee. Animals were kept in a controlled flora environment and sacrificed according to established guidelines. NFSA is a spontaneous, poorly immunogenic murine fibrosarcoma that arose in the C3H strain and has been described previously (10). NFSA[MART] and NFSA[Neo] were developed by transfection of the NFSA line with a plasmid carrying the MART-1 and Neo genes or a plasmid with only the Neo gene, respectively. A RT-PCR-generated 400-bp product of MART-1 cDNA was cloned into the pUC19 plasmid, sequenced to confirm the identity of the PCR product obtained, and inserted into the pBcCMV expression plasmid (Invitrogen, Carlsbad, CA). MARTpRcCMV plasmid (2.5 μg) was mixed with 15 μl of N[1-(2-dioleoyl)oxylprop]-N,N,N-trimethylammoniummethyl sulfate (Boehringer Mannheim, Indianapolis, IN) in 20 mM HEPES buffer (Sigma, St. Louis, MO) and incubated with 1 x 10^6 tumor cells at 37°C for 6 h, after which the medium was replaced with fresh culture medium. Forty-eight h later, the transfected population was subjected to selection with 0.5 mg/ml G418 (Geneticin; Life Technologies, Inc., Gaithersburg, MD) in RPMI 1640 containing 10% FCS (Gemi Products, Calabasa, CA) and 1% (v/v) penicillin, streptomycin, and fungizone (Gemicin) and incubated at 37°C in 5% CO2. After three to four in vitro passages under G418 selection, tumor cells were injected in the flanks of C3H mice. Developing tumors were passed in vivo and periodically checked for MART-1 and Neo expression by RT-PCR. The number of NFSA[MART1] cells from a single-cell suspension of in vivo growing tumors required for tumor development in 50% of mice (TD50) was determined using a previously established protocol (10). SVEC is a murine endothelial cell line in the H-2b background. SVEC(MART1) and SVEC(Neo) were created by the same method used for NFSA sublines.

RNA Analysis. Total cellular RNA was isolated using TRIzol (Life Technologies, Inc.). Each RNA sample was treated with DNase (Stratagene, La Jolla, CA), reverse-transcribed with random hexamer primers, and then subjected to PCR with either MART-1, neomycin resistance, or murine adenine phosphoribosyltransferase-specific PCR primers to confirm the expression of the MART-1 and neomycin resistance transgenes as well as semiquantify the cDNA yield in each reverse-transcribed sample. The primers used were as follows: MART-1, 5'-CCCTTTTCTCTCTTAGAATGTGGTACCCTTTACTAA-3' and 5'-TGGGACAGCTCTAGCTGAGCGTCTAATAGGT-3'; adenine phosphoribosyltransferase, 5'-ACTCCAGGCTCTTGTTTGG-3' and 5'-ATCCCAATAGCACCCTCTTCTG-3'; Neo, 5'-GGTGGAGAGGCTTACCGGT-3' and 5'-CACAGGAGGAGGAGGCTGGT-3'.

Construction of Recombinant AdVs. Seed viral stocks of a recombinant Ad vector (RR5), which does not contain a transgene insert, were generously provided by Drs. Robert Meidell and Michael Barry (University of Texas Southwestern, Dallas, TX). RR5 is an El-deleted replication-deficient AdV type-5 vector. We constructed the recombinant Ad vector AdVMART1, which contains human MART-1 cDNA driven by the CMV enhancer/promoter. The MART-1 cDNA, previously cloned into the pUC19 plasmid, was subcloned into Ad vector pAdTrack-C mv CMV/Neo. The Ad vector RMAdMART1 was used to infect the cell line SVEC (H-2b) background, SVEC(MART1) and SVEC(Neo) were created by the same method used for NFSA sublines.
cloned into the shuttle vector pAC-CMVpLPa. The recombinant AdVMART1 vector was made by cotransfection with pM17, a plasmid containing the E1-deleted AdV type-5 genomic sequences, and pAC-CMVpLPa/MART-1 into the 293 human embryonic renal cell line. Homologous recombination between the two plasmids occurs, and recombinant adenoviral capsids are then released into the media. Clones of AdVMART1 were obtained by plaque purification on fresh 293 cells. Viral seed stocks were then obtained by amplification in 293 cells. The titer of each viral stock ranged from 10^{10} pfu/ml to 10^{11} pfu/ml. The titer was determined by plaque assay on 293 cells. Contamination with wild-type recombinant AdV was assessed for each viral stock by plaque assay on HeLa cells and was always found to be negative.

**Construction of Plasmid cDNA for i.m. Immunization.** MART-1 cDNA was cloned as described above, inserted into the VR-1012 plasmid (Vical, La Jolla, CA), and prepared for i.m. injection using an endonuclease-free plasmid prep kit (Qiagen, Santa Clarita, CA).

**Preparation of DCs.** The development of DCs from murine bone marrow progenitor cells has been described previously (5). Briefly, bone marrow cells were cultured overnight in RPMI with 10% FCS and antibiotics in a Petri dish. Cells were replated the following day at 1-2 x 10^6 cells/well in 24-well plates in the presence of murine granulocyte macrophage colony-stimulating factor (100 ng/ml; a generous gift from Amgen, Thousand Oaks, CA) and murine IL-4 (500 units/ml; R&D Systems, Minneapolis, MN). The loosely adherent cells were harvested on day 8 and found to have a distinctive DC-like morphology with cytoplasmic processes and to display a strong mixed lymphocyte reaction-stimulating activity. Flow cytometric analysis of several preparations showed that DCs comprised more than 90% of the large gated cells, as judged by high expression of CD11b, CD11c, CD18, CD44, CD80 (B7.1), CD86 (B7.2), and MHC class I and II antigens.

**AdV Transduction of DCs.** DCs were incubated at 37°C for 2 h with the AdV vector at a MOI of 100 in a final volume of 1 ml of infection media consisting of RPMI 1640 with 2% FCS. Infection was stopped by the addition of 3 volumes of RPMI 1640 with 10% FCS. The DCs were washed extensively before the injection of 5 x 10^6 DCs resuspended in 0.2 ml of PBS (Mediatech, Herndon, VA)/animal. Cell counts were determined using a hemocytometer, and viability was assessed by trypan blue exclusion. In all cases, viability exceeded 95%.

**In Vivo Tumor Therapy Models.** C3H mice were immunized with DCs administered i.v. via lateral tail vein. For tumor implants, a single-cell suspension of 1 x 10^6 cells/animal was isolated from NFSA(MART1) tumors in at least the third in vivo transplant generation. Tumors were surgically removed, decapsulated, and minced. Minced tumors underwent a brief enzymatic digestion (60 min) with DNase 1 (0.1 mg/ml; Sigma) and collagenase D (1 mg/ml; Boehringer Mannheim) in 50 ml of AIM-V media (Life Technologies, Inc.). Cells were washed three times in PBS and resuspended in 0.2 ml of PBS/animal/injection. Injected cells were >90% viable as determined by trypan blue exclusion. For splenic MART-1 i.m. injection, 50 μg of plasmid in 50 μl of PBS were injected into the tibialis anterior muscle of mice once a week for 3 weeks. For AdVMART1 injection, 5 x 10^6 pfu were resuspended in a final volume of 50 μl (i.m. injection) or 200 μl (i.p. injection) for each animal and injected weekly for 3 weeks. The size of tumors was assessed three times weekly. Tumor volume was approximated by the following calculation: \( V = \frac{4}{3} \pi r^3 \) (r, radius). All experiments included five mice/group and have been repeated at least twice.

**Cytotoxicity Assays.** Splenocytes harvested from mice 7 days after the last immunization were restimulated by coculture with irradiated NFSA(MART1) cells (25:1 ratio) in RPMI 1640 with 10% FCS and 10 units/ml IL-2 (Hoffmann-La Roche, Nutley, NJ). Effector cells were harvested 5 days later. Splenocytes from normal unimmunized mice served as controls and were stimulated in vitro in the same manner. For cytotoxicity assays, 2 x 10^5 target cells were labeled by incubation in 2.5 ml of RPMI 1640 plus 10% FCS with 51Cr (100 μCi; Amersham Life Science, Arlington Heights, IL) for 18 h at 37°C, washed extensively, and cocultured at 1 x 10^5 cells/well with effector cells (at the ratios given in the figures) in 96-well round-bottomed plates (200 μl/well) for 4 h at 37°C. The supernatants (150 μl) from triplicate cultures were collected and counted. In monoclonal antibody MHC class I blocking experiments, 10 μl of purified hybridoma supernatant (16-1-2N hybridoma, HB-14; American Type Culture Collection, Rockville, MD) were added to chromated target cells before mixing with effector cells. Data points are expressed as the mean percentage of release of 51Cr from target cells ± SE.

**Statistical Analysis.** Student’s t test was performed to interpret the significance of differences between final tumor volumes of animals immunized with DCs and other immunization strategies (presented as mean ± SE). The comparisons were performed excluding the animals that were completely protected from tumor challenge to give a reliable assessment of the effect of immunization in the tumor growth curves. Two-sided P values are presented for individual comparisons.

**Results**

**Characterization of NFSA(MART1).** NFSA, a poorly immunogenic murine fibrosarcoma syngeneic in C3H mice (H-2b), was stably transfected with human MART-1 cDNA. Expression of MART-1 was stable over a 7-month period of multiple in vitro and in vivo passages as determined by RT-PCR (Fig. 1). The phenotype of tumor line NFSA(MART1) was unchanged compared with that of the wild-type tumor with respect to in vitro doubling time (20 h), expression of MHC class I, and lack of expression of MHC class II. The in vivo TDo50 for NFSA(MART1) is approximately 3 x 10^4 tumor cells, which is only slightly higher than that of NFSA (6 x 10^7 tumor cells; Ref. 10).

**Transduction of Murine DCs by AdVMART1.** We previously reported that E1-deleted AdV vectors are excellent gene transfer vectors for DCs, superior to any physical method tested (7). Murine DCs were differentiated for 8 days in vitro from normal C3H femoral bone marrow progenitor cells in the presence of murine granulocyte macrophage colony-stimulating factor and IL-4. We constructed an AdV vector in which the human MART-1 cDNA was driven by the CMV enhancer/promoter. Transduction of murine DCs with AdVMART1 resulted in MART-1 expression that persisted for at least 5 additional days of cell culture as determined by RT-PCR (Fig. 1). Semiquantitative RT-PCR assessment of the transduction of murine DCs by AdVMART1 indicates a viral dose effect, with higher MART-1 mRNA expression at increasing MOI (Fig. 1).

**Genetic Immunization Using AdVMART1-transduced DCs.** In vivo studies were performed to determine the optimal immunization schedule using murine DCs transduced with AdVMART1.4 We have used our best immunization protocol to directly compare AdVMART1/DC immunization with two other genetic immunization strategies: systemic i.m. AdVMART1 administration and i.m. MART-1 plasmid injection. Groups of naive C3H mice received two i.v. AdVMART1/DC injections (MOI = 100 × 10^7 pfu/DCs), or three weekly i.m. injections of 50 μg of MART-1 plasmid cDNA, or three weekly i.m. or i.p. injections with 5 x 10^8 pfu of AdVMART1. All animals were challenged 1 week after the last immunization with 10^6 NFSA(MART1) cells. A representative experiment is shown in Fig. 2. The DC-based strategy was superior in eliciting protective immunity (i.v. AdVMART1/DC versus i.m. MART-1 cDNA, P = 0.002; i.v. AdVMART1/DC versus i.m. AdVMART1, P = 0.01; i.v. AdVMART1/DC versus i.p. AdVMART1, P < 0.001).

**Immunization with AdVMART1/DCs Generates MART-1-specific CTLs.** In vitro restimulated splenocytes from mice immunized twice with AdVMART1/DCs were able to lyse target cells that expressed MART-1 but were unable to lyse the parental NFSA line. Splenocytes from control unimmunized mice were unable to lyse NFSA(MART1) or the untransfected parental cell line, and the MART-1-specific lysis was blocked with an anti-MHC class I antibody (HB14; Fig. 3). We have performed several additional 51Cr release assays in which splenocytes from AdVMART1/DC-immunized mice were unable to lyse the NFSA(Neo) cell line, which is the NFSA line transfected with an empty plasmid, but lysed another C3H (H-2k) MART-1-transfected target, SVEC(MART1). Furthermore,
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immunization was effective in generating protective immunity, suggesting rapid CTL induction. We therefore attempted to treat microscopically established tumors with either one or two iv. injections of AdVMARTI/DCs. AdVMARTI/DCs were injected i.v. 3 days (five animals) or 3 and 7 days (five animals) after s.c. inoculation of \(10^6\) NFSA(MART1) cells. Empty DCs were injected twice, at 3 and 7 days after tumor implantation. Significant protection was observed with both one and two i.v. treatments with AdVMARTI/DCs. Animals treated twice with empty DCs showed a slight initial delay in the tumor growth curve, but final tumor volumes were equal to those of the control group (Fig. 4).

Discussion

MART-1 is a well-characterized human melanoma-associated antigen (2, 3). A nonapeptide derived from the MART-1 gene presented in the context of the HLA-A2.1 is recognized by the majority of tumor-infiltrating lymphocyte cultures tested (2). We recently cloned and analyzed the MART-1 promoter, and we have shown that it confers MART-1-specific expression on a reporter gene (11). A murine model of genetic immunization against MART-1 was developed to define an optimal genetic immunization strategy. Our model uses a human gene inserted into a highly aggressive and poorly immunogenic murine fibrosarcoma cell line, NFSA (10).

DCs are the most effective APCs for the induction of primary immune responses (4, 5). Their high level of costimulatory molecule expression makes them well suited for antigen presentation and less likely to induce tolerance. Transduced with an AdVMART1 vector, murine DCs express high levels of MART-1 mRNA in a viral dose-dependent fashion, and this high level of expression is maintained for days.
at least 5 days in vitro (Fig. 1). Immunization of mice with these genetically engineered cells has the theoretical advantages of allowing the processing and presentation of the MART-1 antigen by the appropriate restriction elements and the continuous exposure of the murine immune system to endogenously processed MART-1-MHC class I and possibly class II complexes on the surface of DCs.

Our results suggest that the presentation of MART-1-derived peptides by DCs elicits a better protective antitumoral response compared to immunization with plasmid cDNA or the in vivo delivery of the AdVMART1 vector. Both the i.m. immunization with naked DNA and the systemic administration of AdV vector rely on host APCs to present MART-1-derived epitopes (12-14). In this model, direct presentation of antigens by genetically engineered DCs seems to be superior for the generation of tumor-specific immunity. The use of in vivo passaged tumors for tumor challenge avoids the theoretical concern of eliciting culture media or FCS-derived immune responses using cultured DCs. The in vitro cytotoxicity studies provide confirmatory evidence that the immunity generated was MART-1 specific. Splenocytes from AdVMART1/DC-immunized animals were able to lyse only MART-1-expressing tumor cells in a MHC class I-restricted manner.

We were able to significantly protect mice with previously implanted MART-1-expressing tumor cells using AdVMART1/DCs. The s.c. injection of 1 x 10^6 NFSA(MART1) cells into naive mice induces the appearance of a palpable tumor 5-11 days after injection, and this inoculum of tumor cells will kill all of the mice in less than 1 month. This result implies that the AdVMART1/DC treatment can elicit very rapid induction of cytotoxic immune effectors. Recent studies have shown that freshly isolated DCs from both tumor-bearing animals (15) and cancer patients (16) are unable to adequately present antigens to the immune system, whereas DCs cultured in vitro from bone marrow progenitor cells from the same subjects with malignant tumors allow for the restoration of the antigen-presenting function of DCs (15, 16).

Several groups have attempted to use human DCs to stimulate the production of antigen-specific CTLs in vitro. Cultured DCs seem to be superior to other APCs for the generation of specific immune effectors, and genetically engineered DCs are able to induce specific CTLs with fewer in vitro stimulations than when DCs are exogenously pulsed with antigenic peptides (17, 18). The transduction of our AdVMART1 construct into HLA.A2.1 human DCs cultured from peripheral blood mononuclear cells was able to present appropriate MART-1 peptides that were recognized and specifically lysed by previously established CTL lines from MART-1-expressing melanoma patients.²

Results of this murine model cannot be directly extrapolated into human melanoma patients: MART-1 is a differentiation antigen for cells of melanocyte lineage, and tolerance to this self-antigen must be overcome to be able to elicit an effective immune response in melanoma patients, who are likely to have a higher tumor burden than the mice protected in these murine studies. However, there is a large body of evidence that T-cell responses can be generated to this antigen in melanoma patients (14, 17). In summary, we report the successful use of genetically engineered DCs to generate protective antitumor immunity in vivo to a defined human melanoma antigen. This preclinical model supports a gene therapy strategy for human melanoma.

References


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² L. H. Butterfield and B. Mukherji, unpublished observations.


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