Recombinant adenovirus-transduced dendritic cell immunization in a murine model of central nervous system tumor

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Object. Dendritic cells (DCs) are potent antigen-presenting cells that have been shown to play a critical role in the initiation of host immune responses against tumor antigens. In this study, a recombinant adenovirus vector encoding the melanoma-associated antigen, MART-1, was used to transduce murine DCs, which were then tested for their ability to activate cytotoxic T lymphocytes (CTLs) and induce protective immunity against B16 melanoma tumor cells implanted intracranially.

Methods. Genetic modification of murine bone marrow–derived DCs to express MART-1 was achieved through the use of an E1-deficient, recombinant adenovirus vector (AdVMART1). Sixty-two C57BL/6 mice were immunized by subcutaneous injection of AdVMART-1-transduced DCs (23 mice), untransduced DCs (17 mice), or sterile saline (22 mice). Using the B16 murine melanoma, which naturally expresses the MART-1 antigen, all the mice were then challenged intracranially with viable, unmodified syngeneic B16 tumor cells 7 days later. Splenocytes obtained from representative animals in each group were harvested for standard cytotoxicity and enzyme-linked immunospot assays. The remaining mice were followed for survival.

Immunization of C57BL/6 mice with DCs transduced with AdVMART1-DC elicited the development of antigen-specific CTL responses. As evidenced by a prolonged survival curve when compared with control-immunized mice harboring intracranial B16 tumors, AdMART1-DC vaccination was able to elicit partial protection against central nervous system (CNS) tumor challenge in vivo. However, this CNS antitumor immunity was weaker than that previously demonstrated against subcutaneous B16 tumors in which the same vaccination strategy was used.

Conclusions. These data suggest that immune responses generated against CNS tumors by DC-based vaccines may be different from those obtained against subcutaneous tumors.

Key Words • dendritic cell • brain neoplasm • tumor immunity • gene therapy • immunotherapy • melanoma

Dendritic cells are potent antigen-presenting cells with the capacity to acquire and process antigen, migrate to lymphoid organs, and stimulate antigen-specific T-cell responses. They express high levels of MHC class I and class II proteins, as well as important costimulatory molecules necessary for activating naïve T cells. Because of these properties, DCs are potentially powerful adjuvants for tumor antigen immunization.

It is now well recognized that many tumor cells express antigens that may provide potential targets for therapeutic vaccine strategies that boost natural immune-mediated tumor rejection. The identification of genes encoding such cancer-rejection antigens has allowed for the development of novel, more rational antitumor vaccine strategies that focus the immune response on the relevant tumor antigen. In malignant melanoma, several of these antigen genes have been identified and characterized. One of these, MART-1, encodes a 118-amino acid protein with an unknown physiological function and is the immunodominant antigen recognized by most melanoma-specific tumor-infiltrating lymphocytes obtained in patients with histocompatibility leukocyte antigen–A2.1 melanoma.

Several methods have been previously described by which DCs are loaded with tumor antigens to generate antigen-specific antitumor responses. These strategies have included coculturing (pulsing) DCs with acid-eluted tumor peptides, with defined synthetic peptides, with tumor cell–derived total RNA, and with whole tumor lysates. Using such approaches in a variety of different models, it has been shown that effective antigen-specific immunity and antitumor responses can be elicited. Genetically modifying DCs with genes coding for specific tumor antigens has potential theoretical advantages over these other methods. First, gene transfer to DCs en-

Abbreviations used in this paper: AdVMART1 = adenovirus vector coding for MART-1 antigen; CNS = central nervous system; CTL = cytotoxic T lymphocyte; DC = dendritic cells; ELISPOT = enzyme-linked immunospot; FCS = fetal calf serum; IFN = interferon; IL = interleukin; MHC = major histocompatibility complex; PBS = phosphate-buffered saline; PCR = polymerase chain reaction.

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sures that the gene product is constitutively expressed and endogenously processed, leading to the appropriate presentation of epitopes in the context of MHC class I elements for the immune system to recognize. This allows for the activation of MHC class I–restricted CTLs, which is the efferent arm of cell-mediated immune responses against tumors. Second, the use of an unlimited genetic source of tumor antigen eliminates the requirement to generate sufficient tumor cell cultures, tumor homogenates, or tumor total RNA from individually resected tumor specimens. Particularly for tumors within the CNS, the ex vivo purification and expansion of such material derived from small surgically obtained specimens may be a practical limitation. With these advantages in mind, different gene transfer approaches have been explored to genetically modify DCs in antitumor therapy. Among these, the use of replication-incompetent adenoviral vectors has been found to be one of the most efficient in recent studies investigators have shown that genetically modifying DCs with AdVMART1 can successfully generate antigen-specific CTLs in vitro. Furthermore, vaccination with these genetically modified DCs induced protective antitumor immune responses that were sufficiently strong to reject extracranial MART1-expressing subcutaneous neoplasms in vivo. The main objective of this current study is to assess the ability of AdVMART1-DC vaccination to induce similar responses against tumors implanted intracranially within the immunologically privileged CNS. Evaluating the efficacy of immunotherapy against tumors such as melanoma in the CNS is important because of the high incidence of brain metastases in patients with these malignant cancers.

MATERIALS AND METHODS

Mice and Tumor Cell Line

Female C57BL/6 mice (H-2b), ages 5 to 8 weeks, were handled in accordance with the animal care policy of the University of California at Los Angeles. All animal studies were approved by the University of California at Los Angeles Chancellor’s Animal Research Committee. A poorly immunogenic murine melanoma cell line syngeneic in C57BL/6 mice, B16 was used in these experiments. These cells were maintained in routine tissue culture at 37°C/5% CO₂ in Dulbecco’s modified Eagle medium with 10% FCS, and 1% penicillin/streptomycin, and fungizone.

Recombinant Adenovirus

The replication-deficient adenoviral vector used in this study was an E1-deleted vector based on human type 5 adenovirus. The recombinant AdVMART1 was constructed as described previously. Recombinant AdVMART1 contains the 400-bp human MART-1 complementary DNA driven by the CMV enhancer/promoter.

Preparation of DCs

Dendritic cells were prepared from murine bone marrow progenitor cells as described previously. Briefly, bone marrow cells derived from the femurs of syngeneic C57BL/6 mice were cultured overnight in RPMI-1640 with 10% FCS and antibiotic agents (1% penicillin–streptomycin) in a sterile Petri dish. Cells were replated the following day at approximately 10⁶ cells/well in 24-well plates in the presence of 100 ng/ml recombinant murine granulocyte-macrophage colony–stimulating factor and 500 ng/ml murine IL-4. On Day 8, the nonadherent and loosely adherent cells were harvested and found to have distinctive DC-like morphology with cytoplasmic processes. Flow cytometric analyses of representative preparations showed high levels of cells positive for MHC class I and class II antigens, B7.1 (CD80) and B7.2 (CD86), which were comparable with the phenotype of bone marrow–derived DCs used in previous studies (data not shown).

The MART-1–Adenoviral Transduction of DCs

In vitro cultured DCs were transduced in 15-ml conical tubes in a final volume of 1 ml of infection media, consisting of RPMI-1640 with 2% FCS. The virus stock was added at a multiplicity of infection of 100 viral pfu per DC. Incubation was performed at 37°C for 2 hours, after which the DCs were washed extensively and resuspended in 0.2 ml PBS per animal. Morphological characteristics were assessed and cell counts were determined using a hemocytometer. In all cases, DC viability exceeded 95%, as determined by trypan blue exclusion.

The expression of the MART-1 transgene in AdVMART1-transduced DCs was confirmed by reverse-transcriptase PCR. Total cellular RNA was isolated using Trizol per manufacturer’s protocol. Each RNA sample was treated with DNase, reverse transcribed with random hexamer primers, and then subjected to PCR with MART1-specific PCR primers: 5’-CTCTTTTCTCTCTAGACCTGTGCCCTGACCCTACAA-3’ and 5’-TTGT-GCAGAGCTCTAGACGTGCTGCTTATAGGT-3’.

Immunizations and Implantation of Brain Tumors

Groups of naïve C57BL/6 mice received two weekly subcutaneous immunizations in the right flank with 5 × 10⁶ AdVMART1-DCs/mouse, 5 × 10⁵ unmodified DCs/mouse, or PBS. Dendritic cell suspensions were washed extensively with PBS prior to injection. All DC injections were performed in a volume of 0.2 ml PBS/animal.

One week after the last immunization, all animals were challenged intracranially with 500 B16 tumor cells/mouse. The B16 cells were harvested by trypsinization, washed twice with PBS, and resuspended. Using a 10-μl Hamilton syringe, 500 B16 cells in 5 μl PBS were stereotactically implanted into the right frontal lobes of anesthetized C57BL/6 mice by using a Kopf stereotactic frame as previously described. Tumor cells were injected at the bregma, 2 mm to the right of the sagittal suture, and 3 mm deep from the surface of the skull. Animals in each of the vaccination groups were followed for survival.

In Vitro Cytotoxicity Assays

For the in vitro cytotoxicity assays, spleens were harvested from representative mice in each experimental group 1 week after the last immunization. Spleen cells were restimulated for 4 days in vitro with irradiated B16
Cytokine Profile by ELISPOT Assays

The presence of B16-specific effector T cells in the immunized mice was also assessed in ELISPOT assays, as previously described. Briefly, splenocytes obtained in mice in each group were restimulated in vitro with culture with irradiated B16 (responder-to-stimulator ratio 25:1) cells in the presence of 10 U/ml IL-2 for 48 hours. Restimulated splenocytes were then plated in 96-well nitrocellulose filter plates (5 x 10⁴ cells per well) precoated with rat anti-mouse anti–IFN-γ antibody or anti–IL-4 antibody. After incubation for 24 hours at 37°C/5% CO₂, the plates were then washed with PBS, and the presence of cytokine-producing spleen cells was detected by incubation at 4°C with biotinylated goat anti–rat secondary antibody, followed by 100 μl/well horseradish peroxidase avidin-D and 150 μl/well freshly prepared substrate buffer (0.4 mg/ml 3-amino-9-ethyl-carbazole in a total of 50 ml 0.05 mol/L sodium acetate buffer) and 20 μl 30% H₂O₂. The stained spots corresponding with IFN-γ- or IL-4-producing cells were enumerated under a dissecting microscope.

Histological and Immunohistochemical Analysis

The brains and spleens of mice in each experimental group were collected at necropsy for histological and immunohistochemical analysis as previously described. Briefly, mice were perfused with 500 ml saline followed by 500 ml of 4% paraformaldehyde. Tissue specimens were then postfixed in 4% paraformaldehyde overnight and stored at 4°C in 15% sucrose prior to cryosectioning. Sections (10, μm) were made and either stained with hematoxylin and eosin for routine histological inspection or incubated with rat anti–mouse monoclonal CD8 or CD4 antibodies for immunohistochemical examination, as previously described.

Sources of Supplies and Equipment

We purchased female rats from the Jackson Laboratory (Bar Harbor, ME). The poorly immunogenic murine cell line, B16, was obtained from American Type Culture Collection (Rockville, MD). The Dubeczko’s modified Eagle serum was aquired from Gibco-BRL (Gaithersburg, MD), the FCS from Irvine Scientific (Santa Ana, CA), and 1% penicillin/streptomycin, and fungizone from Gemini Products (Calabasas, CA). The AdVMART was provided by Dr. James Economidou, in the Division of Surgical Oncology, at the University of California.

The granulocyte-macrophage colony–stimulating factor was obtained from Immunex (Seattle, WA). We purchased the IL-4 from Genzyme, (Cambridge, MA). Cultured DCs were transduced in conical tubes manufactured by Costar (Acton, MA). Total cellular RNA isolation was performed using Trizol, which was aquired from Life Technologies (Rockville, MD). The DNAase was purchased from Stratagene (La Jolla, CA). The following materials were manufactured by Pharmingen (San Diego, CA): anti–IFN-γ antibody, anti–IL-4 antibody, goat anti–rat secondary antibody, and CD8 and CD4 antibodies. The horseradish peroxidase avidin D was acquired from Vector Laboratories (Burlingame, CA).

Statistical Analysis

Survival estimates and median survival times were determined using the Kaplan–Meier method. Survival data were compared using the Wilcoxon log-rank test. The Student’s t-test was used for calculating the significance of the other data. Statistical significance was determined at the p ≤ 0.05 level.

RESULTS

Transduction of Murine DCs by AdVMART1 Vector

Dendritic cells that we derived from mouse bone marrow exhibited the veiled dendrite morphological features typical of previously described DCs and displayed the characteristic DC surface markers as determined by fluorescence-activated cell sorter analysis (data not shown). Exposure of DCs to recombinant AdVMART1 vector at a multiplicity of infection of 100 reproducibly resulted in detectable MART-1 expression that persisted for the entire 8-day culture period, as determined by reverse-transcriptase PCR (Fig. 1A). Adenoviral transduction did not affect the distribution of DC surface markers or the morphological features of DCs (Fig. 1B).

Generation of Tumor-Specific CTLs

In chromium release assays in vitro, restimulated splenocytes from mice immunized twice with AdVMART1-DCs were able specifically to lyse target B16 cells that endogenously expressed MART-1. Splenocytes derived from control saline-immunized mice were unable to lyse B16 cells, even at high effector to target ratios. The B16-specific CTL response induced in DC-immunized mice was statistically significant compared with that seen in control mice injected with PBS (p = 0.015, Fig. 2).

Specific Cytokine Production by Splenocytes Derived From Immunized Mice

In mice, quantification of specific cytokine-producing cells by ELISPOT has been shown to be a very sensitive way of detecting antigen-specific CTLs. This assay was used to confirm the presence of CTLs specific for B16 antigens, because CTLs against culture media components could have potentially participated in the in vitro lysis of B16 cells demonstrated in the chromium release assays. In these studies, the numbers of MHC class I–restricted CTLs that produced IFN-γ and MHC class II-restricted CTLs producing IL-4 were measured upon restimulation with irradiated B16 for 48 hours. The frequency of cells producing the T₇-type cytokine IFN-γ was significantly higher in the AdMART1-DC–vaccinated mice compared with those that received unmodified DCs or PBS (p = 0.046). The production of the T₇-type cytokine IL-4 was also highest in the AdV-
MART1-DC–immunized mice compared with control groups \( (p = 0.014) \). In agreement with previous findings, higher levels of IL-4–producing T2 cells than IFN-γ–producing T1 cells were activated by B16 restimulation in this vaccination model (Fig. 3). These results extend previous reported finding that vaccination with AdVMART1-transduced DCs does indeed generate the appropriate tumor-specific T-cell cytokine production to elicit CTL activity against B16 tumor cells.\(^{11,44}\)

**Intracranial B16 Tumor Challenge After Immunization With AdVMART1-DCs**

The ability of AdMART1-transduced DCs to induce effector CTLs capable of lysing B16 tumor cells in vitro suggested that AdVMART1-DC immunization might also provide antitumor protection in vivo. In previous studies the authors have shown that immunization of naive C57BL/6 mice with AdMART1-DCs resulted in protection against subsequent subcutaneous B16 tumor challenge.\(^{44}\) As an extension of this observation, we assessed the efficacy of this gene-modified DC-based tumor vaccine in preventing the establishment of B16 tumors in the CNS. Sixty-two C57BL/6 mice were immunized with two weekly subcutaneous injections of AdVMART1 gene–modified DCs (23 mice), untransduced DCs (17 mice), or PBS (22 mice). Seven days later, mice underwent intracranial challenge in the right frontal lobe of the brain with 500 B16 cells. As shown in Fig. 4, a prolonged survival curve was demonstrated in the group of mice immunized with AdVMART1-DCs compared with control mice \( (p = 0.05) \), suggesting a statistically significant level of protection afforded by the AdVMART1-DC administration. Mice in the PBS control group and the untransduced DC group had median survivals of 17 and 18 days, respectively. Mice treated with the AdVMART1 vector–transduced DCs had a significantly longer median survival of 23 days. In two of the mice receiving AdVMART1-DC vaccination complete protection from the intracranial B16 challenge was demonstrated, and there was no evidence of tumor seen on histological examination of the brains of these long-term (>60-day) survivors. However, in contrast to previous findings that eight (23.5%) of 34 mice vaccinated with AdVMART1-DC were completely protected from subcutaneous tumor challenge,\(^{44}\) only two of...
23 (8.7%) similarly immunized mice in this study showed complete protection from intracranial tumor challenge.

Histological Characterization of CNS Tumors

To characterize the intracranial tumors histologically, brains from triplicate tumor-bearing animals immunized with PBS, empty DCs, or AdVMART1-DCs were examined by immunohistochemistry for antibodies against CD8+ and CD4+ T-cell markers. As shown in Fig. 5, immunohistochemical examinations of the brains of both control and AdVMART1-DC–vaccinated animals revealed tumor cells without significant leukocyte infiltration by CD8+ or CD4+ T cells. In contrast, spleens obtained from these animals displayed prominent CD8+ and CD4+ mononuclear cells and served as positive controls for the immunohistochemical analysis of the brain sections (data not shown).

DISCUSSION

It has been convincingly demonstrated in previous studies that vaccination with DCs genetically engineered to express the MART-1 antigen stimulates a potent immune response against MART-1–expressing tumors outside the CNS.44 In this study, we confirmed observations that immunization with AdVMART1–transduced DCs generates tumor-specific CTLs, as measured by in vitro cytotoxicity and ELISPOT assays.11,39 Furthermore, we evaluated the ability of these gene-modified DCs to protect against intracranial tumor challenge. As evidenced by a prolonged survival curve when compared with control mice harboring intracranial tumors, AdMART1-DC vaccination was able to elicit partial protection against CNS tumor challenge in vivo. However, the efficacy of this vaccination strategy seemed to be better against subcutaneous MART1–expressing tumors44 than against the same tumors implanted intracranially, suggesting that the unique immunological milieu of the CNS may play a role in lim-
The concept that the brain is an immunologically privileged site has been supported by animal studies in which the persistence of intracranial tumors was demonstrated to be resistant to systemic immune responses. This has led to the development of various strategies to break CNS tolerance and elicit antitumor immunity. Our results in this present study are consistent with previous findings: although measurable systemic CTL responses can be elicited against CNS tumors, these responses may not be sufficient to control intracranial tumor growth. The concept that the brain is an immunologically privileged site was initially supported by animal studies in which it was demonstrated that the persistence of intracerebral tumor allografts and xenografts in immunocompetent hosts is capable of rejecting the same tumor grafts in the periphery. This notion of CNS immune privilege has been further corroborated clinically by the finding that CNS tumors failed to respond to adjuvant immunotherapy protocols that were otherwise effective in preventing and treating systemic cancers. The mechanisms limiting antitumor immunoreactivity within the brain are not currently understood. Features such as the absence of conventional lymphatic drainage, the presence of the blood-brain barrier, and heightened immunological tolerance may all contribute to the relative immunosuppression within the CNS.

Immunological tolerance is a well-described and distinguishing biological feature of the brain, which may possibly explain the relatively weaker antitumor immune response elicited by MART-1–transduced DCs in our CNS tumor model in C57BL/6 mice. The MART-1 is a nonmelanoma lineage antigen, not a melanoma-specific antigen. Because murine MART-1 is also expressed by melanocytes in this mouse strain, attempts to vaccinate C57BL/6 mice against B16 melanoma cells by using MART-1 as an immunological target requires that tolerance to this self-antigen be overcome. Although it has been shown that genetically modified DCs may be capable of breaking peripheral tolerance to lineage-specific antigens expressed by tumor cells, it is conceivable that CNS tolerance to self-antigens is more difficult to overcome. Because melanocytes have a neural crest origin and are found diffusely in the leptomeninges of the brain and spinal cord, loss of CNS tolerance to normal melanocytes may lead to potentially disastrous consequences such as autoimmune encephalomyelitis. Therefore, an explanation for the diminished efficacy of antigen-specific immune responses within the brain (when compared with other sites) may include a greater level of CNS tolerance for self-antigens to protect the brain from deleterious autoimmunity that is lethal. Although several reports have indicated that antigen-specific T cells may be activated to prevent peripheral tumor growth without concomitant autoimmune destruction of normal tissues expressing the same antigen, the authors of these previous studies did not examine tumors within the CNS. In our immunohistochemical analyses, we found no evidence of T-cell infiltration into brain tumors or normal brain tissues after AdVMART1-DC vaccination. Therefore, the lack of experimental autoimmune encephalomyelitis observed in our DC-treated mice may have been maintained at the cost of effective antigen-specific antitumor responses capable of overcoming CNS tolerance. The mechanisms regulating the recognition of self compared with nonsself (that is, tolerance compared with autoimmunity) in the CNS are incompletely understood and warrant further study.

Although the use of genetic modification of DCs to express a defined tumor antigen gene can serve as a continuous source of activated antigen-presenting cells, this strategy requires the identification of the appropriate tumor-specific or tumor-associated antigens that can best mount an effective antitumor immune response in vivo. To date, defined tumor-rejection antigens have only been identified for a small number of cancers, and none is currently characterized for brain cancers. Furthermore, it is still unclear whether vaccination with multiple tumor antigens or a single defined antigen is superior in eliciting such antitumor immunity. In comparison with other studies of DC-based immunotherapy for intracranial neoplasms in which investigators used unfractionated tumor antigen preparations (that is, acid-eluted surface peptides, tumor homogenates, or tumor messenger RNA), the results of our current AdMART1-DC model demonstrated a weaker level of protection against CNS tumor challenge. One possibility may be that expansion of the antigenic repertoire of activated T cells may improve immunogenicity within the CNS. Although it has been shown in previous studies that DCs pulsed with acid-eluted tumor peptides may be less effective against subcutaneous tumors than genetically engineered DCs expressing a single dominant epitope, results demonstrated in our laboratory and by others indicate that the pulsing of DCs with unfractionated preparations may actually be superior against intracranial tumors. This dichotomy between peripheral and CNS immunotherapy results further supports the hypothesis that both afferent and effector immune mechanisms against brain tumors may be different from those generated against subcutaneous tumors.

CONCLUSIONS

In summary, we have demonstrated that adenoviral-transduced DCs expressing a defined melanoma antigen, MART-1, could generate tumor-specific CTL responses that partially protect immunized animals against an intracranial melanoma tumor challenge. The potency of this antitumor immunity within the CNS, however, was comparably weaker than that generated against subcutaneous tumor challenges in which the same genetic immunotherapeutically.
apy strategy was used. This latter observation underscores the existence of unique mechanisms regulating neuroimmuno-
ology and the fact that results of DC-based immuno-
otherapy trials against systemic cancers cannot be directly
extrapolated into brain tumor models. Further studies
are needed to better acquire understanding and to optimize
the potential of gene-modified DCs against CNS tumors.

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