α-Fetoprotein-specific Genetic Immunotherapy for Hepatocellular Carcinoma


Abstract

The majority of human hepatocellular carcinomas overexpress α-fetoprotein (AFP). Two genetic immunization strategies were used to determine whether AFP could serve as a target for T-cell immune responses. Dendritic cells engineered to express AFP produced potent T-cell responses in mice, as evidenced by the generation of AFP-specific CTLs, cytokine-producing T cells, and protective immunity. AFP plasmid-based immunization generated less potent responses. These novel observations demonstrate that this oncofetal antigen can serve as an effective tumor rejection antigen. This provides a rational, gene therapy-based strategy for this disease, which is responsible for the largest number of cancer-related deaths worldwide.

Introduction

Our improved understanding of antigen processing and presentation by professional antigen-presenting cells, as well as the requirements for induction of T-cell immunity, has spawned the discipline of genetic immunotherapy. DNA-based immunization strategies are capable of generating strong cellular immune responses to a variety of antigens, including tumor antigens.

A major obstacle in developing rational strategies in tumor immunotherapy has been the identification of suitable target tumor antigens. The recent identification of several human melanoma rejection antigens using cytolytic T-cell clones has greatly modified our thinking about self/non-self discrimination and what actually constitutes a genuine tumor antigen (1). Many of these melanoma antigens (MART-1, gp100, and tyrosinase), as well as those associated with other malignancies (carcinoembryonic antigen and prostate-specific antigen), represent normal, nonmutated self proteins (2–4). It is now evident that these normal protein determinants have not induced self-tolerance, can be recognized by the mammalian T-cell repertoire, and, when properly and specifically activated, can mount an effective antitumor response that is essentially an autoimmune response (5–7).

The majority of human HCCs3 overexpress the oncofetal antigen AFP. This $M_{r}$ 70,000 glycoprotein, produced at high levels by the fetal liver, is transcriptionally repressed at birth and is present thereafter at low serum levels throughout life (8). Serum AFP measurements play an important role in the diagnosis and monitoring of HCC. We wished to determine whether the murine immune system, despite being exposed to AFP (at even higher relative levels than humans) throughout life, could generate T-cell responses to this oncofetal antigen. Two powerful genetic immunization modalities, genetically engineered DCs and plasmid DNA, were used to generate AFP-specific T-cell responses in a murine model. Both strategies induced AFP-specific immune immunity, as evidenced by in vivo protection against AFP-producing murine tumors and by the stimulation of AFP-specific CTLs and cytokine-producing T cells.

Materials and Methods

Construction of mAFP Expression Vectors. RT-PCR primers were designed to include the entire mAFP coding region, including the signal sequence. The primers used were 5'-GCCATGAAGTTGAGGTCAACAAA9-3' and 5'-CTCTGCTTCTTCTTAGTATTACGCTA-3'. RT-PCR template total RNA was isolated from Hepa 1-6 cells by the TRZol (Life Technologies, Inc., Gaithersburg, MD) method. The 1.8-kb mAFP cDNA PCR product was initially cloned into pCR3.ineo (Invitrogen, San Diego, CA) according to manufacturer’s instructions for T-A cloning to generate stable transfectants. The 1.8-kb mAFP cDNA and the 400-bp MART-1 cDNA (9) were subcloned into expression vector VR1012 (Vical, San Diego, CA) by blunt-end cloning for i.m. immunization. The mAFP cDNA was also subcloned into pA-CMV-pLpA (AdV shuttle vector), which was used to generate the virus AdVmAFP as described previously (10).

Mice and Cell Lines. Female 5–6-week-old C57BL/6 mice (H-2b) were purchased from The Jackson Laboratory (Bar Harbor, ME) and handled in accordance with the animal care policy of the University of California, Los Angeles. EL4 is a spontaneous murine lymphoma that arose in C57BL/6 mice, and both Hepa 1-6 and BW1/5 are derivatives of BW756 (11). EL4 and Hepa 1-6 were obtained from the American Type Culture Collection (Rockville, MD), and BW1/5 was obtained from Dr. John Papanicolaou (University of Texas, Galveston, TX). Murine cell lines were maintained in vitro in DMEM (Life Technologies, Inc.) with 10% FCS (Omega Scientific, Tarzana, CA) and 1% (v/v) penicillin, streptomycin, and fungizone (Gemini Bioproducts, Calabasas, CA). EL4(mAFP) was developed by stable transfection of the EL4 cell line with pCR3.1 mAFPneo by lipofection (DMRIE-C; Life Technologies, Inc.). Transfected cells were maintained under constant G418 selection (0.5 mg/ml; Life Technologies, Inc.) in RPMI 1640 (Life Technologies, Inc.) complete media.

Mice were immunized once with 5 × 10³ DCs/mouse administered s.c. in the left flank or 100 µg plasmid/mouse administered i.m. in the left anterior tibialis muscle and challenged 14–21 days later with 7.5 × 10³ to 5 × 10³ EL4(mAFP) cells/mouse on the right flank and EL4 on the left flank. Cells used for tumor challenge were obtained from single cell suspensions of progressively growing tumors in syngeneic mice to avoid the confounding effects of media and serum-derived products on in vivo protection studies (9). Cell suspensions were washed extensively and injected into mice in a final volume of 0.2 ml PBS/animal. Tumor incidence and volume were assessed three times weekly using calipers. Data are presented as mean volume ± SE, as described previously (9).

Preparation of DCs and Adenoviral Transduction. DCs were differentiated from murine bone marrow progenitor cells following the method of Inaba et al. (12), with modifications (9). Day +8 nonadherent and loosely adherent cells contained DC aggregates with a high level of major MHC class I and II, B7.1 (CD80), B7.2 (CD86), CD11c, and CD18, and CD4-positive cells that were superior stimulators of a mixed lymphocyte reaction (data not shown). In vitro cultured DCs were transduced in 15-ml conical tubes (Costar, Acton, MA) in a final volume of 1 ml of RPMI with 2% FCS to which the virus stock was added at a MOI of 100 viral plaque-forming units/DC. Transduction

Received 3/17/99; accepted 5/13/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 Supported by NIH/National Cancer Institute Grants PO1 CA5926, RO1 CA 77623, RO1 CA 79976, T32 CA75956, and K12 CA 79805 and the Stacy and Evelyn Kesselman Research Fund (all to J. S. E.); Tumor Immunology Training Grant CA09120-22 (to C. M. V.); and the Fondo de Investigacion Sanitaria 97/5485 (to A. R.).

3 To whom requests for reprints should be addressed, at Division of Surgical Oncology, UCSF, University of California Los Angeles Medical Center, Los Angeles, CA 90095-1782; Phone: (310) 825-2644; Fax: (310) 825-7575; E-mail: jcconomy@medsch.ucla.edu.

3 The abbreviations used are: HCC, hepatocellular carcinoma; AFP, α-fetoprotein; DC, dendritic cell; mAFP, murine AFP, RT-PCR, reverse transcription-PCR; AdV, adenovirus; MOI, multiplicity of infection; IL, interleukin; ELISPOT, enzymelinked immuno SPOT.
was carried out for 2 h at 37°C, and then the DCs were washed extensively and resuspended in 0.2 ml PBS/animal for injection into mice. Cell counts were determined using a hemocytometer, with viability assessed by trypan blue exclusion. Viability exceeded 95% in all cases.

**In Vivo Monoclonal Antibody Ablation.** CD4 and CD8 T-cell subpopulations were depleted by i.p. injection of protein G-purified hybridoma supernatants. A total of 0.25 mg/mouse/injection of anti-CD8 (clone 2.43; ATCC TIB 210) or anti-CD4 (clone GK1.5; ATCC TIB 207) purified antibody was injected on days 5, 3, and 1 before tumor inoculation and every 6 days thereafter (13). Adequacy of CD4+ and CD8+ T-cell depletion was monitored by flow cytometric analysis of splenocytes at the day of tumor challenge using FITC-labeled anti-CD4 antibody (clone CT-CD4; Caltag, Burlingame, CA) and Phycoerythrin-labeled anti-CD8a antibody (Ly-2; clone 53–6.7; PharMingen, San Diego, CA). Ablated mice had >90% decrease in CD4 and CD8 cells in the spleen after in vivo depletion (data not shown).

**Cytotoxicity Assays.** For in vitro microcytotoxicity assays, splenocytes were harvested 10–14 days after the last immunization, depleted of RBCs by hypotonic lysis, restimulated in vitro with irradiated EL4(mAFP) cells (25:1 ratio) + 10 units/ml IL-2, and assayed in a standard 4-h chromium release test (9). Samples were tested against their own maximum and spontaneous release for each different condition.

**Cytokine Profile by ELISPOT.** ELISPOT assays were performed as follows. Briefly, RBC-depleted splenocytes were restimulated in vitro by culture with irradiated EL4(mAFP) or EL4 (25:1 responder:stimulator ratios) cells with 10 units/ml IL-2 for 48 h. Restimulated splenocytes were added in duplicate in 3-fold dilutions to 96-well mixed cellular plates (multiscreen filtration system; Millipore, Bedford, MA) precoated with anti-IFN-γ or anti-IL-4 antibody (PharMingen). After a 24-h incubation at 37°C, plates were washed and incubated at 4°C with a secondary biotinylated antibody and then developed the next day by adding freshly prepared substrate buffer. Spots were counted under a dissecting microscope.

**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 mAFP, neomycin resistance (Neo), or murine adenine phosphoribosyltransferase (APRT)-specific PCR primers to confirm the expression of the mAFP and neomycin transgenes and well as semiquantitatively the cDNA yield in each reverse-transcribed sample. The primers used for mAFP were identical to the cloning primers above, and the others were as follows: APRT, 5′-ACTCCAGGGGCTTCCTTTGTT-3′ and 5′-ATCCA-CATTGACCACTCTCTG-3′; and Neo, 5′-GGTGGAGAGGCTATTCG-3′ and 5′-GATGAAAGGGCGATGCTG-3′.

**Statistical Analysis.** Results of in vivo studies are presented as the mean and SE of tumor volumes in each treatment group (9). Mice completely protected from a tumor challenge are presented separately from mice that did develop tumors. Significance is calculated using the t test (or the rank sum test in case of failing the Kolmogorov-Smirnov test) for normality (excluding mice that did not develop tumors). Each study included five mice/group and has been repeated at least twice. Tumor appearance and growth to 10 mm in diameter was calculated by the Kaplan-Meier method, and differences between immunized and control mice were calculated by the Mantel-Haenszel test.

**Results and Discussion**

**Generation of mAFP-expressing Plasmid, AdV, and Cell Lines.** The 1.8-kb mAFP cDNA was isolated from the murine HCC cell line Hepa 1-6 by RT-PCR and subcloned into both a eukaryotic plasmid expression vector, VR1012 (pmAFP), and an E1-deleted replication-incompetent AdV vector (AdVmAFP). In both vectors, the mAFP gene is driven by the cytomegalovirus enhancer-promoter (9). A surrogate murine HCC cell line was constructed by stably transfecting the EL4 lymphoma cell line with mAFP. This AFP-producing cell line [EL4(mAFP)] thus served as a T-cell target for both in vivo and in vitro investigations and could be directly compared with the parental, untransfected control, EL4 (Fig. 1a). Similar results were observed when another syngeneic murine tumor, B16 melanoma, was stably transfected with mAFP in a similar fashion (data not shown).

**Genetically Engineered DC-based Immunization.** The DC-based AFP immunization strategy proved superior in generating strong immune responses. We recently reported that human DCs transfected with an AdV expressing the human MART-1 melanoma antigen properly process and present the HLA-A2.1-restricted immunodominant peptide and can stimulate MART-1 peptide-specific human T-cell responses in vitro (14). We also reported that murine DCs transfected with the MART-1 gene can generate potent MART-1-specific T-cell responses in mice, as evidenced by in vivo tumor protection and the generation of MART-1-specific CTLs (9). Therefore, we applied this proven methodology to the putative mAFP tumor antigen. C57BL/6J murine DCs were generated from bone marrow precursors by cell culture in granulocyte macrophage colony-stimulating factor and IL-4 over the course of a week. These DC-enriched cultures had characteristic DC morphology, phenotype, and biology (12, 15). After transduction with AdVmAFP, these DCs expressed mAFP mRNA in a viral dose-dependent manner, as assessed by RT-PCR (Fig. 1a). DCs transduced with AdVmAFP were used to immunize naive mice to a subsequent challenge of EL4(mAFP). Mice receiving one or several weekly s.c. immunizations of AdVmAFP/DC were partially or completely protected from a challenge of EL4(mAFP) (Fig. 1b) but not EL4 (Fig. 1c). Untransduced DCs and DCs transduced with an empty RR5 AdV vector (Fig. 1b) or with the Escherichia coli β-galactosidase gene (data not shown) were all ineffective in generating protective immunity. We noted in our MART-1/DC model that protection required the participation of both CD8 and CD4 T cells. Likewise, in vivo ablation of either T-cell subset completely abrogated AdVmAFP/DC-induced immunity (Fig. 1d), effectively ruling out non-T-cell mechanisms and implying that both MHC class I- and II-restricted responses are being generated. The requirement for MHC restriction is further supported by the observation that only AdVmAFP-transduced syngeneic (C57BL/6; H-2b) DCs but not allogeneic (C3H/Sem/Kam; H-2k) DCs induced protective immunity (data not shown). Splenocytes from immunized mice contained mAFP-specific (MHC class I-restricted) CTLs, as measured by short-term chromium release assays (Fig. 1f) and specifically released the Th1-type cytokine IFN-γ in ELISPOT assays (Fig. 1e). Our cumulative experience with this model includes 12 separate experiments using a total of 140 mice, in which 12 of 89 (13.5%) immunized mice were completely protected from tumor challenge with EL4(mAFP) cells during the 40-day observation period (P = 0.01, χ²). Significantly delayed tumor growth was noted in the remainder of immunized mice (time to reach 10 mm in diameter was 14 days compared to 18.5 days in controls; P < 0.001). None of the AdVmAFP/DC-immunized mice (even multiply immunized animals) challenged with EL4 cells (n = 25) had altered tumor growth compared with controls. We have observed similar but less impressive protection using murine HCC tumors, probably because these cell lines, all of which have been derived from the spontaneous murine HCC line BW7756 and propagated since 1957, do not express detectable levels of MHC class I or II. The only available AFP-producing murine HCCs are BW113 and its derivative cell line, Hepa 1-6.

**DNA-based Immunization.** In a parallel effort, we examined the efficacy of plasmid DNA-based genetic immunotherapy using i.m. injections of AFP plasmid mAFP (pmAFP). Protection with plasmid immunization was less reproducible than with transduced DCs. After receiving as little as one weekly i.m. injection or up to four weekly i.m. injections of pmAFP, naive C57BL/6J mice generally showed some degree of protection from EL4(mAFP), but not after identical immunization with a plasmid encoding the human MART-1 melanoma antigen (pMART; Fig. 2a). Mice were not protected from the parental EL4 tumor (Fig. 2b). In 10 separate experiments using a total of 104 mice, 4 of 54 (7.4%) immunized mice were completely protected from EL4(mAFP) (P = not significant). In the remainder of these mice, tumor growth delay was somewhat prolonged [time to reach 10 mm in diameter, 17 days (immunized mice) and 19.5 days (control); P < 0.001]. Efforts to improve the DNA-based immuniza-
AdVmAFP/DC-ablated, M3 protected from the parental EL4 tumor. A parental EL4 tumor (5 × 10^6) was lysed neither EL4(mAFP) cells (Fig. 2a) nor EL4 cells (itrio). Splenocytes from AdVmAFP/DC-immunized mice were cytotoxic for EL4(mAFP) cells (Fig. 2b) and released IFN-γ when restimulated for 48 h with irradiated EL4(mAFP) or EL4 cells that had a higher EL4(mAFP)-stimulated IFN-γ release as measured by ELISPOT assay than did splenocytes from control or empty DC-treated mice. Data are expressed as the number of spots/one million cells. AdVmAFP/DC-immunized mice are not protected from the parental EL4 tumor. A parental EL4 tumor (5 × 10^6 inoculum) challenge grew progressively in control mice (■), but not for the parental EL4 cells (□). c, frequencies of IFN-γ-producing immune splenocytes. Splenocytes were restimulated for 48 h in vitro with irradiated EL4(mAFP) or EL4 cells and had a higher EL4(mAFP)-stimulated IFN-γ release as measured by ELISPOT assay than did splenocytes from control or empty DC-treated mice. Data are expressed as the number of spots/one million cells. d, induction of AFP-specific CTLs after AdVmAFP/DC immunization. Splenocytes were restimulated from mice immunized with AdVmAFP/DC (same conditions as described in c) and assayed for lytic activity in a standard 4-h chromium release assay. Splenocytes from AdVmAFP/DC-immunized mice were cytotoxic for EL4(mAFP) cells (■), but not for the parental EL4 cells (▲; P = 0.003). Splenocytes from unimmunized mice lysed neither EL4(mAFP) cells (□) nor EL4 cells (itrio).

These experiments clearly demonstrate that both AFP-transduced DCs and AFP plasmid immunization can generate effective T-cell immune responses in vivo. These findings are consistent with the observations made with many other self antigens that have been shown to serve as effective tumor rejection antigens (23, 24). The fact that the T-cell repertoire has not been deleted of these self-reactive clones and can be activated by antigen-engineered DCs is remarkable. Presumably, the overexpression of these self antigens by tumors (MART-1, gp100, MAGE, carcinoembryonic antigen, and now AFP) renders them susceptible to T-cell-based immune responses (25, 26). This represents the first report that this oncofetal antigen can serve as a target for cell-mediated immune responses. This novel observation provides a solid rationale for developing AFP-directed genetic immunotherapy for the treatment and possible prevention of HCC, a disease responsible for the largest number of cancer deaths with an annual global incidence of 1.2 million (27).

ACKNOWLEDGEMENTS

We thank Drs. Eli Sercarz and Mitchell Kronenberg for helpful discussions, Dr. Scott Nelson for pathological assessments, and Angela Chen and Roy Lau for technical assistance.
Fig. 2. AFP plasmid-based generation of AFP immunotherapy. a, AFP plasmid-immunized mice are partially protected from a challenge with EL4(mAFP) cells. Mice received one i.m. injection of 100 μg of either pmAFP plasmid (•) or a plasmid (pMART) expressing the human MART-1 melanoma cDNA (○) or no treatment (control; □). Two weeks after immunization, mice were challenged with 7.5 × 10⁴ EL4(mAFP) cells. Delayed tumor growth was noted in AFP plasmid-immunized mice compared with both untreated controls (P = 0.008) and mice immunized with pMART (P = 0.003). b, AFP plasmid-immunized mice are not protected from a challenge with parental EL4 tumor. Mice identical to those in a were challenged with parental EL4 cells, and no difference in tumor growth was noted. c, induction of AFP-specific CTLs in AFP plasmid-immunized mice. Mice received 2 weekly injections of 100 μg of pmAFP or no treatment. Ipsilateral lymph node lymphocytes were harvested 2 weeks after the final immunization, restimulated for 72 h with irradiated EL4(mAFP) cells, and assayed for lytic activity in a standard 4-h chromium release assay. Lymphocytes from pmAFP-immunized mice lysed EL4(mAFP) (○) but not EL4 (□; P = 0.0002), whereas lymphocytes from control mice lysed neither target (□) and △. d, frequency of IFN-γ-producing draining lymph node lymphocytes. Ipsilateral lymph node lymphocytes retrieved from mice immunized with pmAFP (restimulated in vitro with EL4(mAFP) or EL4 cells for 48 h) had a higher EL4(mAFP)-stimulated IFN-γ release as measured by ELISPOT assay than control or pMART-treated mice lymphocytes.

References


α-Fetoprotein-specific Genetic Immunotherapy for Hepatocellular Carcinoma


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/13/3064

Cited articles
This article cites 23 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/13/3064.full#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/13/3064.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.