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Development of an allogeneic whole-cell tumor vaccine expressing xenogeneic gp100 and its implementation in a phase II clinical trial in canine patients with malignant melanoma

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Abstract A xenogeneic melanoma-antigen-enhanced allogeneic tumor cell vaccine (ATCV) is an appealing strategy for anti-cancer immunotherapy due to its relative ease of production, and the theoretical possibility that presentation of a multiplex of antigens along with a xenogeneic antigen would result in cross-reaction between the xenogeneic homologs and self-molecules, breaking tolerance and ultimately resulting in a clinically relevant immune response. In this study, we evaluated the efficacy of such a strategy using a xenogeneic melanoma differentiation antigen, human glycoprotein 100 (hgp100) in the context of a phase II clinical trial utilizing spontaneously arising melanoma in pet dogs. Our results demonstrate that the approach was well tolerated and resulted in an overall response rate (complete and partial response) of 17% and a tumor control rate (complete and partial response and stable disease of > 6 weeks duration) of 35%. Dogs that had evidence of tumor control had significantly longer survival times

than dogs that did not experience control. Delayed type hypersensitivity (DTH) to 17CM98 canine melanoma cells used in the whole cell vaccine was enhanced by ATCV and correlated with clinical response. In vitro cytotoxicity was enhanced by ATCV, but did not correlate with clinical response. Additionally, anti-hgp100 antibodies were elicited in response to ATCV in the majority of patients tested; however, this also did not correlate with clinical response. This approach, along with further elucidation of the mechanisms of tumor protection after xenogeneic immunization, may allow the development of more rational vaccines. This trial also further demonstrates the utility of spontaneous tumors in companion animals as a valid translational model for the evaluation of novel vaccine therapies.

Keywords Tumor cell vaccine · Allogeneic · Immunotherapy · Melanoma · Dog · Glycoprotein-100

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Introduction

Melanoma is the most common oral tumor in the dog [6, 35], occurring most frequently on the hard palate and the maxillary gingiva [22]. This spontaneously arising tumor in dogs is similar biologically to malignant melanoma in humans [36]. Despite advances in surgery, radiation and chemotherapy, approximately 75–80% of dogs treated by conventional means will die within 1 year of diagnosis, and survival with distant disease is limited to a median of 165 days [3, 10, 19, 29]. Similarly, subungual melanomas of the digits are generally aggressive tumors that grow rapidly, are locally invasive, and metastasize [1, 30]. At present, the treatment of choice is aggressive local surgical excision. Radiation therapy has shown palliative benefit, prolonging life in some patients [3, 24]. Chemotherapy agents examined for efficacy in malignant melanoma include dacarbazine, doxorubicin, cisplatin, and carboplatin [7, 21, 27]. Poor results have been observed with all standard protocols.

These findings clearly demonstrate the need for new strategies to treat oral and subungual melanomas. From a comparative standpoint, oral melanoma in dogs is similar in biological behavior to oral melanoma and advanced cutaneous melanoma of the acral lentiginous form in people [2, 26].

Considerable evidence exists that the immune system can modulate the progression and metastatic behavior of cancer. Adoptive immunotherapy and active immunizations using tumor vaccines exploit the fact that many tumors express unique antigens on their surfaces against which an immune response can be generated. Ideally, antibodies or T-cells generated against cancer cell-specific proteins would selectively target only those tumor cells displaying the appropriate antigen. Melanoma cells express lineage-specific antigens that can be recognized by the immune system and mediate tumor cell destruction [28, 32]. Recent studies have shown that vaccination with xenogeneic tumor-specific antigens or DNA can elicit an anti-tumor response in mice and dogs with advanced tumors, including malignant melanoma [4, 8, 9, 11, 34, 39]. A recombinant xenogeneic protein could serve as a potent vaccine antigen by breaking immune tolerance through cross-reaction between the xenogeneic and self-antigen. The use of xenogeneic antigens in vaccines provides an effective means to induce both a cellular and humoral response to otherwise poorly immunogenic self-proteins [34, 40].

Advances in molecular detection of non-mutated self-proteins expressed on melanoma cells have given a significant boost to the study of novel cancer vaccines by identifying candidate antigens for the induction of an anti-tumor response. One such antigen, glycoprotein 100 (gp100), is expressed on 90% of cells of melanocytic origin, suggesting its potential as an ideal antigen target for vaccination immunotherapy designed to break tolerance or ignorance to self-antigens, leading to *in vivo* cytolytic T-cell activity [14]. Several studies have shown that gp100 vaccination stimulates HLA class I restricted cytolytic T-cell activity in humans [31, 37]. Studies in mice using adenoviral vectors to encode gp100 indicated that using a xenogeneic antigen (human gp100) induced greater immune protection and anti-tumor activity against murine B16-F10 melanoma than was observed in mice treated with murine gp100 [17, 23]. Currently, the most commonly used techniques for gene transfection of tumor cells involve viral delivery systems. Although effective, these techniques are laborious, time consuming, potentially toxic, and associated with immunogenicity that can preclude repeated administration. In contrast, utilizing gene gun particle-bombardment, in which plasmid DNA is precipitated onto gold beads and “shot” into target cells is an attractive and safe alternative to viral gene delivery and has been utilized for vaccine development in normal dogs and dogs with spontaneously arising melanoma [12, 13]. Murine studies using particle-mediated vaccination with human gp100 (hgp100) DNA resulted in a greater level of protection from tumor challenge

and a significant suppression of tumor growth in mice with established tumors [25]. Studies have shown that administration of xenogene DNA tumor vaccines result in the induction of T cells with primary specificity for epitopes in the xenogene, but which still possess sufficient T-cell receptor plasticity to recognize the self-tumor antigen [8]. These findings provide the immunologic rationale for using human gp100 in dogs with melanoma.

In this study we describe the development of a xenogeneic melanoma-antigen-enhanced (hgp100) allogeneic tumor cell vaccine (ATCV) and its implementation in a treatment protocol designed to evaluate efficacy in the context of a phase II clinical trial utilizing spontaneously arising melanoma in pet dogs. We hypothesized that this ATCV would result in an anti-tumor immune response leading to tumor regression and prolonged survival. Additionally, we assessed several surrogates of immune stimulation in vaccinated dogs; both *in vivo* and *ex vivo*.

Materials and methods

Cell lines

The canine melanoma cell line 17CM98 was established in our laboratory. The canine melanoma cell lines CML-1, CML-6M and CML-10 were provided by Dr. Lauren Wolfe, Auburn University, Auburn, AL. The human melanoma cell line M21 was provided by Mark Albertini at the University of Wisconsin Comprehensive Cancer Center (Madison, WI). The canine and human osteosarcoma cells lines, D17 and MG-63, were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Minimal Essential Medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat activated fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin-streptomycin (Mediatech, Herndon, VA), 2 mM L-Glutamine (Mediatech), 1 mM sodium pyruvate (Mediatech) and non-essential amino acids (Sigma, St. Louis, MO).

RT-PCR analysis of melanoma-associated antigen expression

Total RNA was isolated from four canine melanoma cell lines (17CM98, CML-1, CML-6M and CML-10), one human melanoma cell line (M21), and a canine (D17) and human (MG-63) osteosarcoma cell line using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The total RNA was then further purified using the RNeasy Mini kit (Qiagen, Valencia, CA). Purified total RNA was assayed for gp100 mRNA expression by reverse transcription-PCR (RT-PCR) using oligonucleotide primers (Table 1) designed in our laboratory by aligning homologous regions from human, mouse, and horse gp100 sequences. Briefly,

Table 1 Oligonucleotide primer sequences used for RT-PCR

Antigen	Primer sequence
Canine gp100	5'-CCTGGATTGTGTTCTGTATCG-3' 5'-CACCAGCAAGATGCCACGA-3'
Canine tyrosinase	5'-GGAAGAAGAATGCTGGTGGCTG-3' 5'-TGGCAGGTCCGATTGTAAGAC-3'
Canine MART-1	5'-GCAGGGATTGGAATCTTGACAGTG-3' 5'-CTTGAAGAGGCAGTTTGCTGTGC-3'

The annealing temperature for all primer pairs was 56°C.

3 µg of total RNA was reverse transcribed into cDNA using random oligo(dT) and reverse transcriptase. Control samples that did not contain reverse transcriptase were also included to rule out genomic DNA contamination. The isolated cDNA was then subjected to PCR using the above mentioned primers. The PCR mixture (HotStarTaq kit, Qiagen) containing the isolated cDNA was amplified under the following conditions: one cycle of 94°C for 15 min; 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 10 min. The expected PCR products for gp100 mRNA (399 bp) were size fractionated onto a 1% agarose gel and stained with ethidium bromide. Sequence analysis of the PCR product confirmed gp100 amplification. Similarly, RT-PCR was used to detect MART-1, and tyrosinase mRNA expression in the canine melanoma cell lines using primers designed in our laboratory (Table 1). The 17CM98 cell line, developed from a metastatic lymph node in a dog with histologically confirmed malignant oral melanoma, was chosen for allogeneic vaccine production based on expression levels of several melanoma-associated antigens (see Results).

Transfection of 17CM98 cells with hgp100 cDNA for ex vivo studies

The full-length gene encoding hgp100 (2 kb) had been previously cloned into an expression plasmid (pCDNA3.0; Invitrogen), and generously provided to us by Dr. Steven Rosenberg (National Institutes of Health). Following methods reported previously [5, 12, 13], 175 µg of cDNA was precipitated onto 70 mg of microscopic gold particles, mixed with polyvinylpyrrolidone, and the slurry was deposited onto the lumen of tefzel plastic cartridges and dried with nitrogen. These conditions result in a delivery of 0.5 mg gold and 1.25 µg of cDNA per transfection. 17CM98 canine melanoma cells were grown in Minimal Essential Media (MEM) supplemented with L-glutamine, non-essential amino acids, penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were harvested, washed, and resuspended in 10 ml of MEM. The cells were then irradiated to a total dose of 100 Gy using a ¹³⁷Cs irradiator. Following irradiation, the cells were washed in Hank's Balanced Salt Solution (HBSS) and resuspended

at a concentration of 1.0×10⁸ cells/ml. The cells were then spotted onto a 100 mm Petri dish in 20 µl aliquots and spread into ~2–3 cm diameter circles. The Accell gene gun was used to bombard the 17CM98 cells with hgp100 cDNA-coated gold particles as previously described [12, 13]. Flow cytometric analysis was used to document transgene expression. Briefly, hgp100 and mock-transfected cells (gold bead only) were transferred to flasks containing growth media and incubated for 24 h at 37°C, 5% CO₂. Following incubation, adherent cells were harvested, permeabilized, and stained with mouse anti-gp100 antibody (HMB45, Dako labs) and secondary GAM-FITC antibody. Cells were then evaluated for fluorescence using flow cytometry.

In vivo studies

Animals

Thirty-four privately owned pet dogs referred to the University of Wisconsin Veterinary Medical Teaching Hospital (UW-VMTH) between 2000 and 2003 for treatment of spontaneously arising, histologically confirmed malignant melanoma were included in this study. Dogs were treated as patients of the UW-VMTH and in accordance with the "NIH Guidelines for Care and Use of Laboratory Animals". Protocol approval was obtained from the Institutional Animal Care and Use Committee and the Campus Biosafety Committee. Twenty-four dogs were treated at UW-VMTH and 10 were subsequently treated at referring practices. All dogs had advanced (WHO stage II, tumors 2–4 cm diameter, negative nodes; III, tumor > 4 cm and/or positive nodes; or IV, distant metastatic disease) gross macroscopic primary or metastatic (lymph node and/or pulmonary) disease (25 oral; 8 subungual; one cutaneous) present at the time of vaccine initiation. All dogs were free of complicating concurrent disease conditions, had not received any corticosteroids, or non-steroidal anti-inflammatory medications for 3 weeks prior to entry and had a modified ECOG performance score of 0 or 1 (0 = normal, fully active, able to perform at pre-disease level; 1 = restricted in activity from pre-disease level, but able to function as an acceptable pet; 2 = compromised, severely restricted in activity level, ambulatory only to the point of eating, sleeping, and consistently defecating and urinating in acceptable areas; 3 = disabled, must be force fed, unable to confine urination and defecation to acceptable areas; 4 = dead). None of the dogs had received prior chemotherapy. Signed study and necropsy consent forms were obtained from each dog's owner prior to initiation of treatment.

Complete clinical staging included a physical examination, complete blood count (CBC) with platelet count, serum biochemistry profile, urinalysis, thoracic radiographs, abdominal ultrasound if indicated, photographs and three-dimensional measurements of the tumor. Heparinized venous whole blood (30 ml) and 5 cc of

serum were collected for performance of the immune assays described subsequently.

In all dogs, biopsy of the primary tumor and/or metastatic lesion was performed under general anesthesia followed by cytological assessment to insure that the tissue contained melanoma cells. The remainder of the harvested tumor tissue was aseptically digested mechanically and enzymatically (hyaluronidase 200 U/ml, DNase I 100 U/ml, collagenase 200 U/ml, Sigma, St. Louis, MO), creating a single cell suspension. Red blood cells were lysed with ACK lysing buffer (Bio-Whittaker, Walkersville, MD). The cells were added to flasks along with the appropriate growth media, allowed to grow to confluence, and passaged 3–4 times in order to obtain a pure population of melanoma cells. Melanoma cells were harvested, viability and total cell number assessed, washed and frozen in cell freezing medium (Sigma, St. Louis, MO) in 5×10^5 cell aliquots to be used for targets for *ex vivo* analysis as described below.

Vaccine preparation

On the day of vaccination, 2×10^7 cryopreserved 17CM98 cells were thawed, suspended in 10 ml of MEM with 10% FBS (MEM10%), and irradiated to a total dose of 100 Gy (10,000 rad) using a ^{137}Cs irradiator. Following irradiation, the cells were centrifuged at 1,400 rpm for 7 min and resuspended in 10 ml of HBSS. The cells were transfected using the method described above. The transfected cells were washed twice in saline and resuspended in 0.2 ml of 0.9% sodium chloride. The cells were drawn up into an insulin syringe and stored at 4°C until time of injection (within 2 h).

Vaccination protocol

Dogs were to receive eight vaccinations according to the protocol outlined in Fig. 1. Each vaccination consisted of 2×10^7 cells (hgp100-ATCV) administered intradermally into a site on the left hemithorax (0.2 ml/site) as follows: once weekly for 4 weeks then every other week for four additional treatments. Three dogs received additional boost vaccinations following this protocol (see Table 2). For dogs with documented progressive disease (i.e., 25% or greater increase in tumor volume or new lesions) at 2 months following initiation of vaccine treatment (allowing sufficient time for the development of an immune response), ATCV treatment was discontinued and alternate available treatment options were discussed with the dog's owner.

Response evaluations

Performance status, complete physical examination and tumor measurements were determined at each treatment. Thoracic radiographs and ultrasound (if indicated) was performed every two months. Heparinized venous blood

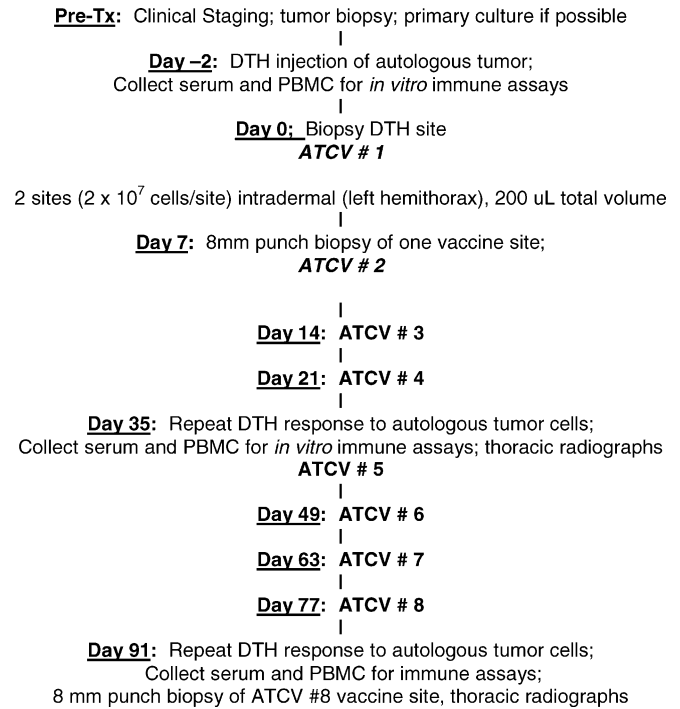


Fig. 1 Vaccination protocol used in 34 dogs with advanced stage, spontaneously arising malignant melanoma

(30 ml) and serum (5 ml) was collected at treatments 4 and 8 for performance of immune assays described subsequently. Complete postmortem examinations were obtained on nine dogs at the time of death or humane euthanasia.

Response criteria

Tumor volume was determined from three-dimensional measurements and calculated using the formula: $V = \pi/6 \times lwh$, where l , w , and h represent tumor diameters in three mutually orthogonal planes. Response was recorded as follows: Complete Response (CR)—complete regression of tumor; Partial Response (PR)—at least a 50% decrease tumor volume; Stable Disease (SD)—less than 50% decrease or not more than 25% increase in tumor volume for a minimum of 6 weeks; Progressive Disease (PD)—at least a 25% increase in tumor volume or the appearance of a new lesion. Only CR and PR were used to determine objective response rate. CR, PR and SD were included to determine tumor control rate. At the time of submission, all cases achieving PR were also confirmed using the RECIST standard (PR being at least a 30% reduction in the longest measured diameter). The time to progression (TTP) was measured from the time measurement criteria are met for CR/PR/SD (whichever was first recorded) until the first date that recurrent or progressive disease was objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started).

Table 2 Characteristics of pet dogs with melanoma treated with hgp100-ATCV

ID	Site of primary tumor	Primary tumor present? ^a	Metastasis present ^a	Clinical stage	TTP (days)	OS (days)	Status	Best response	Vaccines received	Enhanced PBMC cytotoxic activity	Anti-hgp100 antibody detected	DTH ^c response
1	Oral	Yes	None	2	23	357	DD	PD	8/8	Yes	Yes	1/1
2	Oral	No	Lung	4	94	154	DD	SD	8/8	–	No	0/1
3	Oral	Yes	None	2	239	337	DD	SD	8/8	No	Yes	1/2
4	Oral	No	Lung	4	183	417	DD	PR	8/8	Yes	Yes	1/2
5	Oral	Yes	Lung	4	0	126	DD	PD	8/8	–	–	–
6	Oral	Yes	Lung	4	0	95	DD	PD	8/8	No	No	0/0
7	Subungual	No	Lung + LN	4	0	39	LTFU	PD	5/8	–	–	–
8	Subungual	No	Lung	4	0	228	DD	PD	5/8	–	–	–
9	Subungual	Yes	Lung	4	–	89	DO	PR	8/8	Yes	Yes	1/1
10	Oral	Yes	None	2	0	75	DD	PD	8/8	Yes	Yes	–
11	Oral	Yes	LN	3	0	26	DD	PD	3/8	–	–	–
12 ⁺	Oral	Yes	None	2	–	843	Alive	CR	8/8	Yes	Yes	2/3
13	Oral	No	LN	3	63	76	DD	SD	6/8	–	–	1/1
14	Oral	No	Lung + LN	4	93	187	DD	PR	8/8	–	–	–
15	Oral	No	Lung + LN	4	0	58	DD	PD	6/8	–	–	–
16	Cutaneous	Yes	None	2	405	434	DD	SD	8/8	–	No	1/2
17	Oral	No	Lung + LN	4	0	60	DD	PD	6/8	–	–	–
18	Oral	Yes	LN	3	22	63	DD	PD	5/8	–	–	–
19	Oral	No	Lung + LN	4	0	125	LTFU	PD	4/8	–	–	–
20	Subungual	Yes	None	2	0	63	DD	PD	4/8	–	–	–
21	Oral	Recurrence	None	2	0	119	LTFU	PD	8/8	No	No	0/0
22	Oral	Yes	LN	3	21	33	DD	PD	4/8	–	–	–
23	Subungual	Yes	Lung	4	0	164	LTFU	PD	8/8	No	No	–
24	Oral	Yes	LN + skin	4	0	20	LTFU	PD	2/8	–	–	–
25	Oral	Yes	None	2	0	91	LTFU	PD	8/8	–	–	–
26	Oral	Yes	None	2	14	153	DD	PD	8/8	Yes	Yes	–
27	Oral	Yes	Lung	4	0	27	DD	PD	4/8	–	–	–
28	Subungual	Yes	None	2	168	855	Alive	SD	8/8	–	No	2/3
29	Oral	Yes	None	2	0	128	DD	PD	8/8	–	No	–
30 ^c	Subungual	No	LN	3	170	229	DD	SD	8/8	Yes	Yes	1/2
31	Oral	Yes	LN	3	0	27	DD	PD	4/8	–	–	–
32	Oral	Yes	No	2	0	24	DD	PD	3/8	–	–	–
33	Subungual	Yes	Lung + LN	4	584	652	DD	PR	8/8	Yes	Yes	–
34 ^c	Oral	Yes	LN	3	124	127	DD	PR	8/8	–	–	–

PD progressive disease, DD dead due to disease, DO dead due to other cause, SD stable disease, PR partial response, CR complete response, LTFU lost to follow-up after disease progression

^aPresent at initiation of treatment

^bThree dogs received additional boost vaccinations (#12—3 additional vaccinations, q 6 mo; #30—5 additional vaccination,

q 2 weeks; #34—1 additional vaccination 2 weeks after vaccination #8)

^cDTH score pre-vaccination over score post-vaccination

Assessment of *in vivo* immune response

In vivo immune responses were evaluated in a subset of 12 dogs, by performing DTH tests. The DTH assessments were performed immediately prior to vaccine initiation, and after the eighth vaccination (Fig. 1). Briefly, an early passage of 5×10^5 cryopreserved 17CM98 melanoma cells were thawed, washed several times in saline, and suspended in 200 μ l of sterile 0.9% NaCl. The cell suspension was injected intradermally in the skin over the ventral thorax. Forty-eight hours later, 8-mm Keyes-type skin punch biopsies were obtained from the injection site, and hematoxylin and eosin-stained sections were evaluated histologically by a board-certified pathologist (R.R.D.) using coded samples. The character and severity of cellular infiltrates was assessed by means of a semi-quantitative scoring system incorporating extent of polymorphonuclear and lymphocytic infiltrate, presence

of edema, and disruption of collagen bundles [12]. Inflammation was scored as follows: Grade 0 = no inflammatory response, Grade 1 = mild response, Grade 2 = moderate response; Grade 3 = marked inflammation. A positive response was defined as an increase in the degree of inflammation (i.e., an increase in Grade as defined above) in the post-treatment DTH biopsy tissue compared to pre-treatment.

Ex vivo investigations

Cytotoxicity assay

Peripheral blood mononuclear cells (PBMC) from a subset of 12 dogs in this study were collected prior to the first vaccine treatment, at treatment week 5 of the vaccination protocol, and again at completion of vaccina-

tions to assess ATCV-induced, specific anti-melanoma immune activity. PBMC were cryopreserved and the immune response to allogeneic (17CM98) or autologous tumor cells (when available) were evaluated using a cytotoxicity assay comparing PBMC from before vaccination and after the last vaccination (i.e., day 91). In only four cases were primary cultures successful; therefore, all assays but one were performed with allogeneic targets. PBMC were co-cultured in the presence of IL-2 (100 U/ml) with irradiated 17CM98 tumor cells in 6-well plates for 5 days in order to expand reactive lymphocytes. PBMC co-cultured with irradiated fibrosarcoma cells (FIB-I) were used as a negative control. Previously, we have demonstrated that FIB-I does not express gp100 (data not shown). PBMC were removed and an 18-h CTL assay using allogeneic or autologous target cells was performed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), which quantitatively measures lactate dehydrogenase release upon target cell lysis. A 20% or greater increase in cytotoxicity over pre-treatment baseline was used to define enhanced cytotoxicity.

Anti-hgp100 antibody assay

Circulating anti-hgp100 antibodies were measured in a subset of 16 dogs using a cell-based immune assay technique developed by Ziad Jabbar in Dr. Mark Albertini's laboratory, University of Wisconsin Comprehensive Cancer Center. Briefly, serum from hgp100 ATCV treated dogs and β -gal treated normal laboratory dogs was diluted, placed into glass tubes, and incubated for 30 min with Chinese hamster ovary cells (CHO) that had been transfected to express hgp100. CHO cells were washed, centrifuged, and resuspended in 2% paraformaldehyde for 30 min. Following fixation, cells were washed, and permeabilized with 0.1% saponin for 15 min. After making the appropriate dilutions, 50 μ l of CHO cells were incubated with 50 μ l of serum. As a positive control, anti-hgp100 antibody (HMB45, Dako Labs) was substituted instead of serum. CHO cells were pelleted, washed, and incubated with secondary antibodies: GAM-FITC (B-D labs) for controls or an intermediate mouse anti-canine IgG antibody (QED Bioscience Inc) followed by secondary GAM-FITC (for patient samples), and subjected to flow cytometric analysis. Previously, this technique has been shown to detect circulating anti-hgp100 antibodies in human patients immunized with gp100 (Albertini et al. unpublished data).

Statistical analysis

Median time to progression (TTP) and overall survival (OS) times for dogs in this study were determined from survival curves generated using the Kaplan–Meier method which accounts for dogs that are alive, lost to follow-up (LTFU), or have died from unrelated causes

at the time of analysis (these cases are treated statistically as censored data, however they are included in the analysis using this method). The Log-rank test was used to determine differences in survival between responders and non-responders. To determine whether clinical stage, in vivo DTH, in vitro cytotoxicity assays, and production of anti-hgp100 antibodies correlated with tumor control, Fisher's exact test (2-tailed, 90% C.I.) were performed. For all statistical analyses, $P < 0.05$ was considered significant.

Results

In vitro studies

Native expression of melanoma-associated antigens in canine melanoma cells

Using RT-PCR, canine gp100 mRNA was detected in varying levels in all four canine melanoma cell lines tested, and was only weakly expressed in cells of non-melanoma origin (Fig. 2). Additionally, PCR analysis demonstrated that 17CM98 canine melanoma cells contain multiple melanoma-associated antigens, making this cell line an excellent candidate to be used as an ATCV (Fig. 3).

Transfection of 17CM98 cells with hgp100: Flow cytometric analysis determined that transfected 17CM98 cells expressed hgp100 when compared to mock-transfected controls (Fig. 4). A transfection rate of 12% was observed.

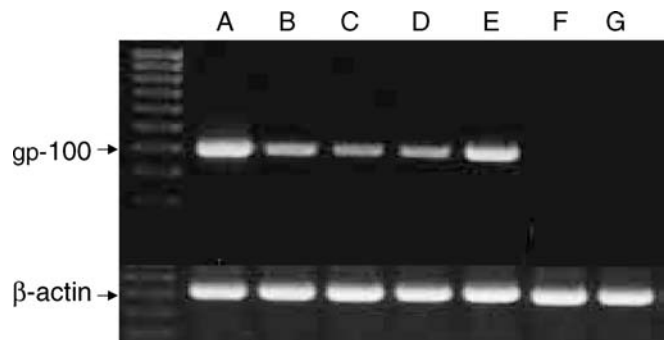


Fig. 2 Detection of canine gp100 mRNA expression in canine melanoma cells. Total RNA samples purified from a human melanoma M21 cell line (positive control; lane A), four canine melanoma cell lines (lanes B, C, D, E), and two osteosarcoma cell lines (human, lane F; canine, lane G) were subjected to reverse transcription-PCR analysis. The expected PCR products for gp100 mRNA (399 bp) were size fractionated onto a 1% agarose gel and stained with ethidium bromide. Sequence analysis of the PCR product confirmed gp100 identification. gp100 transcripts were strongly expressed in melanocytic cell lines (lanes A–E) and weakly expressed in cells of non-melanocytic origin (lanes F and G). PCR amplification of β -actin was used as an internal control

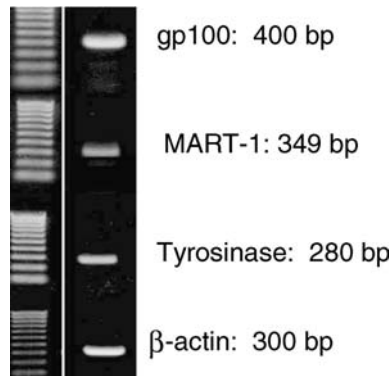


Fig. 3 Detection of canine gp100, MART-1, and tyrosinase mRNA expression in the canine melanoma cell line, 17CM98. Total RNA was purified from several different canine melanoma cell lines and subjected to RT-PCR analysis. The expected PCR products for various melanoma antigens were size fractionated onto a 1% agarose gel and stained with ethidium bromide. Pictured is a representative gel of the canine melanoma cell line 17CM98 that strongly expressed three melanoma-associated antigens: gp100, MART-1, and tyrosinase

In vivo studies

All dogs tolerated hgp100-ATCV vaccination well. The mean age of dogs entered into this trial was 12 years (median 11.5, range 6–16) and the mean weight was 26.1 kg (median 26.7, range 6–52). There were 25 dogs diagnosed with oral melanoma, eight dogs with subungual melanoma, and one dog with cutaneous melanoma. Thirteen dogs were stage II, 7 Stage III and 14 were stage IV. Of these 34 dogs, there were eight Labrador retrievers, four Cocker spaniels, three Golden retrievers, three Chow, three mix breed, three each of Airedale and Newfoundland, and one each of Standard poodle, Miniature poodle, Doberman, Schnauzer, Australian shepherd, Cairn terrier, Rottweiler, Sharpei, and Beagle.

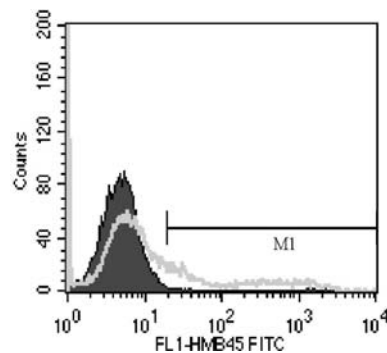


Fig. 4 Flow cytometric analysis in canine melanoma cells (17CM98) transfected with hgp100. 17CM98 cells were transfected with hgp100 using the Accell gene gun and transferred to flasks containing growth media. Adherent cells were harvested the next day, permeabilized, stained with anti-gp100 HMB45 antibody, and secondary GAM-FITC antibody. A transfection efficiency of 12% hgp100-positive cells was observed in 17CM98 cells transfected with hgp100 (line) when compared with mock-transfected cells (filled peak)

Table 2 shows the survival characteristics, clinical stage, and follow-up information on the 34 dogs in this trial. Adverse reactions were limited to mild induration and erythema at the site of vaccination. Systemic toxicities, vitiligo, or autoimmune reactivity against normal melanocytes were not observed with the exception that one dog (#12) developed depigmentation of his oral tumor and some degree of depigmentation of the oral mucosa. This same dog had a complete response to ATCV and remains free of disease at the time of this report (853 days post-initiation of vaccine treatment). Of the 34 dogs that were entered into this trial, 25 died due to progressive disease, two are alive as of the manuscript preparation date (one had a complete response and remains free of disease, and one had a partial response defined as greater than a 50% reduction in tumor volume), six were lost to follow-up (after progressive disease was noted), and one dog died of an unrelated cause (histologic evidence of regional lymph node metastasis was found at the time of death). Objective evidence of tumor regression (one complete response and five partial responses) was observed in six of the 34 dogs (17.6% objective response rate), as determined by three dimensional tumor measurements. Additionally, six dogs experienced SD for a minimum of six weeks for a tumor control rate (CR/PR/SD) of 35.3%. Tumor stage did not correlate with the likelihood of tumor control (Fisher's; II vs. IV, $P=0.71$; II vs. III/IV, $P=1.00$; II/III vs. IV, $P=0.73$; III vs. IV, $P=1.00$). Table 3 presents time to progression and overall survival times. Figure 5a, b show the Kaplan–Meier-generated curves for time to progressive disease (median = 170 days) for dogs that had tumor control (CR/PR/SD), and overall survival time for dogs having tumor control (CR/PR/SD) or no response. Dogs experiencing tumor control survived significantly longer than dogs having no response (337 days vs. 95 days; $P=0.0014$).

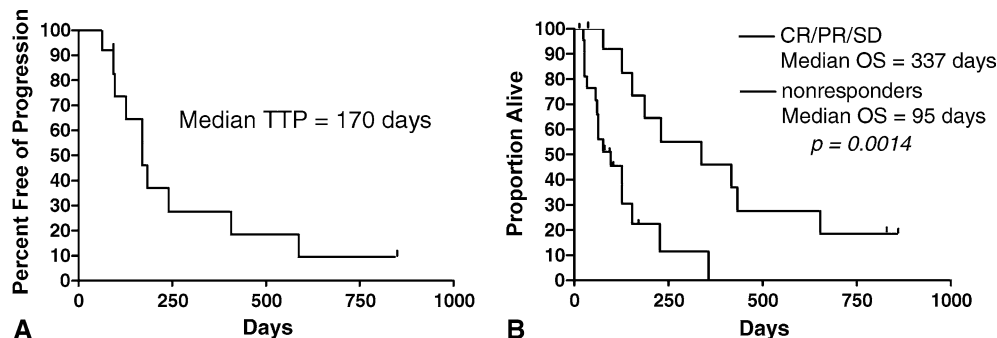
DTH responses occurred more commonly in dogs that had evidence of clinical response

Histopathologic evaluation of vaccination sites demonstrated prominent poly- and mononuclear infiltrates, including neutrophils, lymphocytes and macrophages. Positive DTH responses (as defined by an increase in the designated Grade of inflammation post-treatment compared to pre-treatment) were observed post-treatment in

Table 3 Time to progression (TTP) and overall survival times for dogs in study

Group	TTP (days)	OS (days)	<i>P</i> value when OS is compared to non-responders
CR/PR/SD	170	337	$P=0.001$
CR/PR	183	417	$P=0.010$
Non-responders	–	95	–
All dogs	–	153	$P=0.078$

Fig. 5 Kaplan–Meier curves for (a) time to progression (TTP) for dogs achieving tumor control (CR/PR/SD) and (b) overall survival time for dogs experiencing tumor control (CR/PR/SD; $n=12$) or no response ($n=22$) after receiving hgp100-ACTV



2/3 dogs evaluated that had exhibited tumor regression, in 5/6 dogs evaluated that had stable disease for a minimum of 6 weeks, and in 0/3 dogs that had progressive disease while undergoing vaccine treatment. A positive DTH correlated with the likelihood of tumor control (Fisher's; $P=0.046$). Three dogs received additional treatment after progression of disease. These were all non-responders to the vaccine and additional treatment did not markedly affect OS, likely due to advancement of disease.

Ex vivo studies

Peripheral blood mononuclear cells (PBMC) have enhanced cytotoxic activity against autologous tumor cells following hgp100-ATCV treatment in dogs with melanoma In vitro cytotoxic activity of PBMC against autologous or allogeneic tumor targets was evaluated prior to initiation of treatment and post-vaccine therapy (i.e., day 91) in 12 dogs. Eight of the 12 dogs showed enhanced cytotoxic activity post-treatment compared to pre-treatment (Table 1). Figure 6 shows the results of a representative assay in a treated dog. Enhanced cytotoxicity after vaccination did not correlated with the likelihood of tumor control (Fisher's; $P=0.546$).

Anti-hgp100 antibodies were detected in the majority of dogs following ATCV Vaccine treatment resulted in detectable anti-hgp100 antibodies in serum of 9 of 16 dogs receiving gp100-ATCV assayed (see Table 1). The presence of anti-hgp100 antibodies after vaccination did not correlated with the likelihood of tumor control (Fisher's; $P=0.615$).

Discussion

Several vaccination strategies have been or are being developed with the goal of breaking tolerance or self-ignorance, resulting in controlled autoimmunity against tumor-associated antigens. In this study, we describe the development of a xenogeneic melanoma-antigen-enhanced (hgp100) allogeneic tumor cell vaccine and its implementation in a treatment protocol designed to

evaluate efficacy in the context of a spontaneously arising melanoma model in pet dogs. We chose an allogeneic whole-cell approach due to the ease of producing cells in quantity, previous documentation of their use as effective stimulators of anti-melanoma immunity in humans and dogs [12, 13, 14, 33], the demonstration that 17CM98 cells expressed three melanoma-associated antigens known to be important in mediating anti-tumor immunity in humans (gp100, MART-1, tyrosinase), and the likelihood that additional unknown tumor associated antigens would be presented. It was hypothesized that presentation of these antigens along with xenogeneic hgp100 would result in cross-reaction between the xenogeneic homologs and self-molecules and possibly facilitate epitope spreading, a phenomenon shown to occur with other gp100 vaccine constructs [18, 37]. The 12% expression efficiency we achieved is similar to that previously reported for canine melanoma cell lines using the gene gun particle-bombardment approach [12, 13].

The 17.6% objective response and 35.3% tumor control rates compare favorably with other vaccine approaches in dogs and humans with advanced stage melanoma [4, 13, 33, 38]. In addition, the overall sur-

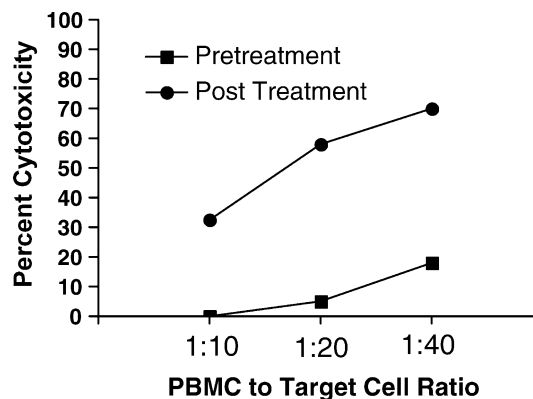


Fig. 6 PBMC cytotoxic activity against allogeneic tumor cells pre- and post-ATCV treatment. PBMC collected pre-treatment and at the time of the eighth vaccine treatment were co-cultured for 18 h with autologous melanoma cells from the dog's primary tumor. Following incubation, cytolytic activity was measured using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). PBMC cytotoxic activity pre and post hgp100-ATCV treatment. At PBMC to tumor cell ratios of 10:1, 20:1 and 40:1, there was a marked increase in percent cytotoxicity by PBMC post-treatment, compared to pre-treatment

vival of dogs in this study, despite having macroscopic disease present, compares well with historical median survivals reported for dogs with advanced-stage melanoma that were rendered microscopic with surgery [10, 19, 20]. Interestingly, clinical stage did not correlate with tumor control in this study; however, the lack of correlation observed may be due to the inclusion of dogs with a primary melanomas at varying sites (i.e., digital, oral, and cutaneous).

A major hurdle in designing, implementing, and assessing clinical trials involving anti-cancer vaccines has been integrating methods of measuring the effects of a particular strategy on the immune system of patients who have or have not experienced a clinical response. The end-points of clinical efficacy (e.g., survival, time to progression/recurrence, and CR/PR rate) obviously describe a biological response and the fact that dogs achieving a response in this study had significantly longer overall survival times than non-responders provides further proof of biologic efficacy. However, responders represent the minority in currently available vaccine approaches and having reliable surrogate immune measures would provide valuable information regarding the ability of a vaccine approach to modulate a particular arm of the immune system and help us to understand why one patient responds to one approach while another, seemingly similar patient, does not. In the present study, we chose to monitor DTH response, PBMC cytotoxicity and the presence of anti-hgp100 antibodies before and at the end of the vaccination protocol. Only DTH response correlated with the likelihood of a clinical response, something that has been previously reported in melanoma vaccine trials [15, 16]. A DTH response may serve as a measure of lymphocyte trafficking to tumor antigens; however, it is a non-specific measure that does not tease out precise information on immune effectors and mechanisms. While we documented enhanced PBMC cytotoxicity and anti-hgp100 antibodies in dogs following vaccination they did not correlate with clinical efficacy. In the latter case, we are indeed asking the wrong question, as the production of antibodies against xenogeneic antigens should be relatively easy. The real question of interest would be to document the presence of antibodies generated against the canine homolog of gp100, which we did not perform. In current human melanoma vaccine trials, more recently validated functional immune assays, including enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining with flow cytometry (CFC), are being assessed. Our laboratory has recently validated a commercially available ELISPOT assay for canine IFN- γ that will be utilized in future trials.

Currently, based on the information gained from the trial reported here and previous work reporting efficacy following vaccination with a GM-CSF transfected canine melanoma vaccine [13], we are studying a dual-transfected 17CM98 cell line that expresses both hgp100 and canine GM-CSF. We feel this combination ap-

proach along with further elucidation of the mechanisms of tumor protection after xenogeneic immunization may allow the creation of more efficacious vaccines.

This trial also demonstrates the utility of spontaneous tumors in companion animals as a valid translational model for the evaluation of novel vaccine therapies. The dog offers an excellent research model due to its large size, spontaneous development of tumors, intact immune system, rapid assessment of therapeutic activity, similar response to environmental influences, and biological similarity to humans. Tumor burdens in dogs with oral cancers are more similar to humans than the experimentally-induced tumor volumes found in many murine models and canine patients are generally less heavily pretreated than human patients entering clinical trials [36].

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