

Evaluation of a Novel Tumor Vaccine in Dogs with Hemangiosarcoma

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Background: Hemangiosarcoma (HSA) is a highly metastatic and often rapidly fatal tumor of dogs. At present, adjuvant chemotherapy is the only proven effective treatment for dogs with HSA, though the benefits from chemotherapy are modest. Administration of immunotherapy together with chemotherapy has also been reported to improve survival in dogs with HSA. Therefore, we evaluated safety and immunologic responses to a novel tumor vaccine administered together with doxorubicin chemotherapy in dogs with different stages of HSA.

Hypothesis: That tumor vaccination could be safely and effectively combined with doxorubicin chemotherapy for treatment of dogs with HSA.

Animals: Twenty-eight dogs with various stages of HSA were enrolled in the study.

Methods: The HSA vaccine was prepared with lysates of allogeneic canine HSA cell lines mixed with an adjuvant composed of liposome-DNA complexes. Dogs received a series of 8 immunizations administered over a 22-week period, and most also received chemotherapy. Clinical adverse effects were noted, immune responses were measured by enzyme-linked immunosorbent assay (ELISA) and flow cytometry, and survival times were calculated.

Results: The most common adverse effects observed in vaccinated dogs also treated with doxorubicin chemotherapy were diarrhea and anorexia. Vaccinated dogs were found to mount strong humoral immune responses against a control antigen and, most dogs also mounted antibody responses against canine HSA cells. Thirteen dogs with stage II splenic HSA that received the tumor vaccine plus doxorubicin chemotherapy had an overall median survival time of 182 days.

Conclusions: We conclude that an allogeneic tumor lysate vaccine is safe in dogs with HSA and can elicit humoral immune responses in dogs that are receiving concurrent doxorubicin chemotherapy.

Key words: Adjuvant; Allergenic; Cancer; Immunize; Liposome; Neoplasia; Vaccination.

Hemangiosarcoma is an aggressive and highly metastatic tumor of dogs. The tumor is thought to arise from transformed endothelial cells and typically occurs in the spleen, right heart, or skin.^{1–5} The most common cause of death in dogs with hemangiosarcoma (HSA) is tumor rupture and hemorrhage, which is often complicated by coagulation abnormalities.^{6–8}

Survival times for dogs with the most common form of HSA (splenic HSA) are typically less than 2–3 months after splenectomy alone.^{4,9–13} Several studies report a modest survival benefit from the addition of adjuvant doxorubicin (DOX)-based chemotherapy of HSA after surgical splenectomy.^{10,12–14} A trial of combination therapy with minocycline, a putative angiogenesis inhibitor, and DOX chemotherapy failed to show a survival benefit over treatment with DOX alone.¹⁰ However, administration of the immune stimulant liposomal muramyl tripeptide phosphatidylethanolamine in combination with DOX-based chemotherapy did show a significant survival advantage compared with dogs treated with chemotherapy alone,¹⁵ suggesting that immunotherapy combined with chemotherapy may have utility in the treatment of HSA in dogs.

Targeted immunotherapy is promising as new treatment option for cancer in humans and companion animals. Unfortunately, there are currently few effective immunotherapeutics available for use in dogs with cancer. We have investigated a novel immune stimulant, composed of cationic liposome and DNA complexes (LDC), for use in cancer immunotherapy. We previously reported that LDC were very potent activators of innate immunity and antitumor activity in mice.^{16–19} More recently we also reported that intravenous administration of LDC with the IL-2 gene elicited immune activation and significantly prolonged survival times in dogs with metastatic osteosarcoma.²⁰ Moreover, we also recently reported that infusion of LDC could inhibit tumor angiogenesis and cause tumor regression in some dogs with soft tissue sarcoma.²¹

We hypothesized therefore that LDC could also be used as an effective cancer vaccine adjuvant in dogs. This hypothesis was based in part on a recent report in which we found that LDC had potent adjuvant properties in mice.²² In addition, we also reported that LDC could be used as a vaccine adjuvant for immunization of dogs with refractory atopic dermatitis and that immunization with the LDC-based vaccine could reduce clinical signs and suppress the Th2 polarization of the T cells from atopic dogs.²³ In addition, we showed recently that in cancer-bearing dogs, the LDC adjuvant could be used recently to elicit humoral immune responses even in dogs receiving chemotherapy.²⁴ Others have also reported that liposomes complexed with DNA can be used to produce effective vaccine adjuvants.^{25–27} However, LDC-based tumor vaccines have not been previously evaluated in dogs. Therefore, we conducted an open, phase I study of an allogeneic tumor vaccine based on the LDC adjuvant in 28 dogs with HSA. The purposes of the study were to assess safety and immunologic responses in dogs re-

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ceiving concurrent doxorubicin chemotherapy and also survival times in a subset of dogs with Stage II HSA.

The tumor vaccine was found to be safe in dogs with HSA, including many dogs treated concurrently with chemotherapy, with the major adverse effects being transient diarrhea and vomiting. The vaccine was found to elicit humoral immune responses against control antigens and HSA tumor cells in most dogs that were vaccinated while receiving concurrent DOX chemotherapy. The humoral immune responses that were elicited appeared to be largely tumor specific inasmuch as humoral responses were not observed against irrelevant tumor cell lines. We conclude therefore that additional studies of tumor vaccination as adjuvant therapy for dogs with HSA are warranted.

Materials and Methods

Vaccine Preparation

The HSA vaccine was prepared with lysates from 2 canine HSA cell lines (DEN-HSA and Fitz-HSA). The endothelial derivation of one of these cell lines (DEN-HSA) was described recently.²⁸ Tumor lysates were prepared from equal numbers of pooled HSA cells, which were washed extensively in phosphate-buffered saline (PBS) to remove any residual fetal bovine serum (FBS), then resuspended in distilled water and subjected to 4 freeze-thaw cycles, followed by sonication.^a The cell lysate solution was then centrifuged at $1,500 \times g$ and filtered through a 0.22- μ m filter, adjusted to a final protein concentration of 5 mg/mL, and stored at -80°C until used.

The LDC vaccine adjuvant was prepared as described previously.¹⁶ In brief, equimolar amounts of the cationic lipid 1,2-diacyl-3-trimethylammonium propane (DOTAP) and cholesterol^b were dissolved in chloroform, then dried down to a thin film in round-bottomed tubes in a vacuum desiccator.^c Liposomes were prepared by rehydration in a 10% sucrose solution, followed by filtration, as described previously.²⁹ Liposome-DNA complexes were prepared by adding plasmid DNA (noncoding plasmid DNA with <0.05 EU/mL endotoxin content; Althea Technologies^d) to liposomes in 10% sucrose to form LDC, as described previously.¹⁶ The vaccine was then prepared by adding tumor lysate to the liposome-DNA complexes at a concentration of 5 mg tumor lysate antigen per 4 mL LDC, followed by gentle mixing. In addition, 50 μ g keyhole limpet hemocyanin (KLH)^e was added to each vial of vaccine as a control antigen. The vaccine was aliquoted to vials (4 mL/vial) and frozen to -80°C , after which the vials were lyophilized. After lyophilization, the vials were capped and stored at -20°C until used.

Patients

Twenty-eight dogs with HSA (2 with stage I, 18 with stage II, and 8 with stage III disease; Table 1) were recruited by participating oncologists in the United States (see list of participating veterinarians in Acknowledgements section) to participate in this open-label phase 1 study. The protocol for these studies was approved by the Animal Care and Use Committee and the Clinical Review Board at Colorado State University. A histologic diagnosis of HSA was required for entry into the study. Patients were excluded from participation if they had serious concurrent medical diseases, including renal failure or hepatic disease, or had been treated in the preceding 2 weeks with systemic corticosteroids. Staging and administration of chemotherapy was performed according to standard of practice at each participating institution. Dogs were staged as follows: stage I, tumor <5 cm diameter and confined to the primary tumor site; stage II, tumor

Table 1. Characteristics and staging of patients enrolled in the hemangiosarcoma vaccine trial.

Tumor Stage	Number
Stage I	
Spleen	1
Skin	1
Stage II	
Spleen	17
Skin	1
Stage III	
Spleen	3
Skin	2
Heart	1
Kidney	2

>5 cm diameter, with SC invasion, tumor rupture (eg, splenic HSA), regional lymph node spread, or all 3; stage III, tumor with measurable metastases, or multicentric disease. Follow-up assessment for adverse effects and treatment responses was done by telephone survey of participating veterinarians, veterinary technicians, or both at each practice.

Survival times and disease-free intervals were calculated for the subset of 13 study dogs with stage-II splenic HSA that received the tumor vaccine plus DOX chemotherapy. In addition, we compiled historic data (cases evaluated from 1996–2006) from 2 participating institutions (Colorado State University and Veterinary Referral Center of Colorado) for 24 dogs with stage-II splenic HSA that were treated only with DOX chemotherapy. The survival times and disease-free interval data for these 24 historic control dogs were calculated and compared with survival times and disease-free interval times for study dogs.

Vaccine Administration Schedule

Patients enrolled in the study were scheduled to receive a series of 8 tumor vaccines, administered over a 22-week period. The first 5 vaccines were administered once every other week, while the remaining 3 vaccines were administered once monthly. The vaccine was administered intraperitoneally, based on data in mice that indicated superior immune responses after vaccination by that route.²² The vaccine was rehydrated at room temperature to a 10 mL final volume and administered in a single intraperitoneal injection site. Dogs weighing >10 kg received 10 mL of vaccine, while dogs of <10 kg body weight received 5 mL of vaccine. Treated dogs were monitored in the hospital for adverse effects for the first 6–8 hours after administration of the first vaccine, in particular, for signs of gastrointestinal (GI) disturbances (nausea, vomiting, diarrhea), abdominal pain (vocalization, licking injection site; looking at abdomen), and fever (body temperature).

Determination of KLH Antibody Responses

Humoral immune responses to vaccination were assessed by means of serum samples obtained prevaccination and again at week 8 of the study (after the third vaccine) from 6 patients with stage-II splenic HSA. Within this group of 6 patients, 5 were treated concurrently with DOX and vaccine, while 1 dog (Dog 2) was treated with DOX before vaccination. Serum was stored frozen at -20°C before immunologic evaluation. Anti-KLH antibody responses were assessed by an enzyme-linked immunosorbent assay (ELISA), as described previously.²⁴ In brief, KLH-specific antibody titers were determined with ELISA plates coated with KLH at a concentration of 5 μ g/mL. Plates were preblocked with nonfat

dried milk before incubating serum samples. Serum samples were diluted 1:8,000 in PBS and 1% bovine serum albumin (BSA) before addition to the ELISA assay. Plates were washed and incubated with goat-anti-dog immunoglobulin (IgG) conjugated to horseradish peroxidase (HRP), then washed again and incubated with substrate solution. The optical density of the wells in the plate was determined with an automated optical density reader.^f

Flow Cytometric Assessment of Anti-HSA Antibody Responses

The effect of vaccination on development of antibody responses against canine HSA cell lines was assessed by flow cytometry to detect cell surface antibody responses. The 2 canine HSA cell lines (DEN-HSA and Fitz-HSA) used to prepare the vaccine were also used for the serologic studies. For flow cytometric analysis, serum samples were diluted 1:100 in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FBS and 0.1% sodium azide^e) (dilution based on prior assay optimization experiments; data not shown) and then incubated with viable HSA cells. The HSA cells were detached by brief trypsinization, and then added, at a concentration of 1×10^5 cells per well in a 96-well round-bottomed plate, to wells containing prediluted test serum, and incubated for 30 minutes at 4°C. The cells were then washed twice in FACS buffer, incubated with prediluted phycoerythrin (PE)-conjugated goat anti-dog IgG antibody^g for 20 minutes at 4°C, then washed, and then fixed in 1% paraformaldehyde^e before analysis. Controls included HSA cells incubated with secondary antibody only and cells incubated with serum from control dogs without cancer.

Additional controls for tumor specificity of humoral responses included assessment of antibody binding to 3 different irrelevant canine tumor cell lines. These cell lines included 1 canine melanoma cell line (developed in the laboratory) and 2 canine osteosarcoma cell lines (D-17 and Abrams, kindly provided by Dr G. MacEwen, University of Wisconsin-Madison). These cell lines were incubated with patient prevaccination and postvaccination serum diluted to the same concentrations as used for analysis of HSA cell lines.

Cell surface binding of dog antibodies was assessed with a Cyan flow cytometer^h and data analysis was done by Summit software.^h Gates for determining the degree of antibody binding were set on live cells based on forward and side-scatter characteristics. Analysis gates for each sample were adjusted to allow approximately 1–2% positive events, based on the degree of autofluorescence observed in each of the unstained cell lines. Positive responses to vaccination were defined as at least a doubling of the percentage of antibody-positive cells when prevaccination and postvaccination serum samples were compared between the same dogs by the same cell line.

Statistical Analyses

Disease-free intervals and overall survival times were calculated for 13 dogs with stage-II splenic HSA that received treatment with tumor vaccine plus DOX chemotherapy. Four of the 17 stage-II HSA dogs in the study population (Table 1) were excluded from this analysis because, although they were vaccinated with the tumor vaccine, they were not treated with DOX chemotherapy. In addition, survival times and disease-free intervals were also calculated for 24 historic control dogs with stage-II splenic HSA that were treated with DOX only. Comparison of survival and disease-free interval times between study dogs and historic control dogs was done by Kaplan-Meier curves generated by GraphPad software.ⁱ Comparison of prevaccination and postvaccination antibody titers was done by the Wilcoxon signed rank test. A *P* value of <.05 was considered significant for all statistical analyses performed in this study.

Table 2. Adverse effects associated with immunization with an hemangiosarcoma (HSA) tumor lysate vaccine combined with doxorubicin chemotherapy in dogs with HSA.

Adverse Effect	No. Affected	% Affected
Diarrhea	5/28	18
Anorexia	3/28	11
Vomiting	2/28	7
Fever	2/28	7
Abdominal pain	1/28	4
Vaccine site reaction	1/28	4
Weakness, tremors	1/28	4

Results

Patient Population Characteristics

Twenty-eight dogs were enrolled in the study and complete follow-up was obtained for all of the animals (Table 1). Of these 28 dogs, 21 dogs had primary splenic HSA (1 stage I, 17 stage II, 3 stage III), 4 dogs had primary cutaneous HSA (1 stage I, 1 stage II, 2 stage III), 2 dogs had primary renal HSA (stage III), and 1 dog had primary cardiac HSA (stage III). Five dogs (1 with stage I disease, 3 with stage II disease, and 1 with stage III disease) received the vaccine but did not receive chemotherapy. Of the 17 dogs with stage-II splenic HSA that received the HSA vaccine, 3 did not receive any chemotherapy, 1 was treated with low-dose cyclophosphamide therapy, and 13 dogs received DOX chemotherapy. Two of the stage-II dogs treated with DOX chemotherapy received the VAC protocol (DOX plus cyclophosphamide and vincristine). The HSA vaccine was administered concurrently with chemotherapy protocols, generally on alternating weeks.

Toxicity and Adverse Effects

The vaccine was well tolerated by most dogs enrolled in the study. The most common adverse effect observed (see Table 2) was mild to moderate diarrhea that occurred after at least one treatment in 5 of 28 treated dogs (2 dogs, grade I toxicity; 3 dogs, grade II), as determined by the Veterinary Cooperative Oncology Group (VCOG) toxicity scale.³⁰ Other adverse effects included mild to moderate anorexia in 3 treated dogs (1 grade I, 2 grade II toxicity). Other adverse effects noted included mild to moderate vomiting in 2 dogs (1 grade I and 1 grade II toxicity), mild fever in 2 treated dogs (2 grade I toxicity), a mild vaccine site reaction in 1 dog (grade I toxicity), abdominal pain in 1 dog (grade I toxicity), and 1 dog that exhibited weakness and muscle tremors (grade I toxicity). None of the adverse effects necessitated in-hospital treatment or discontinuation of the vaccine. It should be noted that many of these same adverse effects (especially diarrhea) also occur in dogs treated with DOX chemotherapy alone.^{31–33} Therefore, it was not possible from the design of this study to ascertain whether the toxicity observed was due to the effects of vaccination alone or to chemotherapy alone. Nevertheless, the observed incidence of GI adverse

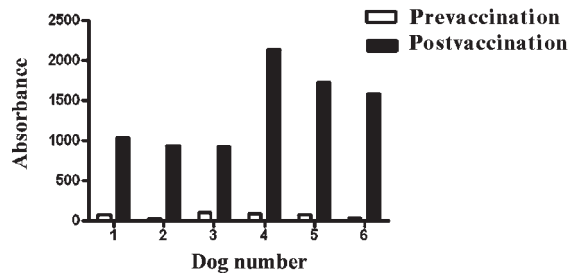


Fig 1. Development of antibody responses to a control antigen in 6 dogs after immunization with tumor vaccine and chemotherapy. Serum was obtained from 6 dogs with stage-II splenic HSA that were vaccinated and treated with DOX chemotherapy. Serum was obtained before immunization and after immunization with the HSA vaccine, which contained 50 μg KLH in addition to HSA lysate antigens. Serum was assessed for anti-KLH antibodies by ELISA, as described in Methods section. When prevaccine and postvaccine anti-KLH titers were compared, vaccinated dogs developed significant ($P < .03$) antibody responses. DOX, doxorubicin; HSA, hemangiosarcoma; KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay.

effects in this study (17%) is consistent with the incidence noted with DOX alone, suggesting that the combination of vaccination with DOX chemotherapy did not increase the overall incidence of adverse effects typically observed with DOX chemotherapy alone.³²

Vaccine-Induced Antibody Responses against a Control Antigen

A control antigen (KLH) was incorporated into the vaccine to allow assessment of humoral immune responses elicited by the vaccine. Serum samples obtained from 6 dogs before vaccination and after the third vaccination were assessed for anti-KLH antibody titers. All 6 vaccinated dogs evaluated mounted strong antibody responses against the KLH antigen incorporated into the vaccine, and there was a significant difference ($P = .03$) when prevaccination and postvaccination antibody titers were compared (Fig 1). These results indicated therefore that the LDC vaccine adjuvant was capable of eliciting strong humoral immune responses against a novel antigen (KLH) in dogs with cancer treated concurrently with DOX chemotherapy.

Generation of Immune Responses against HSA Cells

We next assessed the ability of the HSA lysate vaccine to elicit antibody responses against canine HSA cells. This was done with flow cytometry to assess prevaccination and postvaccination serum samples for the presence of antibodies against cell surface determinants expressed on 2 different canine HSA cell lines. Positive responses were considered to be those where the percentage of positive staining cells was at least 2 times greater in postvaccination samples than in prevaccination serum samples from the same dog. Based on this analysis, we found that 5 of 6 dogs had an increase in antibody responses against the DEN-HSA cell line, while 4 of 6 dogs had an increase in antibody responses against the

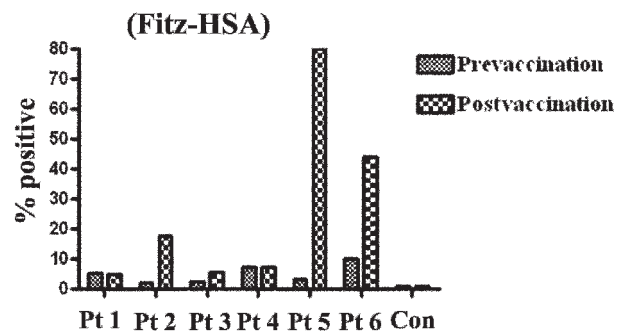
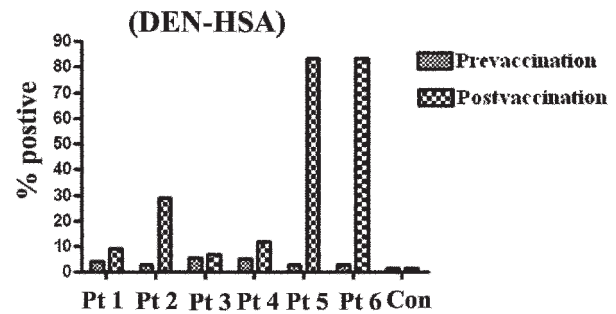


Fig 2. Development of antibody responses against HSA cells after vaccination with tumor vaccine. Serum was obtained before immunization and after vaccination with the HSA tumor lysate vaccine and development of antibody responses against cell surface determinants on 2 different canine HSA cell lines (DEN-HSA and Fitz-HSA) was assessed with flow cytometry, as described in Methods section. There was a significant difference ($P = .05$) in prevaccine and postvaccine titers against DEN-HSA cells, while the difference in prevaccine and postvaccine titers against Fitz-HSA cells did not reach the level of significance ($P = .12$). See Figure 1 for key.

Fitz-HSA cell line (Fig 2). There was a significant difference ($P = .05$) when prevaccination and postvaccination antibody responses against the DEN-HSA cell line were assessed, but the differences in prevaccination and postvaccination responses were not significantly different ($P = .12$) against the Fitz-HSA cell line. Because of the relatively small number of samples evaluated, we were unable to correlate antibody responses with survival times (see below). The fact that dogs mounted stronger and more consistent antibody responses against the foreign antigen KLH than against HSA tumor cells most likely reflects the greater degree of immune tolerance elicited against tumor antigens than against foreign antigens such as KLH.

To control for induction of nonspecific antibodies by the tumor lysate vaccine, we also assessed binding of prevaccination and postvaccination serum samples to 3 non-HSA canine tumor cell lines (1 canine melanoma cell line, 2 canine osteosarcoma cell lines). These cells were prepared and incubated with serum for flow cytometry exactly as described for the HSA cell lines. We found that there was no reactivity against either of the 2 canine osteosarcoma cell lines when prevaccination

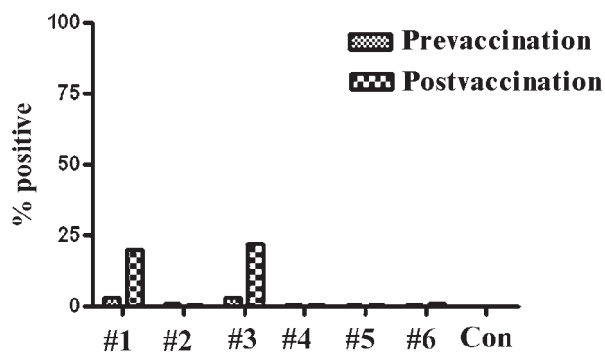


Fig 3. Assessment of antibody responses against irrelevant tumor cell lines. Serum from 6 dogs vaccinated with the HSA vaccine and reactivity against 3 non-HSA cell lines was assessed by flow cytometry as described in Methods section. Immune reactivity against 2 canine osteosarcoma cell lines (Abrams OSA and D17 OSA) and 1 canine melanoma cell line (Talsky MM) was assessed. Antibody binding to the osteosarcoma cell lines was not observed (data not shown), while 2 of 6 vaccinated dogs (patient No. 1 and No. 3) had reactivity against the Talsky melanoma cell line. HSA, hemangiosarcoma.

and postvaccination serum samples were evaluated by flow cytometry (data not shown). Against the canine melanoma cell line, there was increased binding of postvaccination serum from patients No. 1 and No. 3, while there was no increased binding by postvaccination serum from the other 4 patient serum samples (Fig 3). These data suggest therefore that the majority of the increase in antibody binding to HSA cell lines was due to induction of tumor-specific humoral immune responses. However, since there was reactivity of 2 patient serum samples against a canine melanoma cell line, we cannot completely exclude the possibility that some of the reactivity observed against HSA cell lines may have been owing to induction of humoral immune responses against alloantigens. Alternatively, the melanoma cells may have expressed tumor antigens also expressed by the HSA cells.

Survival Times and Disease-Free Interval in Dogs with Stage-II HSA

The median disease-free interval for 13 study dogs with stage-II splenic HSA treated with vaccine and DOX chemotherapy was 146 days, and their median overall survival time was 182 days. The median disease-free interval for the 24 historic control dogs with stage-II splenic HSA treated with DOX chemotherapy only was 126 days and their median overall survival time was 133 days. Comparison of overall survival times revealed a statistically significant increase in survival times in study dogs compared with historic control dogs, whereas disease-free intervals were not significantly different between the 2 groups (data not shown).

Discussion

Several important findings emerged from the studies reported here. First, we found that a novel tumor lysate vaccine with a potent vaccine adjuvant (liposome-DNA

complexes) was well tolerated in dogs with a variety of different stages of HSA. The most common adverse effects were transient diarrhea and anorexia. These adverse effects have also been reported previously in studies where the LDC were administered intravenously to dogs for gene delivery or nonspecific tumor immunotherapy.^{20,21} However, these adverse effects are often noted in dogs treated with DOX chemotherapy alone.³² Therefore, it is not clear from this study which adverse effects were elicited by the vaccine and which by DOX chemotherapy. However, it is apparent that an overall increase in the expected frequency of possible DOX-related toxicity was not observed. Another important finding was that the HSA tumor lysate vaccine was capable of eliciting humoral immune responses against canine HSA cell lines and against a novel antigen incorporated into the vaccine in dogs that were receiving concurrent DOX chemotherapy. Finally, administration of the vaccine together with DOX chemotherapy may have increased survival times over treatment with chemotherapy alone, although these last results will require confirmation in a prospective, randomized trial.

Tumor vaccines can be prepared by means of several different technologies, and each has advantages and disadvantages when used for designing vaccines for use in canine cancer patients. Autologous whole tumor cell vaccines have been evaluated extensively in humans, but with the exception of granulocyte macrophage colony stimulating factor (GM-CSF) transfected tumor cell vaccines, this approach has not been extensively adopted.³⁴⁻³⁶ Autologous tumor cell vaccines have major drawbacks related to difficulty in establishing cell lines and preparing vaccines in a timely and efficient fashion. Because few true tumor antigens have been identified in tumors of dogs, and none in the case of HSA, recombinant tumor antigen vaccines are unlikely to be of much use in the immunotherapy of HSA in dogs. Moreover, clinical experience in human tumor vaccine trials suggests that whole tumor cell vaccines are likely to be more effective than single tumor antigen vaccines, despite the greater difficulty associated with the preparation of whole cell vaccines.³⁴

Dogs with melanoma have been successfully immunized by means of DNA vaccination against the human melanoma antigen tyrosinase.³⁷ In addition, an allogeneic dog melanoma cell line transfected with human glycoprotein100 has also been used to vaccinate dogs with melanoma.^{37,38} However, similar human tumor antigens are not available for use against other common tumors of dogs, such as lymphoma, mast cell tumor, or HSA. Dendritic cell (DC) vaccines have received a great deal of attention over the past several years and there is one report of the use of DC vaccination against cancer in dogs.³⁹ However, the preparation of DC vaccines is quite labor intensive and costly and requires use of autologous DC, along with autologous tumor cell lysates, thereby severely limiting their applicability in veterinary medicine.

Allogeneic tumor vaccines prepared from canine tumor cell lines are an attractive alternative to autologous tumor vaccines. This approach holds promise

because many tumors of the same histiotype may share common antigens, so that vaccination against one tumor may elicit cross-reactive immune responses against other tumors of the same type.^{34,40,41} An allogeneic tumor lysate vaccine for melanoma has been evaluated in a number of clinical trials in humans and has shown clinical benefit in an adjuvant setting.^{42,43} Vaccines prepared with tumor cell lysates are particularly promising because of the ease of preparation, storage, and shipping of the vaccine. An allogeneic whole melanoma cell vaccine in dogs has shown promise in an early clinical trial.³⁸

Vaccines prepared with tumor lysates typically require use of strong vaccine adjuvants to elicit both cell-mediated and humoral immune responses against protein antigens. Prior studies in our lab have shown that LDCs can function as strong vaccine adjuvants in mice and also in dogs with atopy.^{22,23} Therefore, we designed a tumor lysate HSA vaccine for dogs that used pooled tumor lysates prepared from 2 canine HSA cell lines, in conjunction with the LDC adjuvant platform. The vaccine was administered by the intraperitoneal route because vaccine studies in mice demonstrated that this was the most effective route for eliciting T-cell and antibody responses.²²

The HSA vaccine was evaluated in dogs with a variety of different stages of HSA to determine safety and immunologic effectiveness. Some of adverse effects that developed in dogs in this study were those that we have previously associated with activation of innate immunity by the LDC component of the vaccine.²⁰ In addition, a number of adverse effects observed, especially GI adverse effects such as diarrhea, are also commonly associated with DOX chemotherapy.^{31,32} Therefore, it was impossible from the design of this study to fully distinguish vaccine-induced adverse effects from those elicited by treatment with DOX alone.

While development of anti-HSA antibodies after vaccination was observed in most dogs in this study, the specificity of the immune response was not determined. For example, it is possible that the antibody response was directed against allogeneic major histocompatibility complex (MHC) antigens on the HSA cells, rather than against novel HSA tumor antigens. Arguing against this possibility is the fact that the vaccine was prepared with cell lysates, which are unlikely to contain MHC molecules that are typically associated with the cell membrane. Moreover, the tumor lysates were extensively washed to remove proteins that might have been present in the FBS used to culture the cells. In addition, we did not observe antibody recognition of 2 unrelated canine osteosarcoma cell lines by pretreatment or posttreatment serum from HSA-vaccinated dogs, again arguing against recognition of allogeneic antigens (Fig 3). However, when a canine melanoma cell line was evaluated as a target for antibody binding, an increase in immunoglobulin binding by postvaccination serum was observed in 2 of the 6 vaccinated dogs (Fig 3). This response against the melanoma cell line could represent an immune response against alloantigens or the presence of cross-reactive

antigens shared between the HSA and melanoma cell lines. Assessment of humoral immune responses against autologous HSA cell lines, which was not possible in this study, would be necessary to help further resolve this issue.

The development of immune responses against normal endothelial cells is a theoretical concern with the use of vaccines prepared with HSA cell lines. This is because the HSA tumor arises from transformed endothelial cells, and canine HSA cell lines have been shown to express normal endothelial cell antigens.^{3,28} However, we did not observe adverse effects that might be expected with the development of anti-endothelial immune responses, such as coagulopathies or thrombotic events.

Survival times in dogs treated with tumor vaccine plus DOX chemotherapy were also compared with those of disease-matched historic control dogs with stage-II HSA. The median survival time for 13 dogs treated with vaccine plus DOX was 182 days, whereas the median survival time for the 24 historic control dogs treated with DOX only was 133 days. The survival time of 182 days found in the current study is longer than survival times reported with DOX only therapy in some studies¹⁵ but is shorter than reported in other studies for dogs with stage-II HSA treated with DOX or other chemotherapies.^{10,13,14} The reasons for these discrepancies are not fully apparent but may be related to differences in staging of the tumors or to differences in case selection. The increase in survival times observed in vaccine plus DOX-treated dogs suggests a positive interaction between the tumor vaccine and DOX chemotherapy. However, it is also important to note that statistical comparison of unrelated data sets such as these may introduce unintended bias and error into study results. Therefore, before these survival and disease-free interval results can be accepted, a randomized prospective trial comparing dogs treated with DOX alone to dogs treated with DOX plus vaccine would be necessary.

This study did not fully address the effect of timing of administration of the vaccine relative to timing of administration of DOX chemotherapy. Studies in mouse tumor models have shown that timing of administration of chemotherapy can have a significant effect on the efficacy of tumor vaccination.⁴⁴ For example, DOX administration on the same day as or within 7 days after administration of tumor vaccine was optimal for enhancing antitumor immunity, whereas administration of DOX 7 days before vaccination significantly inhibited tumor immunity. In our case, because DOX chemotherapy was given in repeated cycles, it was impossible to achieve the exact optimal timing of administration of DOX suggested in mouse studies. Additional studies in dogs would be required to more completely address these timing issues.

In summary, the results presented here, combined with results from a recent study by our group examining the effects of chemotherapy on immune responses in dogs with cancer, suggest that administration of DOX chemotherapy does not significantly impact generation

of humoral immune responses to vaccination in dogs with cancer.²⁴ The data also suggest that vaccination combined with chemotherapy may improve overall survival times. A previous study of the immunotherapeutic liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) also found an additive effect of immunotherapy when combined with chemotherapy for HSA in dogs.¹⁵ Therefore, additional studies investigating the use of combined immunotherapy and chemotherapy for treatment of particularly aggressive tumors of dogs, such as HSA and osteosarcoma, are warranted. Critical questions remaining to be addressed include determining which chemotherapeutic drugs can be most effectively combined with immunotherapy, the optimal timing of chemotherapy with immunotherapy, and whether tumor-specific immunotherapy (ie, tumor vaccines) is more effective than nonspecific immunotherapy when combined with chemotherapy.

Footnotes

- ^a Sonicator, Microson XL, Misonix Inc, Framingham, NY
^b Cholesterol and DOTAP, Avanti Polar Lipids, Alabaster, AL
^c Vacuum desiccator, Virtis Benchtop lyophilizer, Virtis Co, Gardiner, NY
^d Plasmid DNA, Althea Technologies, San Diego, CA
^e KLH, FACS buffer, and paraformaldehyde, Sigma-Aldrich Chemical Company, St Louis, MO
^f Optical density reader, Multiskan Ascent, Thermo Lab Systems, Waltham, MA
^g Goat anti-dog IgG antibody, Jackson ImmunoResearch, West Grove, PA
^h Cyan flow cytometer and Summit software, DakoCytomation, Ft Collins, CO
ⁱ GraphPad software, GraphPad Software Inc, San Diego, CA

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