

Of Mice and Men (and Dogs): development of a xenogeneic DNA vaccine for canine oral malignant melanoma

Review Article

Philip J. Bergman^{1,*}, Jedd D. Wolchok²

¹Bright Heart Veterinary Centers

²Ludwig Center for Cancer Immunotherapy, Memorial Sloan-Kettering Cancer Center

***Correspondence:** Philip J. Bergman DVM, MS, PhD, Diplomate ACVIM-Oncology, Chief Medical Officer, Bright Heart Veterinary Centers, 80 Business Park Drive, Suite 110, USA; Tel: 914-219-5415 (office); Fax: 914-219-5417; e-mail: pbergman@brighthousevet.com

Adjunct Associate Faculty Member, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021

Key words: Canine Oral Malignant Melanoma, Biologic Behavior, Stage, size, Grade and Histologic Parameters, Treatment

Abbreviations: human tyrosinase, (huTyr); Kaplan-Meier, (KM); median survival times, (MST); murine GP75, (muGP75); murine tyrosinase, (muTyr); Radiation therapy, (RT)

Received: 30 January 2008; electronically published: October 2008

Presented in the Theilen Tribute Symposium at UC Davis 31st May- 1st June 2008.

Summary

Melanoma is the most common oral malignancy in the dog. Oral and/or mucosal melanoma is generally considered an extremely malignant tumor with a high degree of local invasiveness and metastatic propensity. The WHO staging scheme for dogs with oral melanoma is based on size and metastasis, with stage I = < 2 cm. diameter tumor, stage II = 2 to < 4 cm. diameter tumor, stage III = \geq 4 cm. tumor and/or lymph node metastasis and stage IV = distant metastasis. Median survival times for dogs with oral melanoma treated with surgery are approximately 17-18, 5-6 and 3 months with stage I, II and III disease, respectively. Significant negative prognostic factors include stage, size, evidence of metastasis and a variety of histologic criteria. Standardized treatments such as surgery, coarse-fractionation radiation therapy and chemotherapy have afforded minimal to modest stage-dependent clinical benefits and death is usually due to systemic metastasis. Numerous immunotherapeutic strategies have been employed to date with variable clinical efficacy; however, the use of xenogeneic DNA vaccines as outlined herein may represent a leap forward in clinical efficacy. Oral melanoma is a spontaneous syngeneic cancer occurring in outbred, immunocompetent dogs and appears to be a more clinically faithful therapeutic model for human melanoma; further use of canine melanoma as a therapeutic model for human melanoma is strongly encouraged. In addition, the development of an expanded but clinically relevant staging system incorporating the aforementioned prognostic factors is also strongly encouraged.

I. Introduction

The most common oral malignancy in the dog is melanoma (Todoroff and Brodey, 1979; Goldschmidt, 1985; Wallace et al, 1992; Smith et al, 2002). Oral melanoma is most commonly seen in Scottish terriers, golden retrievers, poodles and dachshunds (Goldschmidt, 1985; Hahn et al, 1994). Oral melanoma is primarily a disease of older dogs without gender predilection, but may be seen in younger dogs (Harvey et al, 1981; MacEwen et al, 1986; Hahn et al, 1994). Melanomas in dogs have extremely diverse biologic behaviors depending on a large

variety of factors. A greater understanding of these factors significantly helps the clinician to delineate in advance the appropriate staging, prognosis and treatments. The primary factors which determine the biologic behavior of an oral melanoma in a dog are site, size, stage and histologic parameters (Bostock, 1979; Harvey et al, 1981; MacEwen et al, 1986; Hahn et al, 1994; Spangler et al, 2006). Unfortunately, even with a comprehensive understanding of all of these factors, there are melanomas which have an unreliable biologic behavior; hence the desperate need for additional research into this relatively

common, heterogeneous, but frequently extremely malignant tumor. Molecular biological aspects of melanoma have been previously reviewed (Modiano et al, 1999; Sulaimon and Kitchell, 2003). This communication will assume the diagnosis of melanoma has already been made and will focus on the aforementioned biologic behavior parameters, the staging and the treatment of canine oral melanoma.

II. Biologic behavior

The biologic behavior of canine oral melanoma is extremely variable and best characterized based on anatomic site, size, stage and histologic parameters. On divergent ends of the spectrum would be a 1.0 cm haired-skin melanoma with an extremely low grade likely to be cured with simple surgical extirpation, in comparison to a 5.0 cm high-grade malignant oral melanoma with a poor to grave prognosis. Similar to the development of a rational staging, prognostic and therapeutic plan for any tumor, two primary questions must be answered; what is the local invasiveness of the tumor and what is the metastatic propensity? The answers to these questions will determine the prognosis, and to be discussed later, the treatment.

III. Site

The anatomic site of melanoma is highly, though not completely, predictive of local invasiveness and metastatic propensity. Melanomas involving the haired-skin which are not in proximity to mucosal margins often behave in a benign manner (Goldschmidt, 1994; Smith et al, 2002). Surgical extirpation through a lumpectomy is often curative, but histopathological examination is imperative for delineation of margins as well as a description of cytologic features. In haired-skin melanomas exhibiting histopathologic criteria of malignancy, please see the grade discussion below.

Oral and/or mucosal melanoma has been routinely considered an extremely malignant tumor with a high degree of local invasiveness and high metastatic propensity (Bostock, 1979; Harvey et al, 1981; Goldschmidt, 1985; MacEwen et al, 1986; Hahn et al, 1994). This biologic behavior is extremely similar to human oral and/or mucosal melanoma (Vail and MacEwen, 2000; Smith et al, 2002). Melanoma is the most common oral tumor in the dog; additional neoplastic differentials include squamous cell carcinoma, fibrosarcoma, epulides/odontogenic tumors and others (Bradley et al, 1984; Harvey, 1985; Kosovsky et al, 1991; Wallace et al, 1992; Goldschmidt, 1985; Smith et al, 2002). Melanomas in the oral cavities of dogs are found in the following locations by order of decreasing frequency: gingiva, lips, tongue and hard palate. While most melanomas are pigmented, amelanotic oral melanomas are noted clinically and have been previously reported (Choi and Kusewitt, 2003). In canine oral/mucosal melanomas with histological reporting suggestive of a benign lesion, the reader is referred to the grade discussion below.

The anatomic sites that split the opposite ends of the prognostic spectrum of generally benign-acting haired-skin vs. typically malignant and metastatic oral/mucosal melanomas include melanomas of the digit and foot pad.

While these anatomic sites are not the primary focus of this discussion, dogs with melanoma of the digits without lymph node or further metastasis treated with digit amputation are reported to have median survival times of ~ 12 months, with 42-57% alive at 1 year and 11-13% alive at 2 years (Marino et al, 1995; Henry et al, 2005). Unfortunately, metastasis from digit melanoma at presentation is reported to be ~ 30-40%, and the aforementioned median survival time of only a year with surgery suggest that subsequent distant metastasis is common even when no metastasis is found at presentation/digit amputation. The prognosis for dogs with melanoma of the foot pad has not been previously significantly reported; these authors have found this anatomic site to be similar in metastatic propensity and prognosis to digit melanoma. Interestingly, human acral lentiginous melanoma (plantar surface of the foot, palms of the hand and digit) has an increased propensity for metastasis (Marino et al, 1995).

IV. Size and stage

For dogs with oral melanoma, primary tumor size has been found to be extremely prognostic. The WHO staging scheme for dogs with oral melanoma is based on size and metastasis, with stage I = < 2 cm. diameter tumor, stage II = 2 cm. to < 4 cm. diameter tumor, stage III = 4 cm. or greater tumor and/or lymph node metastasis and stage IV = distant metastasis. MacEwen and colleagues reported median survival times (MST) for dogs with oral melanoma treated with surgery to be approximately 17-18, 5-6 and 3 months with stage I, II and III disease, respectively (MacEwen et al, 1986). More recent reports suggest stage I oral melanoma treated with standardized therapies including surgery, radiation and/or chemotherapy have a MST of approximately 12-14 months, with most dogs dying of distant metastatic disease, not local recurrence (Freeman et al, 2003; Proulx et al, 2003). Other investigators have found dogs with stage I oral melanoma to have median progression-free survival times of 19 months similar to MacEwen and colleagues in 1986.

A variety of limitations exist with the present WHO staging scheme for canine oral melanoma. First, the size of the tumor is not standardized to the size of the patient. Therefore, a 1.5 cm oral melanoma without lymph node metastasis is a stage I melanoma in a Rottweiler, as well as a Chihuahua. Further investigations with standardization to patient size are hereby encouraged. In addition, the histologic appearance and other histologically-based indices of melanomas are not accounted for in the present WHO staging scheme and proposed alternate schemes incorporating histological criteria have unfortunately not gained widespread use for canine melanoma. For these reasons and others, various investigators have pursued other prognostic factors in canine oral melanoma in order to possibly develop alternative staging systems. These investigations have continued to find size to be extremely prognostic, but have also found the following negative prognostic factors: lesser degree of extirpation and incomplete surgical margins, location (caudal mandibular and rostral maxillary do more poorly), tumor mitotic index > 3, and bone invasion/lysis (Hahn et al, 1994; Proulx et

al, 2003). Prospective investigations including these variables into an expanded WHO staging system are hereby strongly encouraged.

The staging system for canine non-oral melanoma is less well defined to date. Henry and colleagues utilized the WHO TNM system for canine digital tumors, which defines T1 = tumor < 2 cm and superficial, T2 = tumor 2-5 cm. and minimum invasion, T3 = tumor > 5 cm. or invading subcutis and T4 = tumor invading fascia or bone (Henry et al, 2005). They reported that metastasis free interval was significantly inversely associated with T stage across all digit tumors. When specifically examining dogs with digit melanoma, there was 1 dog with T2, 5 dogs with T3 and 4 dogs with T4 tumors. Further studies defining staging schemes for canine non-oral melanoma with clinical variables and outcomes are also encouraged.

V. Grade and histologic parameters

Histopathologic grading of a tumor by the pathologist delineates degree of malignancy and grading systems vary across tumor types. The histological grade is commonly predictive of survival, metastatic rate and other clinical variables in a wide variety of tumors across species, including canine melanoma (Bolon et al, 1990; Powers, 2001; Smith et al, 2002). For example, in haired-skin melanomas exhibiting multiple histopathologic criteria of malignancy, such as increased mitotic rate, invasiveness and/or poor differentiation, metastatic propensity is increased and the prognosis is reduced due to variability in outcomes post-operatively. Bostock et al reported that 45% of dogs with malignant skin melanomas died within one year, whereas 8% of dogs with "benign" skin melanomas died from their disease (Bostock, 1979). Furthermore, 10% of dogs with haired-skin melanoma with a mitotic index of 2 or less died from their tumor 2 years after surgery compared to > 70% dogs dying from a tumor with a mitotic index of 3 or more. Dogs with haired-skin melanomas within 0.5 - 1 cm of mucosal margins have been minimally investigated to date; the first author has had multiple patients with histologically benign, haired-skin melanoma within 1 cm. of a mucosal margin develop subsequent distant metastatic disease; additional investigation into patients with peri-mucosal melanoma is therefore encouraged.

The most exhaustive review of histologic findings in canine melanocytic neoplasms was recently published by Spangler and Kass in 2006. In this paper, 384 dogs with melanoma or melanocytoma (IDEXX submissions) had their tumors comprehensively histologically examined and statistically tested for association with malignant behavior (recurrence and/or metastasis) and median survival time. Significant negative prognostic factors included metastasis (ie stage as discussed above), size/tumor volume and a variety of histologic criteria such as mitotic index, nuclear atypia, tumor score, presence of deep inflammation, intralesional necrosis and junctional activity. As expected, these investigators also found three primary anatomic-location mortality groups: 1) oral (19% of samples), 2) feet and mucosal surface of lips (19% of samples) and 3) cutaneous (59% of samples). Too few ocular melanomas were investigated to make recommendations.

Unexpectedly, 32% of dogs with oral melanoma did not have malignant behavior according to their criteria (no recurrence, no metastasis and alive at the end of study or dead due to competing causes). This author sees no reason why oral melanomas may not occasionally behave in a benign fashion; however, 32% is a markedly higher frequency from all previous reports, thereby warranting further study. Similarly, the number of benign-acting oral melanomas was relatively small (n=22) and a variety of factors such as lack of necropsy, type of follow-up, lack of reporting of number of lost to follow-up cases and lastly the large number of cases disqualified for inclusion because of poor differentiation, may have lead to an increased frequency of benign-acting cases. Similarly, the first author has seen in excess of 15 dogs in the last 5 years with a previous histopathologic diagnosis of benign oral melanoma present with distant metastasis. This is consistent with Bostock in 1979 reporting three of seven dogs with "benign" oral melanoma going on to die of their disease (Bostock, 1979).

Spangler and Kass also reported that 38% and 12% of feet/mucosal surface of lips and cutaneous melanocytic tumors, respectively, behaved in a malignant fashion (Spangler et al, 2006). Four percent and 27% of those dogs that died of a foot/lip and cutaneous melanoma, respectively, had a tumor score which would have predicted benign behavior. Upon further review of those cases, there were no attributes found that would allow for prediction of malignant behavior. This suggests that additional testing is needed beyond routine light microscopy for delineation of malignant vs. benign behavior for canine cutaneous melanoma. Laprie et al reported the use of Ki-67 expression via immunohistochemistry in 68 canine cutaneous melanomas (Laprie et al, 2001). This group found that the predictive value of Ki-67 proliferative index (97%) was greater than the predictive value of classical histology (91%) for biologic behavior in canine cutaneous melanoma. This strongly suggests that the use of Ki-67 immunohistochemistry and possibly other proliferative markers (e.g. AgNOR and others) in canine cutaneous melanoma should be seriously considered after the histopathologic diagnosis is made.

VI. Staging

The staging of dogs with melanoma is relatively straightforward (**Figure 1**). A minimum database should include a thorough history and physical exam, complete blood count and platelet count, biochemical profile, urinalysis, 3 view chest films and local lymph node aspiration with cytology whether lymphadenomegaly is present or not. Williams and Packer reported in dogs with oral melanoma that ~ 70% had metastasis when lymphadenomegaly was present, but more importantly ~ 40% had metastasis when no lymphadenomegaly was present (Williams and Packer, 2003). Additional considerations should be made for abdominal compartment testing (e.g. abdominal ultrasound) in all cases of canine malignant melanoma, especially in cases with potentially moderately to highly metastatic anatomic sites such as the oral cavity, feet or mucosal surface of the

lips, as melanoma may metastasize to the abdominal lymph nodes, liver, adrenal glands and other sites. The use of sentinel lymph node mapping and lymphadenectomy has been proven to be of diagnostic, prognostic and clinical benefit in human melanoma (Leong et al, 2006). Relatively few investigations have been reported to date for sentinel lymph node mapping and/or excision for dogs with malignancies (Yudd et al, 1999; Herring et al, 2002; Nwogu et al, 2002; Wells et al, 2006) and these authors strongly encourage additional investigation in this area and specifically with canine melanoma.

VII. Treatment

The treatment for dogs with melanoma without distant metastatic disease on staging starts with local tumor control. This is generally best completed through surgical extirpation due to its speed, increased curative intent and reduced cost compared to other modalities. The dose of surgery is generally based on the anatomic site of the melanoma, with cutaneous melanomas usually requiring lumpectomy and all other sites requiring more aggressive and wide excision. While large resections such as partial mandibulectomy or maxillectomy carry an inherent level of morbidity, owner satisfaction rates are routinely considered high. It cannot be overstated the importance of complete staging when contemplating larger resections; the presence of distant metastatic disease would attenuate the use of more radical surgical procedures and convert the patient to medical and/or palliative care options.

Radiation therapy (RT) plays a role in the treatment of canine melanoma when the tumor is not surgically resectable, the tumor has been removed with incomplete margins and/or the melanoma has metastasized to local lymph nodes without further distant metastasis. The use of smaller fractions of RT (e.g. 3-4 Gy) given daily to every other day can allow for a greater total dose and fewer chronic RT reactions; however, melanoma appears comparatively resistant to these types of fractionation schemes (Banks and Morris, 1975; Proulx et al, 2003). Coarse fractionation schemes for canine melanoma

utilizing 6-9 Gy weekly to every other week to a total dose of 24-36 Gy have been reported by a variety of investigators with complete remission rates of 53-69% and partial remission rates of 25-30% (Bateman et al, 1994; Blackwood and Dobson, 1996; Theon et al, 1997; Freeman et al, 2003; Proulx et al, 2003). Unfortunately, recurrence and/or distant metastasis were common in all of these studies. Other modalities reported for local tumor control as case reports and/or case series have included intralesional cisplatin implants, intralesional bleomycin with electronic pulsing and many others, but widespread use has not been reported to date (Theon et al, 1991; Kitchell et al, 1994; Spugnini et al, 2006).

In dogs with melanoma in the aforementioned anatomic sites predicted to have a moderate to high metastatic propensity, or dogs with cutaneous melanoma with a high tumor score and/or increased proliferation index through increased Ki-67 expression, the use of systemic therapies is warranted. Rassnick and colleagues reported an overall response rate of 28% using carboplatin for dogs with malignant melanoma (Rassnick et al, 2001). Unfortunately, only one dog had a minimally durable complete response (~150 days), and the rest were non-durable partial responses. Similarly, Boria et al reported an 18% response rate and median survival time of 119 days with cisplatin and piroxicam in canine oral melanoma (Boria et al, 2004). Other reports using single agent dacarbazine, melphalan or doxorubicin suggest poor to dismal activity (Aigner et al, 1983; Ogilvie et al, 1989; Page et al, 1991). More recently and importantly, two studies suggest that chemotherapy plays an insignificant role in the adjuvant treatment of canine melanoma (Proulx et al, 2003; Murphy et al, 2005). While it can be argued that the studies performed to date to evaluate the activity of chemotherapy in an adjuvant setting for canine melanoma have been suboptimal due to a variety of reasons, the extensive human literature in this specific setting suggests melanoma is an extremely chemotherapy resistant tumor (O'Day and Boasberg, 2006). It is clear that new approaches to the systemic treatment of this disease are desperately needed.

Figure 1. Traditional World Health Organization (WHO) TNM-based staging scheme for dogs with oral melanoma.

T:	Primary Tumor
	T1 Tumor < 2 cm in diameter
	T2 Tumor 2 – 4 cm in diameter
	T3 Tumor > 4 cm in diameter
N:	Regional Lymph Nodes
	N0 No evidence of regional node involvement
	N1 Histologic/Cytologic evidence of regional node involvement
	N2 Fixed nodes
M:	Distant Metastasis
	M0 No evidence of distant metastasis
	M1 Evidence of distant metastasis

Stage I = T1 N0 M0
 Stage II = T2 N0 M0
 Stage III = T2 N1 M0 or T3 N0 M0
 Stage IV = Any T, Any N and M1

Immunotherapy represents one potential logical systemic therapeutic strategy for melanoma. A variety of immunotherapeutic strategies for the treatment of human melanoma have been reported previously, with typically poor outcomes due to a lack of breaking tolerance. Immunotherapy strategies to date in canine melanoma have used autologous tumor cell vaccines (with or without transfection with immunostimulatory cytokines and/or melanosomal differentiation antigens), allogeneic tumor cell vaccines transfected with interleukin 2 or GM-CSF, liposomal-encapsulated non-specific immunostimulators (e.g. L-MTP-PE), intralesional Fas ligand DNA, bacterial super-antigen approaches with granulocyte macrophage colony-stimulating factor or interleukin 2 as immune adjuvants and lastly canine dendritic cell vaccines loaded with melanosomal differentiation antigens (MacEwen et al, 1986; Dow et al, 1998; Helfand et al, 1994; Hogge et al, 1999; MacEwen et al, 1999; Bianco et al, 2003; Gyorffy et al, 2005; Alexander et al, 2006) Although these approaches have produced some clinical anti-tumor responses, the methodologies for the generation of these products are expensive, time consuming, sometimes dependent on patient tumor samples being established into cell lines and fraught with the difficulties of consistency, reproducibility, and other quality control issues.

The advent of DNA vaccination circumvents many of the previously encountered hurdles in vaccine development. DNA is relatively inexpensive and simple to purify in large quantities. The antigen of interest is cloned into a bacterial expression plasmid with a constitutively active promoter. The plasmid is introduced into the skin or muscle with an intradermal or intramuscular injection. Once in the skin or muscle, professional antigen presenting cells, particularly dendritic cells, are able to present the transcribed and translated antigen in the proper context of major histocompatibility complex and

costimulatory molecules. Although DNA vaccines have induced immune responses to viral proteins, vaccinating against tissue specific self-proteins on cancer cells is clearly a more difficult problem. One way to induce immunity against a tissue specific differentiation antigen on cancer cells is to vaccinate with xenogeneic (different species) antigen or DNA that is homologous to the cancer antigen. As outlined in cartoon form in **Figure 2**, vaccination with DNA encoding cancer differentiation antigens is ineffective when self-DNA is used, but tumor Immunity can be induced by orthologous DNA from another species (Guevara-Patino et al, 2003).

We have chosen to target defined melanoma differentiation antigens of the tyrosinase family. Tyrosinase is a melanosomal glycoprotein, essential in melanin synthesis. Immunization with xenogeneic human DNA encoding tyrosinase family proteins induced antibodies and cytotoxic T-cells against syngeneic B16 melanoma cells in C57BL/6 mice, but immunization with mouse tyrosinase-related DNA did not induce detectable immunity (Weber et al, 1998). In particular, xenogeneic DNA vaccination induced tumor protection from syngeneic melanoma challenge and autoimmune hypopigmentation. Thus, xenogeneic DNA vaccination could break tolerance against a self tumor differentiation antigen, inducing antibody, T-cell and anti-tumor responses.

From April 2000 to June 2007, approximately 500 dogs with previously histologically confirmed spontaneous malignant melanoma were treated at the Animal Medical Center with xenogeneic DNA vaccinations. Pre-trial evaluation included complete physical examination, a complete blood count and platelet count, serum chemistry profile, urinalysis, lactate dehydrogenase, anti-nuclear antibody, and three-dimensional measurements of the

Xenogeneic DNA Immunization Concept

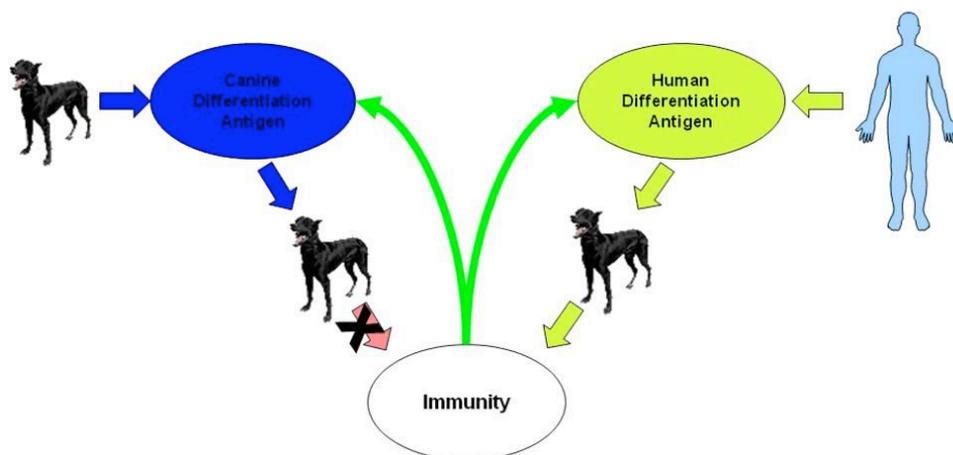


Figure 2. Cartoon outlining the xenogeneic DNA vaccination concept.

primary tumor if present (or maximal tumor size from medical records if patient was treated prior to pre-trial considerations). For evaluation of metastatic disease, 3-view radiographs of the thorax were obtained and regional lymph nodes were evaluated with fine needle aspiration/cytology and/or biopsy/histopathology. All dogs were clinically staged according to the WHO staging system of stage I (tumor < 2 cm diameter), II (tumors 2-4 cm. diameter, negative nodes), stage III (tumor > 4 cm. and/or positive nodes) or stage IV (distant metastatic disease). The numbers of previous treatments with surgery, radiation and/or chemotherapy were recorded. Dogs with WHO stage II, III or IV histologically confirmed malignant melanoma were allowed entrance onto the studies due to the lack of effective available systemic treatments. Due to a strong safety profile, dogs with stage I melanoma were allowed inclusion from 2005 on. Additional entry criteria included: an estimated life expectancy of 6 weeks or more, free of clinically detectable brain metastases, no previous therapy (surgery, radiation and/or chemotherapy) for at least 3 weeks and no serious intercurrent medical illnesses. Written consent for entry onto this trial was obtained from each dog's owner prior to entry onto the study; this consent included request for necropsy upon death due to any reason. These studies

were performed under Animal Medical Center IRB approval (Bergman et al, 2006).

The signalments of dogs on these studies have been similar to those in previously reported CMM studies. No toxicity was seen in any dogs receiving the aforementioned vaccines with the exception of minimal to mild pain responses at vaccination, one muGP75 dog experienced mild aural depigmentation, and one muTyr dog experienced moderate foot pad vitiligo. Dogs with stage I-III loco-regionally controlled CMM across the xenogeneic vaccine studies have a Kaplan-Meier (KM) MST of > 1075 days (median not yet reached) whereas those dogs with stage I-III CMM without local tumor control have a KM MST of 553 days (P = 0.0002; **Figure 3**). The KM MST for stage II-IV dogs on the Phase I trials of huTyr, muGP75 and muTyr are 389, 153 and 224 days, respectively. Dogs which received any melanoma vaccine (ie HuTyr, MuTyr and MuGP75), the KM MST for stage I, II, III and IV CMM was > 939 days (median not reached with 92.8% survival), > 908 days (median not reached, 79% alive at 1 year, 63% alive at 2 years), > 1646 days (median not reached, 77%, 65%, 57% alive at 1, 2, 3 years), and 239 days (40.5% and 18.8% alive at 1 and 2 years), respectively (**Figure 4**). The results from dogs vaccinated with huTyr were published in 2003 (Bergman et al, 2003).

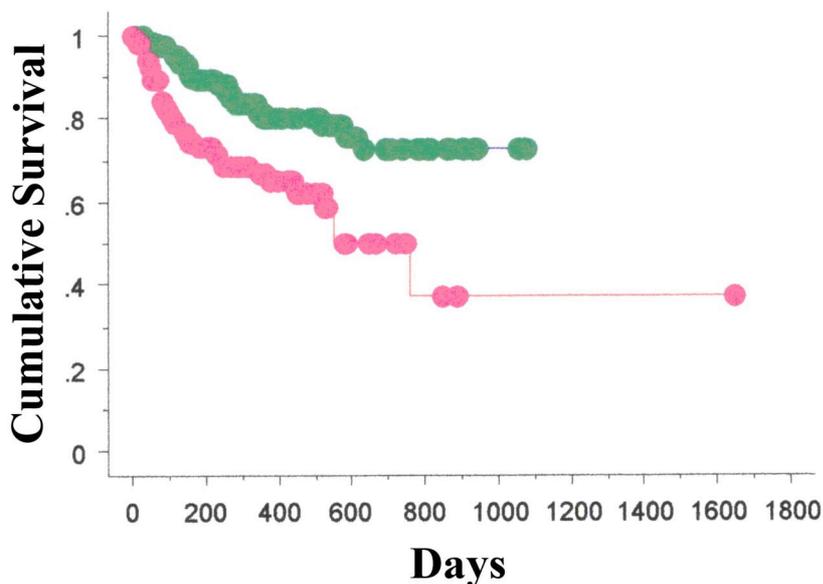


Figure 3. Kaplan-Meier survival curves for dogs with advanced malignant melanoma treated with xenogeneic melanoma vaccinations with or without loco-regional tumor control prior to starting the vaccination protocol. Kaplan-Meier median survival time for those dogs with loco-regional tumor control is > 1072 days (median not reached; green dots = dogs censored) whereas those dogs without loco-regional tumor control is 553 days (pink dots = censored dogs; Log-Rank P = 0.0002). Time as shown on X-axis is in days and percentage cumulative survival on Y-axis.

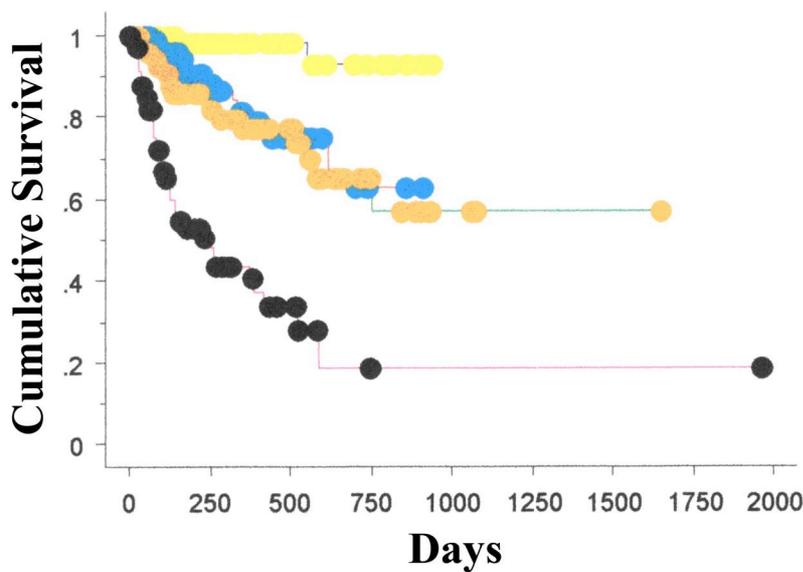


Figure 4. Kaplan-Meier survival curves for dogs with advanced malignant melanoma treated with xenogeneic melanoma vaccinations across WHO stages I, II, III and IV. Kaplan-Meier median survival time for those dogs with stage I disease is > 939 days (median not reached; yellow dots = censored dogs). Kaplan-Meier median survival time for those dogs with stage II disease is > 908 days (median not reached; 79% and 63% alive at 1 and 2 years, respectively; blue dots = censored dogs). Kaplan-Meier median survival time for those dogs with stage III disease is > 1646 days (median not reached; 77%, 65%, and 57% alive at 1, 2, and 3 years, respectively; orange dots = censored dogs). Kaplan-Meier median survival time for those dogs with stage IV disease is 239 days (41 and 19% alive at 1 and 2 years, respectively; black dots = censored dogs). Time as shown on X-axis is in days and percentage cumulative survival on Y-axis.

We have investigated the humoral responses of dogs receiving HuTyr as a potential explanation for the long-term survivals seen in some of the dogs on this study. Utilizing standard ELISA with mammalian expressed purified human tyrosinase protein as the target of interest (kind gift of C Andreoni & JC Audonnet, Merial, Inc.), we have found 3/9 dogs with 2-5 fold post-vaccinal humoral responses compared to pre-immune sera. We have confirmed these findings utilizing a flow-cytometric-based assay of pre- and post-vaccinal sera in permeabilized human SK-MEL melanoma cells expressing endogenous human tyrosinase. Interestingly, the three dogs with post-vaccinal anti-HuTyr humoral responses are dogs with unexpected long-term tumor control (Liao et al, 2006). Additional studies ongoing at Merial are investigating the humoral responses from dogs on the other xenogeneic DNA vaccines as well as T-cell based assays utilizing ELISPOT and intracellular cytokine staining assays.

The results of these trials demonstrate that xenogeneic DNA vaccination in CMM is: 1) safe, 2) develops specific anti-tyrosinase humoral immune responses, 3) potentially therapeutic with particularly exciting results in stage II/III local-regional controlled disease, and 4) an attractive candidate for further evaluation in an adjuvant, minimal residual disease Phase II setting for CMM. A safety and efficacy USDA licensure multi-institutional trial investigating HuTyr in dogs with locally controlled stage II/III oral melanoma was initiated in April, 2006 across 5 sites (P. Bergman, Animal Medical Center; K. Meleo, Seattle; MK Klein, Tucson/Phoenix; S Susaneck, Houston; P Hess, North Carolina State

University). Human trials of xenogeneic tyrosinase DNA vaccination have initiated and are ongoing with promising initial clinical and immunologic assay results (Wolchok et al, 2007). In late March 2007, we received conditional licensure from the USDA for the HuTyr-based canine melanoma vaccine and it became commercially available in June, 2007. This represents the first US-government approved vaccine for the treatment of cancer. As of January 2008, approximately 1500 dogs with malignant melanoma have received the conditionally licensed Merial, Ltd. HuTyr canine melanoma vaccine, and approximately 700 dogs are entered into the internet-based Merial melanoma vaccine followup database (personal communication, Dr. Robert Menardi, Merial Ltd.).

In summary, CMM is a more clinically faithful therapeutic model for HM when compared to more traditional mouse systems as both human and canine diseases are chemoresistant, radioresistant, share similar metastatic phenotypes/site selectivity, and occur spontaneously in an outbred, immuno-competent scenario. In addition, this work also shows that veterinary cancer centers and human cancer centers can work productively together to benefit veterinary and human patients afflicted with cancer. It is hoped in the future that this same vaccine may also play roles in the treatment of melanoma in other species (e.g. horses, cats, humans, etc.) due to its xenogeneic origins, and in melanoma prevention once the genetic determinants of melanoma risk in dogs are further defined. It is easy to see how the veterinary oncology profession is uniquely able to greatly contribute to advances for both canine as well as human melanoma, in

addition to many other cancers with similar comparative aspects across species. These authors believe that the xenogeneic DNA vaccine platform holds promise with other antigen targets and have Phase I and Phase II studies planned utilizing murine CD20 and murine HER2 across the BrightHeart Veterinary Centers network. These authors and the fields of veterinary tumor immunotherapy and veterinary oncology are greatly indebted to the tireless work and seeds laid by the late Dr. Greg MacEwen; he is greatly missed.

Acknowledgment

AMC Staff Doctors, House Officers, Technicians & Dept. of Pathology Staff; Merial, Inc. (Drs. B. Nordgren, T. Leard, R. Menardi, J.C. Audonnet, L. Fischer and C. Andreoni); Bioject Inc. (Dr. R. Stout) and the patients and owners.

References

- Aigner K, Hild P, Breithaupt H, Hundeiker M, Schwemmler K, Henneking K, Illig L, Merker G, Paul E, Brodtkorb J, Jungbluth A (1983) Isolated extremity perfusion with DTIC. An experimental and clinical study. **Anticancer Res** 3, 87-93.
- Alexander AN, Huelsmeyer MK, Mitzey A, Dubielzig RR, Kurzman ID, MacEwen EG, Vail DM (2006) 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. **Cancer Immunol Immunother** 55, 433-442.
- Banks WC, Morris E (1975) Results of radiation treatment of naturally occurring animal tumors. **J Am Vet Med Assoc** 166, 1063-1064.
- Bateman KE, Catton PA, Pennock PW, Kruth SA (1994) 0-7-21 radiation therapy for the treatment of canine oral melanoma. **J Vet Intern Med** 8, 267-272.
- Bergman PJ, Camps-Palau MA, McKnight JA, Leibman NF, Craft DM, Leung C, Liao J, Riviere I, Sadelain M, Hohenhaus AE, Gregor P, Houghton AN, Perales MA, Wolchok JD (2006) Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. **Vaccine** 24, 4582-4585.
- Bergman PJ, McKnight J, Novosad A, Charney S, Farrelly J, Craft D, Wulderk M, Jeffers Y, Sadelain M, Hohenhaus AE, Segal N, Gregor P, Engelhorn M, Riviere I, Houghton AN, Wolchok JD (2003) Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. **Clin Cancer Res** 9, 1284-1290.
- Bianco SR, Sun J, Fosmire SP, Hance K, Padilla ML, Ritt MG, Getzy DM, Duke RC, Withrow SJ, Lana S, Matthiesen DT, Dow SW, Bellgrau D, Cutter GR, Helfand SC, Modiano JF (2003) Enhancing antimelanoma immune responses through apoptosis. **Cancer Gene Ther** 10, 726-736.
- Blackwood L, Dobson JM (1996) Radiotherapy of oral malignant melanomas in dogs. **J Am Vet Med Assoc** 209, 98-102.
- Bolon B, Calderwood Mays MB, Hall BJ (1990) Characteristics of canine melanomas and comparison of histology and DNA ploidy to their biologic behavior. **Vet Pathol** 27, 96-102.
- Boria PA, Murry DJ, Bennett PF, Glickman NW, Snyder PW, Merkel BL, Schlittler DL, Mutsaers AJ, Thomas RM, Knapp DW (2004) Evaluation of cisplatin combined with piroxicam for the treatment of oral malignant melanoma and oral squamous cell carcinoma in dogs. **J Am Vet Med Assoc** 224, 388-394.
- Bostock DE (1979) Prognosis after surgical excision of canine melanomas. **Vet Pathol** 16, 32-40.
- Bradley RL, MacEwen EG, Loar AS (1984) Mandibular resection for removal of oral tumors in 30 dogs and 6 cats. **J Am Vet Med Assoc** 184, 460-463.
- Choi C, Kusewitt DF (2003) Comparison of tyrosinase-related protein-2, S-100, and Melan A immunoreactivity in canine amelanotic melanomas. **Vet Pathol** 40, 713-718.
- Dow SW, Elmslie RE, Willson AP, Roche L, Gorman C, Potter TA (1998) In vivo tumor transfection with superantigen plus cytokine genes induces tumor regression and prolongs survival in dogs with malignant melanoma. **J Clin Invest** 101, 2406-2414.
- Freeman KP, Hahn KA, Harris FD, King GK (2003) Treatment of dogs with oral melanoma by hypofractionated radiation therapy and platinum-based chemotherapy (1987-1997). **J Vet Intern Med** 17, 96-101.
- Freeman KP, Hahn KA, Harris FD, King GK (2003) Treatment of dogs with oral melanoma by hypofractionated radiation therapy and platinum-based chemotherapy (1987-1997). **J Vet Intern Med** 17, 96-101.
- Goldschmidt MH (1985) Benign and malignant melanocytic neoplasms of domestic animals. **Am J Dermatopathol** 7 (Suppl), 203-212.
- Goldschmidt MH (1994) Pigmented lesions of the skin. **Clin Dermatol** 12, 507-514.
- Guevara-Patino JA, Turk MJ, Wolchok JD, Houghton AN (2003) Immunity to cancer through immune recognition of altered self: studies with melanoma. **Adv Cancer Res** 90, 157-177.
- Gyorffy S, Rodriguez-Lecompte JC, Woods JP, Foley R, Kruth S, Liaw PC, Gaudie J (2005) Bone marrow-derived dendritic cell vaccination of dogs with naturally occurring melanoma by using human gp100 antigen. **J Vet Intern Med** 19, 56-63.
- Hahn KA, DeNicola DB, Richardson RC, Hahn EA (1994) Canine oral malignant melanoma: Prognostic utility of an alternative staging system. **J Small Anim Pract** 35, 251-256.
- Harmelin ES, Holcombe RN, Goggin JP, Carbonell J, Wellens T (1998) Acral lentiginous melanoma. **J Foot Ankle Surg** 37, 540-545.
- Harvey HJ, MacEwen EG, Braun D, Patnaik AK, Withrow SJ, Jongeward S (1981) Prognostic criteria for dogs with oral melanoma. **J Am Vet Med Assoc** 178, 580-582.
- Harvey HJ (1985) Oral tumors. **Vet Clin North Am Small Anim Pract** 15, 493-500.
- Helfand SC, Soergel SA, Modiano JF, Hank JA, Sondel PM (1994) Induction of lymphokine-activated killer (LAK) activity in canine lymphocytes with low dose human recombinant interleukin-2 in vitro. **Cancer Biother** 9, 237-244.
- Henry CJ, Brewer WG Jr, Whitley EM, Tyler JW, Ogilvie GK, Norris A, Fox LE, Morrison WB, Hammer A, Vail DM, Berg J; Veterinary Cooperative Oncology Group (VCOG) (2005) Canine digital tumors: a veterinary cooperative oncology group retrospective study of 64 dogs. **J Vet Intern Med** 19, 720-724.
- Herring ES, Smith MM, Robertson JL (2002) Lymph node staging of oral and maxillofacial neoplasms in 31 dogs and cats. **J Vet Dent** 19, 122-126.
- Hogge GS, Burkholder JK, Culp J, Albertini MR, Dubielzig RR, Yang NS, MacEwen EG (1999) Preclinical development of human granulocyte-macrophage colony-stimulating factor-transfected melanoma cell vaccine using established canine cell lines and normal dogs. **Cancer Gene Ther** 6, 26-36.
- Kitchell BE, Brown DM, Luck EE, Woods LL, Orenberg EK, Bloch DA (1994) Intralesional implant for treatment of

- primary oral malignant melanoma in dogs. **J Am Vet Med Assoc** 204, 229-236.
- Kosovsky JK, Matthiesen DT, Marretta SM, Patnaik AK (1991) Results of partial mandibulectomy for the treatment of oral tumors in 142 dogs. **Vet Surg** 20, 397-401.
- Laprie C, Abadie J, Amardeilh MF, Net JL, Lagadic M, Delverdier M (2001) MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma. **Vet Dermatol** 12, 139-147.
- Leong SP, Accortt NA, Essner R, Ross M, Gershenwald JE, Pockaj B, Hoekstra HJ, Garberoglio C, White RL Jr, Chu D, Biel M, Charney K, Wanebo H, Avisar E, Vetto J, Soong SJ; Sentinel Lymph Node Working Group(2006) Impact of sentinel node status and other risk factors on the clinical outcome of head and neck melanoma patients. **Arch Otolaryngol Head Neck Surg** 132, 370-373.
- Liao JC, Gregor P, Wolchok JD, Orlandi F, Craft D, Leung C, Houghton AN, Bergman PJ (2006) Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. **Cancer Immun** 6, 8.
- MacEwen EG, Kurzman ID, Vail DM, Dubielzig RR, Everlith K, Madewell BR, Rodriguez CO Jr, Phillips B, Zwahlen CH, Obradovich J, Rosenthal RC, Fox LE, Rosenberg M, Henry C, Fidel J (1999) Adjuvant therapy for melanoma in dogs: results of randomized clinical trials using surgery, liposome-encapsulated muramyl tripeptide, and granulocyte macrophage colony-stimulating factor. **Clin Cancer Res** 5, 4249-4258.
- MacEwen EG, Patnaik AK, Harvey HJ, Hayes AA, Matus R (1986) Canine oral melanoma: comparison of surgery versus surgery plus *Corynebacterium parvum*. **Cancer Invest** 4, 397-402.
- Marino DJ, Matthiesen DT, Stefanacci JD, Moroff SD (1995) Evaluation of dogs with digit masses: 117 cases (1981-1991). **J Am Vet Med Assoc** 207, 726-728.
- Modiano JF, Ritt MG, Wojcieszyn J (1999) The molecular basis of canine melanoma: pathogenesis and trends in diagnosis and therapy. **J Vet Intern Med** 13, 163-174.
- Murphy S, Hayes AM, Blackwood L, Maglennon G, Pattinson H, Sparkes AH (2005) Oral malignant melanoma - the effect of coarse fractionation radiotherapy alone or with adjuvant carboplatin therapy. **Vet Comp Oncol** 3, 222-229.
- Nwogu CE, Kanter PM, Anderson TM (2002) Pulmonary lymphatic mapping in dogs: use of technetium sulfur colloid and isosulfan blue for pulmonary sentinel lymph node mapping in dogs. **Cancer Invest** 20, 944-947.
- O'Day S, Boasberg P (2006) Management of metastatic melanoma 2005. **Surg Oncol Clin N Am** 15, 419-437.
- Ogilvie GK, Reynolds HA, Richardson RC, Withrow SJ, Norris AM, Henderson RA, Klausner JS, Fowler JD, McCaw D (1989) Phase II evaluation of doxorubicin for treatment of various canine neoplasms. **J Am Vet Med Assoc** 195, 1580-1583.
- Page RL, Thrall DE, Dewhirst MW, Macy DW, George SL, McEntee MC, Heidner GL, Novotney CA, Allen SA, Withrow SJ, et al (1991) Phase I study of melphalan alone and melphalan plus whole body hyperthermia in dogs with malignant melanoma. **Int J Hyperthermia** 7, 559-566.
- Powers BE (2001) The Pathology of Neoplasia. In: Withrow SJ, MacEwen EG, editors. Small Animal Clinical Oncology. Philadelphia: W.B. Saunders, pp: 4-17.
- Proulx DR, Ruslander DM, Dodge RK, Hauck ML, Williams LE, Horn B, Price GS, Thrall DE (2003) A retrospective analysis of 140 dogs with oral melanoma treated with external beam radiation. **Vet Radiol Ultrasound** 44, 352-359.
- Rassnick KM, Ruslander DM, Cotter SM, Al-Sarraf R, Bruyette DS, Gamblin RM, Meleo KA, Moore AS(2001) Use of carboplatin for treatment of dogs with malignant melanoma: 27 cases (1989-2000). **J Am Vet Med Assoc** 218, 1444-1448.
- Smith SH, Goldschmidt MH, McManus PM (2002) A comparative review of melanocytic neoplasms. **Vet Pathol** 39, 651-678.
- Spangler WL, Kass PH (2006) The histologic and epidemiologic bases for prognostic considerations in canine melanocytic neoplasia. **Vet Pathol** 43, 136-149.
- Spugnini EP, Dragonetti E, Vincenzi B, Onori N, Citro G, Baldi A (2006) Pulse-mediated chemotherapy enhances local control and survival in a spontaneous canine model of primary mucosal melanoma. **Melanoma Res** 16, 23-27.
- Sulaimon SS, Kitchell BE (2003) The basic biology of malignant melanoma: molecular mechanisms of disease progression and comparative aspects. **J Vet Intern Med** 17, 760-772.
- Theon AP, Madewell BR, Moore AS, Stephens C, Krag DN (1991) Localized thermo-cisplatin therapy: a pilot study in spontaneous canine and feline tumours. **Int J Hyperthermia** 7, 881-892.
- Theon AP, Rodriguez C, Madewell BR (1997) Analysis of prognostic factors and patterns of failure in dogs with malignant oral tumors treated with megavoltage irradiation. **J Am Vet Med Assoc** 210, 778-784.
- Todoroff RJ, Brodey RS (1979) Oral and pharyngeal neoplasia in the dog: a retrospective survey of 361 cases. **J Am Vet Med Assoc** 175, 567-571.
- Vail DM, MacEwen EG (2000) Spontaneously occurring tumors of companion animals as models for human cancer. **Cancer Invest** 18, 781-792.
- Wallace J, Matthiesen DT, Patnaik AK (1992) Hemimaxillectomy for the treatment of oral tumors in 69 dogs. **Vet Surg** 21, 337-341.
- Weber LW, Bowne WB, Wolchok JD, Srinivasan R, Qin J, Moroi Y, Clynes R, Song P, Lewis JJ, Houghton AN (1998) Tumor immunity and autoimmunity induced by immunization with homologous DNA. **J Clin Invest** 102, 1258-1264.
- Wells S, Bennett A, Walsh P, Owens S, Peauroi J (2006) Clinical usefulness of intradermal fluorescein and patent blue violet dyes for sentinel lymph node identification in dogs. **Vet Comp Oncol** 4, 114-122.
- Williams LE, Packer RA (2003) Association between lymph node size and metastasis in dogs with oral malignant melanoma: 100 cases (1987-2001). **J Am Vet Med Assoc** 222, 1234-1236.
- Wolchok JD, Yuan J, Houghton AN, Gallardo HF, Rasalan TS, Wang J, Zhang Y, Ranganathan R, Chapman PB, Krown SE, Livingston PO, Heywood M, Riviere I, Panageas KS, Terzulli SL, Perales MA (2007) Safety and immunogenicity of tyrosinase DNA vaccines in patients with melanoma. **Mol Ther** 15, 2044-50.
- Yudd AP, Kempf JS, Goydos JS, Stahl TJ, Feinstein RS (1999) Use of sentinel node lymphoscintigraphy in malignant melanoma. **Radiographics** 19, 343-353.

