Clinical Cancer Research

Cancer Therapy: Preclinical

CSPG4-Specific Immunity and Survival Prolongation in Dogs with Oral Malignant Melanoma Immunized with Human CSPG4 DNA

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Abstract

Purpose: Due to the many similarities with its human counterpart, canine malignant melanoma (cMM) is a valuable model in which to assess the efficacy of novel therapeutic strategies. The model is herein used to evaluate the immunogenicity, safety, and therapeutic efficacy of a human chondroitin sulfate proteoglycan-4 (hCSPG4) DNA-based vaccine. The fact that homology between hCSPG4 and cCSPG4 amino-acidic sequences stands at more than 80% provides the rationale for using an hCSPG4 DNA vaccine in the cMM model.

Experimental Design: Dogs with stage II–III surgically resected CSPG4-positive oral MM were subjected to monthly intramuscular plasmid administration, which was followed immediately by electroporation (electrovaccination) for at least 6, and up to 20, months. The immunogenicity, safety, and therapeutic efficacy of the vaccine have been evaluated.

Results: hCSPG4 electrovaccination caused no clinically relevant local or systemic side effects and resulted in significantly longer overall and disease-free survival times in 14 vaccinated dogs as compared with 13 nonvaccinated controls. All vaccinated dogs developed antibodies against both hCSPG4 and cCSPG4. Seven vaccinated dogs were also tested for a cCSPG4-specific T-cell response and only two gave a detectable interferon (IFN)γ response.

Conclusion: Xenogeneic electrovaccination against CSPG4 is able to overcome host unresponsiveness to the "self" antigen and seems to be effective in treating cMM, laying the foundation for its translation to a human clinical setting. *Clin Cancer Res; 20(14); 3753–62. ©2014 AACR.*

Introduction

Melanoma is the sixth most common cancer worldwide and has seen the highest rise in incidence in recent decades (1). In its early stages, melanoma is highly curable with surgery, but approximately one third of all patients with melanoma experience disease recurrence and metastasis; once metastasis has occurred, it is often fatal (2). Patients whose tumors contain a *BRAF* gene mutation may benefit from treatment with mitogen-activated protein kinase pathway inhibitors; however, the development of resistance through multiple distinct mechanisms is a major problem (3). An alternative therapeutic strategy is found in immunotherapeutic approaches using cytokines, such as interferon (IFN)α and IL2 (4, 5), and the administration of antagonist monoclonal antibodies (mAb) that target CTLA-4 (6) and/or PD-1 and PD-L1 (7).

Inherent immunogenicity and the potential role played by immunologic events in melanoma natural history, have stimulated interest in the development and application of cancer vaccines (8). Despite numerous successful studies in murine models, the therapeutic efficacy of cancer vaccines has so far been disappointing (9). This is a reflection of the difficulty met in translating the results from mice to men. To overcome these limitations, the National Cancer Institute's Center for Cancer Research launched the Comparative Oncology Program, in 2003, to foster the use of naturally occurring cancer in pet animals as models of human cancer

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Translational Relevance

The immunogenicity, safety, and therapeutic efficacy of a DNA-based vaccine against chondroitin sulfate proteoglycan-4 (CSPG4) have been assessed in client-owned dogs with stage II–III surgically resected CSPG4-positive oral malignant melanoma (MM). All vaccinated dogs developed an immune response against CSPG4; besides, dogs in this group also showed extended overall and disease-free survival, thus highlighting this CSPG4 vaccination as an alternative option for the management of canine MM (cMM). CSPG4 high expression in a high percentage of human MM, its restricted distribution in normal tissues, and the recognized value of the cMM model in predicting tumor behavior and response to immunotherapy in humans should be instrumental in speeding up the translation of this new management modality to human patients with melanoma, hopefully contributing to improving their survival.

(10). A European initiative with a similar purpose—the LUPA project—has recently been launched (11).

Canine malignant melanoma (cMM) shares many characteristics with its human counterpart, including histologic phenotype, tumor genetics, and clinical biologic behavior as well as the development of recurrent or resistant disease and far-reaching metastasis (12). cMM, thus, represents an attractive translational model for the assessment of the efficacy of novel immunotherapies for the treatment of human MM (13).

A suitable immunotherapeutic target should have a strong oncogenic role, be expressed in malignant cells in a high percentage of patients, and show limited distribution in normal tissues (14, 15). Chondroitin sulfate proteoglycan-4 (CSPG4), an early cell surface progression marker involved in tumor cell proliferation, migration, and invasion meets these criteria (16). The induction of CSPG4-specific antibodies in patients with melanoma that have been immunized with CSPG4 mimics has been associated with significantly longer survival and metastases regression, highlighting the potential clinical relevance of this tumor antigen as a target for antibody-based immunotherapy (17, 18). Interestingly, CSPG4 is highly conserved in its structural and functional properties through phylogenetic evolution. In particular, human CSPG4 (hCSPG4) displays 82% homology and 88% similarity to its canine counterpart in its amino-acidic sequence. Moreover, the frequency of CSPG4 expression in canine and human melanoma lesions is quite similar, being about 60% (19) and 80% (20), respectively.

DNA vaccination has been extensively explored in the field of melanoma immunotherapy, and a number of DNA vaccination clinical trials have been performed in human patients with melanoma; trial results have all been disappointing, probably because of the lack of immunogenicity in DNA vaccines, even when administered with *Salmonella typhimurium* (21–24). Besides, the United States Department of Agriculture (USDA) licensed in 2010 a DNA vaccine (ONCEPT, Merial) in the veterinary field. This represents the first approved anticancer vaccine and is meant for the treatment of cMM. However, its therapeutic efficacy has been recently questioned (25).

The introduction of electroporation to DNA vaccine delivery (electrovaccination) has strongly increased immunogenicity and therapeutic efficacy in both mice and humans (26, 27). Electrovaccination combines the advantages of DNA vaccination and electroporation. Specifically, the former is easy to handle, applicable to a broad population, safe, and induces both cellular and humoral immune responses, whereas the latter enhances the expression of the protein encoded by the immunizing DNA and prolongs the duration of the immune response (28, 29).

These findings and the translational power of veterinary clinical trials have prompted us to test the safety and efficacy of intramuscular electrovaccination of a plasmid encoding for CSPG4 in client-owned dogs with surgically resected stage II–III CSPG4-positive, natural occurring oral MM. Because CSPG4 is a self-antigen with poor, or a lack of, immunogenicity in autologous hosts, we immunized dogs with hCSPG4.

Materials and Methods

Dog enrollment

Dogs were treated according to the European guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC). The Ethical Committee of the University Veterinary Teaching Hospital (Grugliasco, Italy) approved the study; written consent for entry into the study was obtained from dog owners.

Pretreatment work-up included physical examination, blood count, serum biochemistry, and urinalysis. Fine needle aspiration and/or biopsy were used for preoperative tumor diagnosis. Cytology was the initial preoperative procedure adopted to clinically stage the palpable regional lymph nodes (LN), even in case of a not apparent clinicopathologic enlargement; in fact, size has not been considered sufficiently predictive (30). A more objective staging was achieved via the surgical removal of all palpable regional LN at the time of primary tumor resection and their full histologic evaluation. Full tumor staging also included a skull and three-view chest radiography and abdominal ultrasound examination; alternatively, a total body CT scan was performed.

Dogs without concurrent life-threatening diseases and with histologically confirmed oral stage II (2–4-cm diameter, negative LN) and III (>4-cm diameter and negative LN or any tumor size with ipsilateral-positive LN; ref. 31) surgically resected MM with a minimum of 6 months follow-up on June 30, 2013, were included. Primary tumor *en bloc* resection (maxillectomy, mandibulectomy, lip/cheek excision, etc.), with the inclusion—if feasible—of at least 2 cm of macroscopically normal tissue around the tumor, and regional lymphadenectomy were performed. For excision margin evaluation, the cut surface was stained

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with a specific dye (TMD Tissue Marking Dye, Triangle Biomedical Sciences) just after surgery; the sample was then fixed in 10% formalin. The same pathologist evaluated all samples. Those margins with tumor cells reaching the dye were considered as incomplete, whereas those with no evidence of tumor cells within at least 1 mm from the cut surface were considered as "clean" margins. Samples were also immunohistochemically tested for Ki67 expression (polyclonal Ki67 antibody—A-047; DAKO), mitotic index, and nuclear atypia (25, 32, 33). Immunohistochemical analyses of CSPG4 expression on MM samples were performed as previously described (19). Briefly, a total score ranging from 0 to 8 was assigned to each MM sample by adding the value that represented the proportion of CSPG4 positively stained tumor cells (score from 0 to 5) and the average staining intensity of CSPG4-positive tumor cells (score from 0 to 3).

In vivo electroporation

Only dogs with an MM characterized by a CSPG4 score $\geq$3/8 (19) were considered as suitable vaccination candidates. Dogs were vaccinated with a pcDNA3.1 plasmid coding for hCSPG4 generated as previously described (34). Vaccination started 3 to 4 weeks after surgery and was repeated after 2 weeks and then monthly. For vaccination, dogs were anesthetized with fentanyl IV 2 μg/kg (FentanestR; Pfizer) and propofol IV 4 to 7 mg/kg (Propofol Kabi, Fresenius Kabi), and then intubated; anesthesia was maintained with isoflurane (IsofluraneVet, Merial) and 100% oxygen. The hCSPG4 plasmid (500 μg in 200 μL of 0.03% NaCl) was injected into the muscles of the caudal thigh. Two minutes after plasmid injection, nine electric pulses (1 high voltage, amplitude 450 V, length 50 μs, frequency 3 HZ; 1 second pause; eight low-voltage amplitude 110 V, length 20 ms, pause 300 ms) were applied to the injection site using the CLINIPORATOR (Igea). Dogs were monitored for acute, late local or systemic side effects.

At each vaccination, clinical examinations, blood-work, three-view chest radiographs, sera, and peripheral blood mononuclear cells (PBMC) were obtained. Sera were aliquoted and cryopreserved at −80°C until used. PBMC were isolated using density gradient centrifugation columns (Accuspin; Sigma).

Cell lines

B16 cells were stably transfected with either hCSPG4 or cCSPG4 (pcDNA3.1 containing the cDNA coding for cCSPG4 XM_544783.2; GeneScript) plasmids using Lipofectamine (Invitrogen) according to the manufacturer's instructions; these cells are referred to as hCSPG4-B16 and cCSPG4-B16, respectively, and were cultured in the RPMI-1640 medium (Life Technologies), supplemented with 10% heat-inactivated FBS (Life Technologies) and G418 (0.5 mg/mL; Sigma). hCSPG4-positive SK-MEL-28 melanoma cells were purchased from the American Type Culture Collection and authenticated by short tandem repeat profiling. SK-MEL-28 were cultured in DMEM (Life Technologies) supplemented with 10% FBS. Cell lines were routinely checked for contamination by Mycoplasma using the Mycoalert (Lonza) Detection Kit and consistently found to be negative.

CSPG4-specific antibody detection in vaccinated dog sera

Sera collected from dogs at each vaccination were analyzed by ELISA for their ability to selectively bind CSPG4 isolated from the lysates of hCSPG4-B16 and cCSPG4-B16. Cell lysates were obtained by suspending 1×10^7 cells in 1 mL of lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) containing 1% Triton X-100, and an EDTA-free protease inhibitor mix (Roche). Suspensions were extensively vortexed and incubated on ice for 15 minutes and centrifuged for 5 minutes at 13,000 rpm at 4°C. Supernatants were collected and stored at −80°C until used. Protein concentration was determined using an acid protein assay (Pierce Biotechnology).

Of note, 96-well microtiter plates were coated overnight, at 4°C, with a pool of VF1-TP34, VF4-TP108, VF20-T87.41 mAb (4 μg/mL of a 50 mmol/L $NaHCO_3$ buffer, pH 9.6; ref. 35). Plates were blocked in 5% milk in TBST (1X TBS, 0,5% Tween 20) and incubated for 2 hours at room temperature with 1 mg/mL cell lysate from hCSPG4-B16 or cCSPG4-B16 cells diluted in TBST/1% milk powder. Plates were incubated at 4°C overnight at optimal serum dilution (1:50) in TBST/1% milk powder. After washing, bound antibodies were detected using horseradish peroxidase–conjugated sheep anti-dog IgG (AAI32P; Abd Serotech). Color was developed via the addition of 3,3′,5,5′-tetramethylbenzidine substrate and absorbance was measured at 450 nm on an ELISA microplate reader (Bio-Rad).

Immunofluorescence staining of hCSPG4-B16 and cCSPG4-B16 cells with sera was performed by seeding cells on glass coverslips, fixing them with 4% formalin (Sigma) and blocking with PBS supplemented with 1% bovine serum albumin. After 1 hour incubation at room temperature, with an optimal dilution of dog sera (1:10), cells were rinsed twice in PBS and bound antibodies were revealed with FITC rabbit anti-dog IgG antibody (F7884; Sigma). After three rinses with PBS, coverslips were air-dried, mounted with fluoromount mounting medium (Sigma), and visualized using a confocal laser scanning microscopy system equipped with an argon-ion laser (LSM510; Zeiss). Photographs were taken using a CCD-300-RC camera. Images were processed using Adobe Photoshop and Microsoft PowerPoint softwares.

Statistical analysis

The Shapiro–Wilk normality test was used to analyze age and body weight. Normally distributed data are reported as means ± SD. Nonnormally distributed data are reported as median and range. Other variables are expressed as percentages. All other quantitative evaluations were carried out using the Student *t* test. The Kaplan–Meier method was used to estimate disease-free and survival times. Cross tabulations were performed for the evaluation of excision margin status versus disease outcome in the three groups using a

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Table 1. Clinical stage of oral MM from dogs included in the study

Group	Stage II	Stage III
Overall population ($n = 33$)	10 (30.3)[a]	23 (69.7)[a]
Group I[b] ($n = 14$)	5 (35.7)	9 (64.3)
Group II[c] ($n = 13$)	3 (23.1)	10 (76.9)
Group III[d] ($n = 6$)	2 (33.3)	4 (66.7)
Group II+III ($n = 19$)	5 (26.3)	14 (73.7)

[a]Percentage in brackets.
[b]CSPG4-positive MM; vaccinated dogs.
[c]CSPG4-positive MM; nonvaccinated dogs.
[d]CSPG4-negative MM; nonvaccinated dogs.

Table 3. The immunohistochemical score for CSPG4 expression in oral MM from dogs included in the study

CSPG4 score	CSPG4$^+$ population ($n = 27$)	Group I ($n = 14$)	Group II ($n = 13$)
3/8	3 (11.1)[a]	1 (7.1)[a]	2 (15.4)[a]
4/8	6 (22.2)	3 (21.4)	3 (23.1)
5/8	5 (18.5)	3 (21.4)	2 (15.4)
6/8	2 (7.4)	1 (7.1)	1 (7.7)
7/8	10 (37.0)	5 (35.7)	5 (38.5)
8/8	1 (3.7)	1 (7.1)	0 (0.0)

[a]Percentage in brackets.

Fisher exact test. A Wilcoxon rank-sum test was used to compare values of the cCSPG4 expression between groups in disease outcome. Differences in survival distribution were analyzed using the log-rank test. Statistical significance was set at $P < 0.05$. All analyses were conducted in R (36).

Results

Canine population characteristics

Twenty males (60.6%) and 13 females (39.4%) were included (Supplementary Table S1). Twenty-seven had CSPG4-positive and 6 CSPG4-negative MM. Fourteen CSPG4-positive dogs were vaccinated (group I), whereas the remaining CSPG4-positive (group II, 13 dogs) and the CSPG4-negative (group III, 6 dogs) candidates did not undergo vaccination or any other post-surgery treatment. Age and body weight at presentation are reported in Supplementary Table S2. Age did not significantly differ between groups ($P > 0.05$). Postoperative clinical stage data are summarized in Table 1, which shows that stages were similarly distributed in the groups. Histology revealed incomplete excision margins in 3 of 14 group I dogs (21.4%), in 4 of 13 (30.8%) from group II, and 2 of 6 (33.3%) from group III. No significant correlation between the excision margin status and the disease outcome was identified in any of the groups.

The Ki67 index was available for 27 of 33 analyzed cMM (Table 2). It was more than 19.5% in all dogs but one (13.8%) from group I, in all but 2 (18.2 and 19.0%) from group II and in 3 from group III (4, 16.7 and 14.4%). The mitotic index mean in groups I, II and III was 15/10, 28/10, and 17/10 hpf, respectively (Table 2). A nuclear atypia score of more than 30% was recorded in 64.28%, 61.54%, and 33.33% of cMM from groups I, II, and III, respectively (Table 2). The percentages of Ki67 positivity, mitotic index, and nuclear atypia scores showed no statistically significant differences in the three groups and were not correlated with survival. The range of CSPG4 expression in groups I and II (Table 3) was between 3 of 8 and 8 of 8.

hCSPG4 electrovaccination was safe and well tolerated

No local or systemic side effects, except a transient erythema at the injection site, were observed after electrovaccination. Considerable muscle contraction occurred during electric pulse delivery, which subsided immediately; there was an occasional increase in respiratory and heart rates, which subsided after 1 minute. Recovery from anesthesia was uneventful in all dogs. Hematologic and biochemical

Table 2. Histologic and immunohistochemical analysis of oral MM from dogs included in the study

	Threshold	Overall population	Group I	Group II	Group III	Group II+ III
Ki67[a]	<19.5	18.5% (27/33)[b]	9.1% (11/14)[b]	18.2% (11/13)[b]	40.0% (5/6)[b]	25.0% (16/19)[b]
	≥19.5	81.5% (27/33)	90.9% (11/14)	81.8% (11/13)	60.0% (5/6)	75.0% (16/19)
Mitotic Index (MI)[a]	<4/10 hpf	3 (9.1%)	3 (21.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	≥4/10 hpf	30 (90.9%)	11 (78.6%)	13 (100.0%)	6 (100.0%)	19 (100.0%)
Nuclear Atypia	<30.0%	14 (42.4%)	5 (35.7%)	5 (38.5%)	4 (66.7%)	9 (47.4%)
(PLEOM)[a]	≥30.0%	19 (57.6%)	9 (64.3%)	8 (61.5%)	2 (33.3%)	10 (52.6%)

[a]Prognostic factors for cMM as proposed by Bergin et al. (32), Smedley et al. (33), Ottnod et al. (25).
[b]Number of patients for which the data were available.

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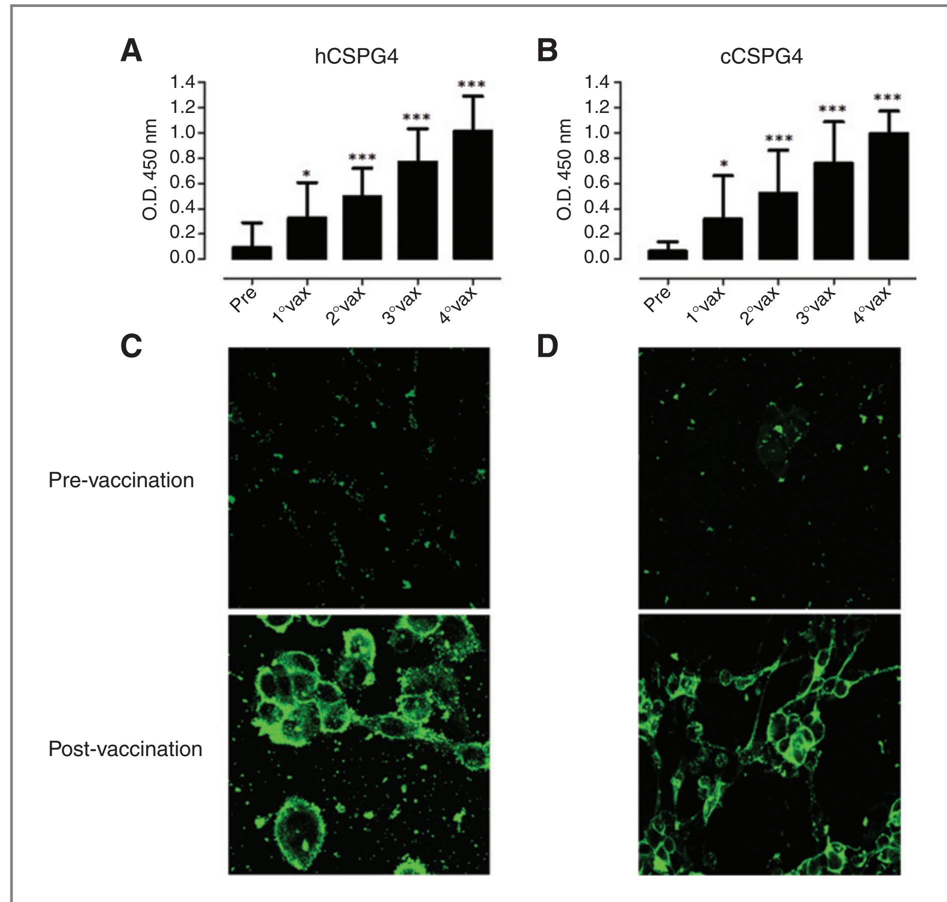

Figure 1. CSPG4-specific antibody response induced by hCSPG4 DNA vaccination. Sera from dogs before and after vaccinations, up to the fourth, were analyzed (dilution 1:50) for the presence of specific anti-human (A) or anti-canine (B) CSPG4 antibodies by ELISA. Results, mean OD $\pm$ SD values of all vaccinated dogs. Student *t* test; *, 0.01; ***, <0.0001. Representative immunofluorescent staining of hCSPG4-B16 (C) and cCSPG4-B16 (D) cells incubated with pre-vaccination serum (top) or post-vaccination serum (bottom) from canine patients; magnification, $\times$63.

parameters did not change over the entire observation period. Moreover, the clinical status, appetite, water intake, and general behavior of all animals remained unaltered until disease progression occurred.

hCSPG4 electrovaccination induced both hCSPG4- and cCSPG4-specific antibodies in all vaccinated dogs

Vaccination-induced immune response was evaluated in PBMC and sera. PBMC collected before vaccination and after the third, were stimulated *ex vivo* with the six peptide-pools from cCSPG4 in ELISPOT for 24 hours, but no significant increases in IFNγ-secreting cells were detected after vaccination (not shown). In the 7 dogs (#2, #3, #4, #7, #8, #10, and #11), from whom it was possible to obtain fresh dendritic cells (DC), PBMC were restimulated with mature DC (mDC) pulsed with cCSPG4-derived peptide pools. The ELISPOT assay only showed a detectable IFNγ response in 2 dogs (#2 and #7) after vaccination, but this response was significantly different from that observed when PBMCs were stimulated with unpulsed mDC or mDC pulsed with an unrelated peptide (Supplementary Fig. S1). These data indicate that vaccination induced a low frequency, if any, of circulating T cells that were cCSPG4 reactive. In contrast, an hCSPG4- and a cCSPG4-specific antibody response was found in the sera of all vaccinated dogs. Sera were tested by ELISA for their ability to bind lysates from hCSPG4-B16 (Fig. 1A) and cCSPG4-B16 (Fig. 1B) cells. Eight (#1, #3, #4, #5, #6, #10, #11, and #13) of 14 dogs showed a measurable increase in hCSPG4-specific antibody titer even after the first vaccination; all but two (#12 and #14) displayed a response after the second vaccination (Supplementary Fig. S2, black lines). The cCSPG4-specific response was detected in 7 (#1, #3, #4, #5, #6, #8, and #11) dogs after the first, and in all but 3 (#7, #12, and #14), after the second vaccination (Supplementary Fig. S2, gray lines). A strong hCSPG4- and cCSPG4-specific antibody response was detected in all vaccinated dogs after the third vaccination. To confirm the ability of sera from vaccinated dogs to recognize native CSPG4 expressed on cells, sera collected after the fourth vaccination were tested, by means of immunofluorescence, for their ability to stain hCSPG4-B16 and cCSPG4-B16 cells. Results were consistent with those found by ELISA; all sera were able to stain the cell membrane of both hCSPG4-B16 (Fig. 1C) and cCSPG4-B16 (Fig. 1D) cells and induce a certain degree of CSPG4 internalization.

An MTT proliferation assay was used to verify whether sera from vaccinated dogs were able to inhibit the proliferation of the CSPG4-positive SK-MEL-28 human melanoma cells. Sera collected after the third and fourth vaccinations were used for this assay, as flow cytometry analysis showed that they contained antibodies able to specifically bind SK-MEL-28 cells (Supplementary

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Fig. S3A). Post-vaccination sera from all vaccinated dogs, except #1 and #14, significantly inhibited SK-MEL-28 cell proliferation as compared with prevaccination sera (Supplementary Fig. S3B). Nevertheless, no statistical correlation was found between the clinical outcome and the anti-CSPG4 serum antibody titer or the ability of the serum to inhibit SK-MEL-28 proliferation.

The lack of a cMM cell line that highly expresses cCSPG4 precludes the assessment of whether sera from vaccinated dogs inhibited cCSPG4-positive melanoma cell proliferation. We have recently generated a cMM cell line starting from the bioptic material obtained from a metastatic LN (OLGA cells). Nevertheless, only about 60% of OLGA cells expressed low amounts of cCSPG4 (not shown). Confocal microscopy on OLGA cells allowed cCSPG4 decorated by hCSPG4-specific mAb (VF1-TP34, VF4-TP108, and VF20-T87.41) and, to a lesser extent, by sera from vaccinated dogs to be detected (Supplementary Fig. S4A and S4B). However, inhibition of OLGA cell proliferation after incubation with anti-CSPG4 mAb was seen only when OLGA cells were pretreated with a DNA methyltransferase inhibitor. This resulted in a transient increase in CSPG4 expression (data not shown).

Prolongation of overall and disease-free survival in hCSPG4 electrovaccinated dogs

The 6- and 12-month survival rates of CSPG4-positive vaccinated dogs (group I) are 100% and 64.3%, respectively. The CSPG4 positivity score was not statistically correlated with survival. Seven of 14 dogs are still alive at the time of writing (273–781 days). Of the remaining 7 dogs, 4 were euthanatized because of MM, 2 because of second tumors (prostatic carcinoma and perianal adenocarcinoma with sublumbar LN metastases), and 1, even though still disease-free, because of unrelated reasons (degenerative orthopedic problems). The 6- and 12-month survival rates of CSPG4-positive nonvaccinated dogs (group II) are 69.2% and 15.3%, respectively. The survival range is 152 to 621 days. Eleven of 13 dogs died because of MM, 1 was euthanatized, even though still disease-free, because of larynx paralysis and 1 was lost to follow-up at 294 days when still disease-free. The 6- and 12-month survival rates of CSPG4-negative nonvaccinated dogs (group III) are 83.3% and 33.3%, respectively. The survival range is 180 to 1,173 days. At the time of writing only 1 of 6 dogs is still alive at 1,173 days, whereas 4 died because of MM and 1 because of multiple organ failure due to advanced age.

Kaplan–Meier curves for median survival time (MST; Fig. 2A and B) and disease-free interval (DFI; Fig. 2C and D) were analyzed. Group I exhibited significantly longer MST than group II ($P < 0.0001$; Fig. 2A), whereas it was not significantly different from group III ($P = 0.13$; Fig. 2A). However, group I MST was significantly longer when compared with the overall nonvaccinated population (group II+III; $P = 0.002$; Fig. 2B). The group I DFI value was significantly longer than group II ($P = 0.01$) but not than group III ($P = 0.6$; Fig. 2C) and group II+III ($P = 0.054$; Fig. 2D). Group II MST and DFI were not significantly different from those in group III ($P = 0.14$ and $P = 0.2$, respectively). A summary of DFI, MST, local recurrences, and lung metastasis is shown in Table 4.

No statistically significant correlation was found between the disease outcome and the excision margin status, the percentages of Ki67 positivity, the mitotic index, the nuclear atypia scores, and the CSPG4 expression.

Discussion

Oral MM is the most common oral malignancy in dogs and is generally locally aggressive and highly metastatic. Regional lymphatic metastases are present in 70% to 80% of dogs at presentation, even in case of clinically normal LN (37). Primary tumor *en bloc* resection and regional lymphadenectomy, and/or radiotherapy are the preferred treatment methods and result in locoregional control of MM in up to 75% of dogs. Disappointingly, the 1-year survival rate is less than 30%, even after adjuvant chemotherapy (38); besides, the addition of radiotherapy to surgery and adjuvant chemotherapy did not result in a significant increase of the disease-free period (39). The USDA has licensed a xenogeneic DNA vaccine against tyrosinase, ONCEPT (Merial), but its efficacy has been recently questioned (25). In fact, although the study by Grosenbaugh and colleagues (40) supported the ability of this vaccine to extend MST in dogs with locally controlled stage II–III oral MM, a further retrospective study by Ottnod and colleagues (25) failed to validate the previous results. This discrepancy could be partially related to the features of the antigen targeted by ONCEPT, tyrosinase, which is expressed by a limited percentage of oral cMM (41) and which does not have a well-recognized oncogenic role in melanoma progression.

The present study has evaluated the immunotargeting of CSPG4, a useful diagnostic biomarker of cMM (19) and an attractive oncoantigen (15), in dogs with surgically resected stage II–III CSPG4-positive oral MM. The age and male prevalence of the canine population presented here were in agreement with the literature (42, 43). Of the 27 CSPG4-positive oral MM-bearing dogs, 14 (group I) were vaccinated, whereas the remaining (group II) and the 6 affected by CSPG4-negative oral MM (group III) were not. The rate of primary tumor local control (noninfiltrated excision margins) at the first surgery was adequately high (72.7% in the whole canine population, 78.6% in group I, 69.2% in group II, and 66.6% in group III). All cMM were immunohistochemically tested for Ki67, mitotic index, and nuclear atypia (32, 33), which have been proposed as markers of poor prognosis (25). These markers were highly expressed in almost all samples, thus indicating the aggressive clinical behavior of most cMM included in the study. However, the expression of these markers was not associated with that of CSPG4 and do not correlate with outcome.

Our results demonstrate the safety of the electrovaccination with a plasmid that codes for hCSPG4 and its ability to induce a specific immune response against both hCSPG4 and the canine ortholog. This immune response resulted in

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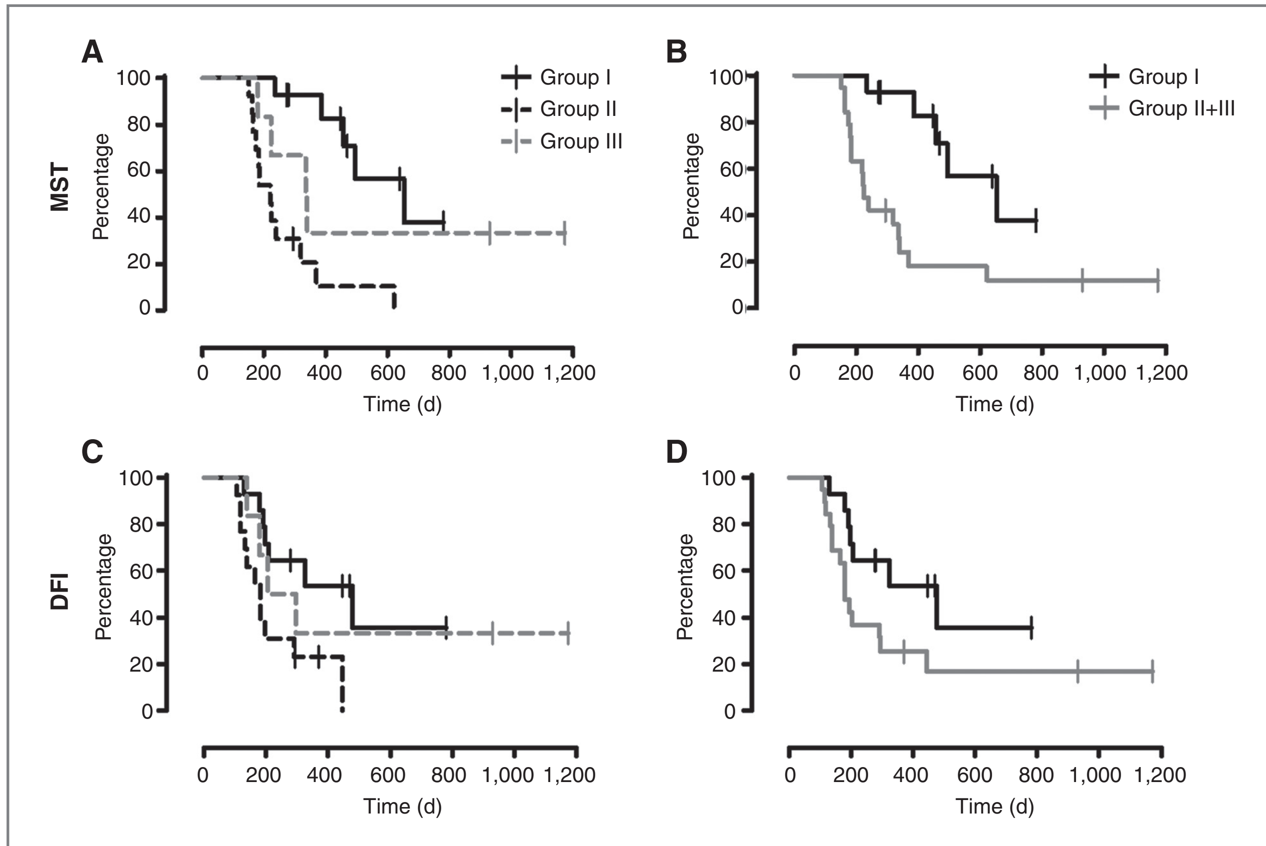

Figure 2. Kaplan–Meier curve comparing MST and DFI in different groups. A, MST (in days) in CSPG4-positive MM, vaccinated dogs (group I, black line), in CSPG4-positive MM, nonvaccinated dogs (group II, black dotted line; log-rank test $P < 0.0001$) and in CSPG4-negative MM, nonvaccinated dogs (group III, gray dotted line; log-rank test $P = 0.13$). B, MST (in days) in CSPG4-positive MM, vaccinated dogs (group I, black line) and in the entire population of nonvaccinated dogs (groups II+III, gray line; log-rank test $P = 0.002$). C, DFI (in days) in CSPG4-positive MM, vaccinated dogs (group I, black line), CSPG4-positive MM, nonvaccinated dogs (groups II, black dotted line; log-rank test $P = 0.01$) and in CSPG4-negative MM, nonvaccinated dogs (group III, gray dotted line; log-rank test $P = 0.6$). D, DFI (in days) in CSPG4-positive MM, vaccinated dogs (group I, black line) and in the entire population of nonvaccinated dogs (groups II+III, grey line; log-rank test $P = 0.054$).

a longer survival over dogs, either with CSPG4-positive or -negative MM, treated with surgery alone. In particular, MST and DFI in vaccinated dogs (group I) were significantly longer than in nonvaccinated CSPG4-positive dogs (group II). The MST of group I was significantly longer even when compared with the entire nonvaccinated control population (group II+III). Together with other promising cancer vaccines recently developed against canine lymphoma, this study supports the importance of active immunotherapy in veterinary oncology (44, 45).

The efficacy of this xenogeneic DNA electrovaccination relies on its ability to induce an antibody response not only against hCSPG4 but, more importantly, against autochthonous cCSPG4. Antibody production against cCSPG4 was

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Table 4. DFI, MST, and the percentage of local recurrence (LR) and lung metastasis (LM) for each group, calculated up to June 30, 2013

Group	DFI (d)	MST (d)	LR	LM
Overall population ($n = 33$)	290 (191-∞)[a]	370 (320-∞)[a]	27.3%	56.7% (17/30)[b]
Group I ($n = 14$)	477 (207-∞)	653 (458-∞)	21.4%	35.7% (5/14)
Group II ($n = 13$)	180 (131-∞)	220 (174-∞)	38.5%	90.0% (9/10)
Group III ($n = 6$)	250 (178-∞)	338 (223-∞)	16.7%	50.0% (3/6)
Group II+III ($n = 19$)	180 (165-∞)	224 (185-∞)	31.6%	75.0% (12/16)

[a]Lower–upper control limits (LCL95%–UCL95%).
[b]Number of affected patients over those for which the data were available.

not synchronous in all dogs, but after the fourth vaccination it was detectable in all patients. A possible mechanism by which antibodies can interfere with the disease is their ability to inhibit CSPG4-dependent proliferation of residual melanoma cells in MM-bearing dogs, which no longer display clinically evident disease. The capacity of vaccination-induced antibodies to inhibit hCSPG4-positive cell proliferation *in vitro* has been displayed in this work. Anti-cCSPG4 antibodies might carry out a similar mechanism *in vivo* because sera from vaccinated dogs can bind cCSPG4 on cMM-derived cells. The availability of a cell line that highly express cCSPG4 would be instrumental in demonstrating that vaccination-induced antibodies interfere with cCSPG4 signaling and, thus, inhibit cMM cell proliferation.

The results here presented overlap those achieved in humans with advanced melanoma that had been immunized with a different type of CSPG4 mimic (18), i.e., the mouse anti-idiotypic mAb MK2-23, which bears the internal image of the mouse mAb 763.74 against a defined CSPG4 epitope (46). A cause–effect relationship between the induction of a CSPG4-specific immune response and a statistically significant DFI and survival prolongation has not been proved in either patients with human or canine melanoma. Nevertheless, CSPG4-specific mAb have been shown to inhibit the proliferation and migration of melanoma cells *in vitro* (20). Furthermore, CSPG4-specific mAb administration to SCID mice grafted with CSPG4-positive human tumors has been found to significantly prolong their survival. These effects reflect the inhibition by CSPG4-specific mAb of signaling pathways associated with cell proliferation (20).

The direct activity of antibodies on melanoma cells may not be the only mechanism that causes the improved clinical outcome observed in our dogs. The ability of each patient to activate antibody indirect activities, such as antibody-dependent cellular and complement-mediated cytotoxicity, may be involved and deserve further investigation.

The downregulation of major histocompatibility complex (MHC) class I molecules is a common mechanism used by melanoma cells to avoid cytolytic attack from fully activated antigen-specific T cells (47), making the role of the T-cell cytotoxic response in fighting melanoma controversial and strictly dependent on the MHC class-I status of cMM in each patient. Here, no specific IFNγ production in response to stimulation with 15-mer peptides derived from cCSPG4 extracellular domains was found. However, it is possible that other T-cell parameters, such as vaccine-elicited T-cell phenotype, the ability to release other cytokines and the presence of cells negatively regulating the immune response, may have an impact. The immunosuppressive microenvironment at the tumor site and draining LN (48, 49) may have locally inhibited the vaccine-induced immune response, allowing local recurrences and/or metastasis to other satellite LN to occur in some dogs.

These data represent a starting point for further investigation into the potential of anti-CSPG4 vaccination against cMM. However, a study involving more dogs is needed to make firm conclusions. A limitation of this study is also that it may seem that more attention was paid to vaccinated as opposed to nonvaccinated dogs; 3 dogs from group I were subjected to a second surgical intervention in the follow-up period. CSPG4 adjuvant vaccination, thanks to its ability to delay expected widespread metastasis development and, therefore, to prolong survival, may have allowed the occurrence of recurrences and further regional lymphatic metastases in group I, despite the initial surgery had apparently locally controlled the tumor in most dogs. These recurrences and LN metastasis were still amenable to surgical resection. This was not the case for nonvaccinated dogs that succumbed to lung or widespread metastasis more rapidly, before they could develop local recurrences. It may also be speculated that the change in the standard progression of oral MM induced by vaccination also resulted in the exposure of long-surviving dogs to a greater risk of dying of a second tumor of a different histotype, as it was observed in 2 patients in group I. However, all these speculations require confirmation through the enrollment of more cases in all treatment groups. Further evaluation is also needed to confirm the lack of statistical correlation between clinical outcome and incomplete excision margin status and CSPG4 expression as this may be due to the low number of dogs included in the study. Finally, the development of recurrences and regional lymphatic metastases despite vaccination emphasizes the need to neutralize the tumor immunosuppressive microenvironment and enhance vaccine efficacy (50). Other adjuvant treatments, such as the administration of mAb against immune check points (7), and even radiotherapy, metronomic chemotherapy, and antiangiogenic therapy may allow CSPG4 vaccination to better control the disease both locally and systemically. The last item to which attention will be addressed in future case enrollment will be the timing of adjuvant vaccination: when to start, when to boost, and how many times.

In conclusion, our data provide the first evidence of the ability of anti-CSPG4 electrovaccination to prolong overall survival in client-owned dogs with oral MM. The extension of follow-up observation, the inclusion of more cases and a deeper evaluation of the induced immune response will help to better evaluate vaccination potential. Nevertheless, our data show CSPG4 vaccination as an alternative option for the management of cMM. Moreover, the strong translational value of the cMM model points to the role that CSPG4 can play as a good immunotherapy target for the management of the high percentage of human melanomas that express this molecule.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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