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Case Report

Treatment of Metastatic Equine Melanoma with a Plasmid DNA Vaccine Encoding *Streptococcus Pyogenes* EMM55 Protein

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ABSTRACT

A 19-year-old castrated male Arab/Quarter horse presented with an extensive history of cutaneous metastatic melanoma. Over a period of 8 months, a total of 8 doses of plasmid DNA vaccine expressing the *Streptococcus pyogenes emm55* gene (pAc/*emm55*) were administered intratumorally at 300 μg/dose via a needless injector. Upon completion of the vaccination protocol, the size of the injected lesions, on average, were reduced by 40.3% from the initial size measurements. Lesions that were not injected were reduced by 47.6%. The overall reduction in total tumor burden was 42.3%. Tumor regression was also associated with the augmentation of antimelanoma IgG antibody response, thus implying that an induction of an effective antimelanoma response would be of great advantage in the management of equine melanoma.

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1. Introduction

Equine melanoma accounts for approximately 4% of the overall number of neoplastic diseases in horses. Melanoma has been reported in horses of various coat colors, with gray and white being the most common. Approximately 80% of gray horses develop melanoma by the age of 15 years. While melanoma can arise anywhere on the body of the horse, most equine melanomas present in the perineal and perianal regions, the ventral surface of the tail, the margin or outer part (pinna) of the ear, and external genitalia. Up to 15% of all equine skin tumors are melanocytic. More than 90% of these tumors are benign at initial presentation and diagnosis and can remain so for a number of years. Approximately two-thirds of these tumors are thought to become malignant and are capable of widespread metastasis [1-3], with a preference for the connective tissue or serosal surfaces of the spleen, liver, and lung. Prognosis is determined by initial tumor staging, histopathology, and available treatment options. There are currently no reliable and consistent therapeutic options available for the treatment of equine melanoma [4,5]. Surgical excision is considered the best clinical option. However, excision of melanoma is rarely curative because of (A) difficulties in achieving a good surgical margin, (B) inability to access the tumors surgically, and (C) rapid recurrence of the disease near the surgical site due to the presence of abnormal melanoblasts [5]. In addition, other treatment options such as chemotherapy (cisplatin) and

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radiation therapy, which are commonly practiced in other species, are of minimal benefit to horses with melanoma. Cimetidine (Tagamet (Prestige Brands, Inc, Tarrytown, NY)), a histamine (H2) receptor antagonist commonly used for the treatment of gastroesophageal reflux disease, has been used in the palliative treatment of both human and equine melanoma [6]. Therefore, for malignant and metastatic equine melanomas, investigation of new therapeutic modalities that might improve survival is imperative. Immunotherapies, especially therapeutic vaccination therapies, offer an additional treatment modality for equine melanoma patients. These therapies are predicated on the immune system's ability to mount an antitumor response. Because most of the tumor antigens are self-antigens and therefore poor immunogens, overcoming tolerance has been a major challenge in cancer immunotherapy. Recently published observations have shown that both local and systemic antitumor responses can be generated in tumor-bearing horses with or without tumor regression. For instance, when plasmid DNA encoding interleukin-18 (IL-18) and IL-12 were injected intratumorally in equine patients with metastatic melanoma, significant tumor regression over the course of the treatment was observed. In this study, approximately 70% of the tumors exhibited peritumoral and/or intratumoral inflammatory infiltrates following treatment [7]. In another study, interferon-γ (IFN-γ) expression was elevated following treatment with an equine IL-12-encoding plasmid DNA. Thirty-six hours after treatment, the plasmid DNA had been eliminated, whereas IFN-γ expression remained elevated [8]. The immunogenicity of a xenogeneic plasmid DNA encoding human tyrosinase, a major tumor antigen in melanoma, has been demonstrated in canine melanoma [9] and is now available as a US Department of Agriculture-licensed product for the treatment of this disease. In a sentinel equine study, normal nontumor bearing horses were vaccinated with the same vaccine, that is, plasmid DNA encoding human tyrosinase. All horses in that study developed positive humoral and cell-mediated immune responses without exhibiting adverse reactions, suggesting its potential future use in horses with melanoma [3]. However, an earlier human phase I vaccination study with tyrosinase in stage II melanoma patients failed to elicit either tyrosinase-specific T-cell or antibody responses after combined intradermal and subcutaneous vaccinations, implying an inherent difficulty in using tumor-specific whole-antigen molecules in therapeutic vaccination therapies [10]. In the present report, a novel plasmid DNA vaccine, pAc/*emm55*, expressing the *emm55* gene derived from *Streptococcus pyogenes* bacteria was used intratumorally to determine its clinical efficacy in an equine metastatic melanoma patient.

The "priming" antigen, Emm55, is a serotyping protein [11] normally expressed on the surface of the bacterium *S. pyogenes* and is highly antigenic but not rheumatogenic [12]. The novel use of Emm55 as a priming antigen for the immune response was recorded in a previous study in which the DNA plasmid containing the *emm55* gene was transfected into canine lymphoma cells, which were subsequently used in a patient-specific immunotherapy for lymphoma [13]. In this study, it was observed that once the plasmid DNA had been introduced into the target cell, Emm55 was expressed on the surface of the transduced tumor cell within 48 hours. Given that the Emm55 antigen is highly antigenic, it was postulated that Emm55 could act as a priming antigen to initiate a strong and highly specific antitumor immune response in a way that was not previously possible. The authors of that study reported quite clearly that there was an induction of both strong humoral and cellular immune responses directed not only to the priming antigen (Emm55) but also to the tumor cells in these patients [13]. The present case study is the first report of the direct intratumor injection of plasmid DNA containing the *emm55* gene in any species as a cancer immunotherapeutic. The needleless injection system used for the direct injection of the DNA construct into the melanoma lesion was the Syrijet injector (Keystone Industries, NJ, USA). This system is customarily used to deliver anesthetic drugs for routine dental procedures and for this study was adapted to deliver the *emm55* gene directly into tumors.

2. Case Details

2.1. History

A 19-year-old castrated male Arab/Quarter horse presented with an extensive history of cutaneous melanoma that had metastasized to the prescapular lymph nodes. At 8 years of age, histopathology confirmed his presenting lesions to be melanoma. For the next 4 years, the patient's disease was stable. Between 12 and 16 years of age, this patient had multiple melanoma lesions and was treated with cimetidine from time to time and also given an injection of an undefined vaccine from Canada at age 14. There was also removal of tumor lesions from the left flank, right hip, and right neck. However, by the age of 16, melanoma recurred at one of the prior surgical sites, and new cutaneous lesions were observed on his tail and neck, within his mane, and at other sites including the perianal region. Some of these lesions progressed to open sores that secreted a dark fluid exudate which later was shown to contain malignant melanocytes. Prior to this current study, the patient had been treated surgically by removing both cutaneous lesions and lesions in the genital region. Results from blood work performed in June 2010 were normal except for a high level of lactate dehydrogenase (LDH), 569 U/L. As the severity of the disease progressed, the option for further surgery was declined, and other treatments were sought. An experimental whole-cell melanoma vaccine from a research center (Veterinary Oncology Services and Research Center, Philadelphia, PA, USA) was initiated as the therapy in September 2010, at the direction of the owner. Ten doses of this vaccine preparation were coadministered with an immunostimulant (EqStim; Neogen, Lexington, KY, USA). By March 2011, no reduction of tumor burden had been noted, and the prescapular lymph nodes were beginning to become enlarged. Blood analysis completed in May 2011 indicated a high LDH level of 606 U/L, implying that the disease was still progressing. By June 2011, the patient developed a fever, and the prescapular lymph nodes continued to enlarge. The exudate fluid removed from the lymph nodes revealed high numbers of melanocytes and was contaminated with

bacteria. The bacterial growth from these lymph nodes was sensitive to both doxycycline and enrofloxacin. The patient was treated with both of these antibiotics throughout August and September 2011. Despite recovering from the fever, the horse was lethargic, his muscle tone atrophied along his top line, and he continued to lose weight despite eating and drinking well. Herbal remedies and treatment with cimetidine (oral and topical) were also used with little or no clinical effect. In August 2011, the vaccine was discontinued as the horse's illness progressed still further. The melanoma continued to progress, and euthanasia was considered. Considering the failure of all previous treatments to control or halt the progression of the melanoma, as well as the severity and late stage of the disease, it was determined in December 2011 that the horse was a candidate for a new and novel form of experimental treatment that involved the direct injection of a DNA cancer vaccine.

2.2. Treatment with Plasmid DNA Vaccine Expressing the S. Pyogenes Emm55 Gene

Under strict veterinary supervision and control, the horse was treated intratumorally with a plasmid DNA vaccine. The plasmid vector pAc/*emm55* consisted of the pAc mammalian expression vector backbone and the 1.6-kb *emm55* gene insert. The *S. pyogenes* Emm55 protein is highly antigenic but nonrheumatogenic [12]. The expression of this bacterial protein on the surface of tumor cells overcomes the inherent self-tolerance to tumor antigens. The use of this approach had been previously optimized for the modification and processing of autologous tumor cells for use in naturally occurring canine lymphoma [13]. In those studies, all vaccinated canine lymphoma patients demonstrated the production of antitumor antibodies and cytotoxic T-cell responses which correlated with improved clinical outcomes such as survival and quality of life.

The treatment, which commenced in December 2011, involved the direct injection of plasmid DNA into cutaneous lesions. Three visible tumor masses were selected for treatment: 1 on the right side of the neck, 1 on the right side of the rump, and 1 on the tail. Two masses, 1 in the region of the mane and 1 on the tail, served as control lesions and did not receive the DNA cancer vaccine. The former 3 melanoma lesions were each injected with 100 μg of pAc/*emm55*, for a total of 300 μg of plasmid DNA in a volume of 200 μL of endotoxin and nuclease-free distilled water, using a needless injector system (Syrijet injector) at each vaccination time point. A total of 8 plasmid DNA vaccinations were administered to each of the 3 lesions. The vaccine doses were administered on days 1, 19, 55, 79, 110, 145, 187, and 255. Responses were studied until the 289th day. The lesions were measured prior to injections. The Syrijet injector is a precision instrument safely used for applying operative and surgical anesthetic for dental procedures. This was the first time this device was used for intratumor delivery of a DNA therapeutic vaccine.

2.3. Regression of melanoma tumor lesions

Visual examinations of the vaccinated sites were performed by the referring veterinary physicians to determine if any vaccine-induced inflammatory reaction(s) had occurred. From these observations, it was determined that there were no inflammatory reactions at any of the injections sites throughout the course of the treatment. The size of the tumor lesions (both injected and noninjected) were measured before and after each of the DNA cancer vaccine administrations. Assessment of tumor size was carried out following published guidelines for the evaluation of immune therapy activity in solid tumors [14]. Briefly, at the baseline and subsequent tumor assessments, all indexed lesions were measured, and the sum of the products of the 2 largest perpendicular diameters (SPDs) was calculated. The SPDs of the lesions were added together to provide the total tumor burden. Table 1 shows all individual measurements of indexed lesions (injected and noninjected). As shown in Table 1, there were fluctuations in the size of individual tumors, which may be due to the ingress and egress of immune cells but resulted in an

Table 1
Measurement of tumor lesions during the study period

Measurement	Injected Lesions (mm)			Noninjected Lesions (mm)	
	Neck	Rump	Tail	Mane	Tail
1 (pre-treatment)	40	160	160	31	112
2	30	144	90	ND	ND
3	30	120	157	ND	ND
4	26	140	83	ND	ND
5	30	250	140	30	87
6	30	168	75	ND	ND
7	23	223	105	26	45
8	19	123	112	30	50
9 (post-treatment)	20	105	90	25	50

ND, not done.
Vaccine doses were administered on days 1, 19, 55, 79, 110, 145, 187, and 255. Responses were studied until the 289th day. Lesions were measured prior to injections. Tumor size was assessed using published guidelines for the evaluation of immune therapy activity in solid tumors [14]. All indexed tumor masses were assessed using a tape measure and expressed in millimeters.

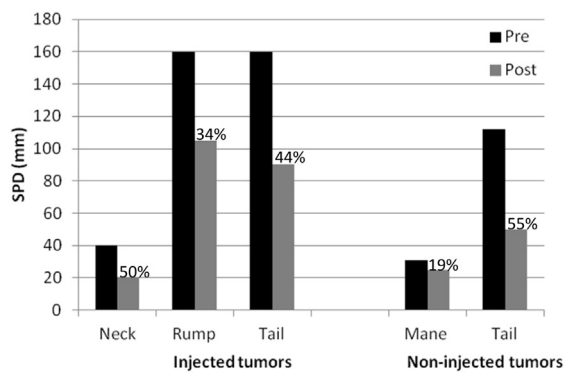

Fig. 1. Regression of tumor lesions is shown during the postvaccination regimen. Vaccine doses were administered on days 1, 19, 55, 79, 110, 145, 187, and 255. Responses were studied until the 289th day. Lesions were measured prior to injections. All indexed lesions were measured, and the percent of reduction of tumor size was calculated for each injected and noninjected lesions before treatment (black bars) and 2 weeks after the eighth vaccination (gray bars). The percent of reductions observed during the postvaccination regimen are indicated above the gray bars. Reductions ranged from 19% to 55% of the original tumor size.

Table 2
Regression of injected and noninjected tumor lesions

Tumor Lesions	Pre-treatment	Post-treatment	% Reduction
Injected	360 mm	215 mm	40.3
Noninjected	143 mm	75 mm	47.6

All indexed lesions were measured, and the sum of the products of the 2 largest perpendicular diameters (SPD) was calculated. The SPDs of the lesions were added to provide the tumor burden. Compared to pretreatment measurements, 2 weeks after the eighth injection, measurements exhibited significant reductions in the size of the tumor lesions. The SPD of injected lesions was reduced by 40.3% and the size of noninjected lesions was reduced by 47.6%.

overall reduction in tumor burden by the conclusion of the treatment. Figure 1 shows the pretreatment and post-measurement of individual indexed lesions along with observed percent of reductions in the mass above each bar. Table 2 summarizes the total reduction in injected and noninjected lesions at the conclusion of the treatment regimen. Based on these data, the SPD of all injected lesions was reduced by 40.3% and that of noninjected lesions was reduced by 47.6%, with an overall reduction in tumor burden of indexed lesions by 42.3%. When we combined all tumor measurements from both the 3 treated lesions and the 2 untreated lesions, pre- and postvaccinations, the reduction observed in tumor size reached statistical significance in a paired *t* test ($P = .0272$).

During the course of this study, it was determined that all of the tumor masses observed stabilized, regressed in size, and ceased leaking the dark melanocyte-containing exudate. The consistency of several of the melanoma lesions went from firm to soft. By the end of September 2012, the patient had gained weight, was alert, and was healthy enough to be ridden.

2.4. Augmentation of Antimelanoma IgG Antibodies Following Vaccinations

In order to ascertain whether the reduction in tumor burden observed correlated with the development of an antitumor immune response, antibody levels were measured using a standard enzyme-linked immunosorbent assay (ELISA). Briefly, prior to each vaccination, peripheral blood was collected in vacuum tubes (Vacutainer; Thermo Fisher Scientific, Marietta, GA) containing sodium heparin and was used for the isolation of plasma and peripheral blood mononuclear cells (PBMCs). Plasma was stored at −30°C. PBMCs were isolated using a single-step gradient (Ficol/Lite; Atlanta Biologicals, Atlanta, GA, USA) and stored in liquid nitrogen. Melanoma lysate was prepared from nonvaccinated, nonindexed lesions by using a mammalian lysis buffer (mammalian cell PE LB buffer; Fisher Scientific, Suwanee, GA, USA). Prior to using as the antigen source for the ELISA, we quantified and diluted the protein concentration of the tumor lysate to a standard concentration of 10 μg/mL for all ELISA assays. Briefly, 96-well ELISA plates were coated with autologous melanoma cell lysate (10 μg/mL) in a volume of 100 μL/well and allowed to bind overnight at room temperature (RT). Following the antigen binding step, the microwells were washed 3 times with a washing buffer (phosphate-buffered saline [PBS] plus 0.05% Tween 20). A blocking buffer (Starting Block Buffer, Thermo Scientific/Pierce, Rockford, IL, USA) was added (100 μL/well), and the plates were incubated at RT for 60 minutes. Sera from the equine melanoma patient was diluted 1:160 in PBS buffer and added to the antigen-coated wells (100 μL/well) in triplicate for 30 minutes at RT. Following the primary antibody step, the wells were washed 3 times with the washing buffer, and a second antibody, alkaline phosphatase-labeled goat anti-horse IgG (Bethyl Laboratories, Montgomery, TX, USA), was added (100 μL/well) at a 1:2,000 dilution and again incubated at RT for 30 minutes. After the incubation with the secondary antibody and the final washing step, alkaline phosphatase (AP) substrate, *p*-nitrophenyl phosphate (PNPP) (Invitrogen, Camarillo, CA, USA) was added, and the color reaction was read using a microplate reader (model 680, Bio-Rad, Hercules, CA, USA) at 405 nm. Data shown in Figure 2 indicate that vaccination by direct injection of the DNA cancer vaccine resulted in the induction of antimelanoma IgG antibody response which increased 2-fold over time and persisted until the end of the study.

3. Discussion and Conclusions

This report describes the clinical outcome in an equine patient with metastatic melanoma following multiple intratumoral injections of a plasmid DNA construct containing the *emm55* gene. Emm55 is an immunogenic protein derived from *S. pyogenes.* Expression of this bacterial protein on the surface of tumor cells causes them to be immunogenic and results in a vigorous antitumor immune response. It has previously been shown that the modification of tumor cells with this gene in naturally occurring canine lymphoma cases resulted in the development of both humoral and cellular responses to the malignancy [13]. In collaboration with a number of veterinary physicians in the state of Florida, autologous tumor cells processed in this manner also have been used to treat various malignancies in both dogs and cats (data not shown). In this current

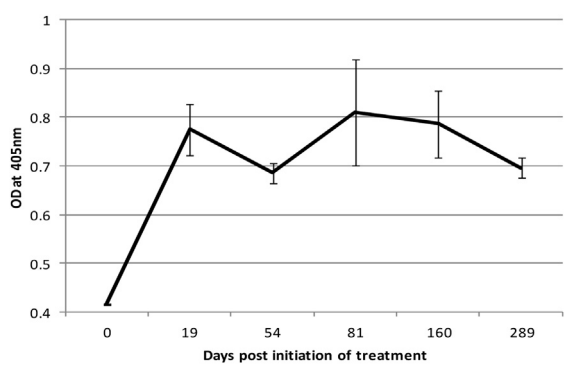

Fig. 2. Augmentation of antimelanoma antibodies is shown during the postvaccination regimen. Vaccine doses were administered on days 1, 19, 55, 79, 110, 145, 187, and 255. Responses were studied until the 289th day. Antibody levels were determined using ELISA. The lysate protein from a noninjected melanoma specimen was used as the antigen source at 10 μg/mL. The ELISA was developed using goat anti-equine IgG antibodies conjugated to AP enzyme and PNPP substrate. The IgG antibody level in the plasma, at a 1:160 dilution in the ELISA assay, was 2-fold increased and sustained over the course of the therapy. The error bars show the SEM of triplicate values. AP, Alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; PNPP, *p*-nitrophenyl phosphate.

study, intratumoral injections of *emm55* plasmid DNA induced a 2-fold increase from baseline of antimelanoma IgG antibody levels that persisted throughout the observation period. This correlated with a 42.3% reduction in total tumor burden of indexed lesions, with injected lesions regressing 34% to 50% from the original tumor size. The observation that there was regression of noninjected lesions (19% to 55%) implied that the immune response induced by the DNA vaccine was systemic and capable of inducing changes in tumor volume in those tumor lesions not given vaccine directly.

Unlike the antibody response observed in this tumor-bearing patient, Lembcke et al [3] found that in naïve nonmelanoma horses, the antibody response was transient. Several melanoma tumor antigens have been identified, with tyrosinase being the most investigated melanoma antigen [15]. In the present study, it is unclear whether the tyrosinase response was dominant in the antimelanoma response. This observation is relevant because the development of antibody response has been associated with positive clinical outcomes. For instance, in advanced canine melanoma, the antibody response induced after 4 biweekly immunizations with xenogeneic human tyrosinase DNA coincided with clinical benefits. Compared with the humoral nonresponders, patients in which antibody was induced exhibited long-term survival, although it did not reach statistical significance due to the small sample size [16]. In human patients with castration-resistant prostate cancer who received sipuleucel-T vaccine, a dendritic cell-based vaccine manipulated to express prostatic acid phosphatase antigen (PAP), an antibody response titer to PAP above 400 was found to be associated with longer survival than in patients in the lower response group [17]. In the study reported here, there was a base level antibody response to the tumor prior to vaccination (optical density [OD], 0.4) which suggests stimulation of an antimelanoma response in this patient, either by the tumor or from previous treatment with the equine immunostimulant, but the disease was progressing. However, following the pAc/*emm55* plasmid vaccination protocol which augmented the antibody response level (OD, 0.8), also exhibited clinical regression of the tumor lesions. Thus, in this patient, the induction of an antimelanoma antibody response was clinically relevant. Further studies using additional equine melanoma patients will determine the significance of the association between antibody response and tumor regression in equine melanoma.

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