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Expression of Leptin and iNOS in Oral Melanomas in Dogs

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Abstract

Background—Oral melanoma (OM) in dogs is an aggressive malignancy, with clinical behavior resembling cutaneous melanomas in humans. Melanoma in humans is promoted by an inflammatory environment which is contributed to by leptin and inducible nitric oxide synthase (iNOS).

Objective—To determine if the patterns of leptin and iNOS expression are similar in OM in dogs and cutaneous melanomas in humans.

Animals—Twenty client-owned dogs.

Methods—Retrospective case study. Immunostaining of the OM tumors from each dog was scored for percentage and intensity of leptin and iNOS expression. Mitotic index was used as an indicator of tumor aggression.

Results—Leptin was detected in 75% of the tumor cells in specimens from eleven dogs. One tumor expressed leptin in 25% of the cells. The intensity of leptin expression was variable with six, nine, and five cases exhibiting low, moderate, and high intensity staining, respectively. OM with the lowest percentage of iNOS positive cells displayed the highest mitotic indices ($p = 0.006$, ANOVA)

Conclusions and Clinical Importance—The expression of leptin is a common finding in melanomas in dogs. These data suggest the possibility of future clinical applications, such as measuring the concentrations of plasma leptin as a screening tool or leptin as a target for therapy. The relevance of iNOS is not as clear in dogs with OM, for which other directed therapeutics might be more appropriate.

Keywords

mucosal tumor; inflammation; prognosis; adipokine

Introduction

Malignant melanoma is an aggressive neoplasm derived from melanocytes populating pigmented tissues. Melanomas develop most frequently on the skin in humans (Fig. 1a); although rarely, tumors will arise from mucosal surfaces such as the anus or the vagina. When advanced, both cutaneous and mucosal melanoma in humans can metastasize

diffusely, resulting in death of the patient. Melanoma occurs in dogs, but the aggressive form arises almost exclusively from the mucosal structures of the oral cavity (Fig.1b), such as the tongue or gingiva, and is generally referred to as oral melanoma (OM).¹ In dogs, tumors arising in fur-covered skin are more likely to behave in a benign manner. Thus, both differences and similarities exist in the natural history of melanoma in dogs and humans. It is possible, however, that valuable clinical and pharmacologic data can be derived from comparisons between the two species.

Inflammation promotes the growth and survival of some malignancies. Although somewhat counter-intuitive, cytokines and other inflammatory mediators can either stimulate or inhibit tumor growth based on their concentrations and concomitant environmental factors. Nitric oxide (NO) is produced at nanomolar levels by melanoma cells where it activates growth-promoting pathways.² Due to the short half-life of NO, however, its synthetic enzyme, inducible nitric oxide synthase (iNOS), is frequently examined as an indicator of NO levels. Human patients whose lymph nodes bear tumor cells expressing high levels of iNOS have significantly shorter survival.³

Leptin is an inflammatory adipokine with multiple functions, such as enhancement of interferon-gamma and interleukin-1 activity in articular disease.⁴ Both leptin and its receptor are present in cultured human melanoma cells.⁵ Leptin mRNA is found in sentinel lymph nodes involved with tumor.

The concept of directing melanoma therapy to the inflammatory environment, while in the early stages of development, is a novel approach with considerable therapeutic potential. In the current study, we examine OM in dogs to determine if the inflammatory processes and targets identified in humans exist in these animals, justifying their participation in clinical trials of new pharmacologic agents.

Materials and Methods

Consent for Use of Archival Materials

Consent for the use of archival material was obtained from the Institutional Review Board of M. D. Anderson Cancer Center, Houston, TX and the Laboratory Sample Use Review Committee of Texas A & M University, College of Veterinary Medicine, College Station, TX. Twenty paraffin blocks of OM were obtained from the pathology archives of Texas A & M University. Four micron sections were prepared for each of the tumors included in this study. Antibody to leptin was obtained from R&D Biotechnology (Minneapolis, MN) and to iNOS from Sigma (St. Louis, MO).

Mitotic index and melanin content

Mitotic index was described as the average number of mitoses per ten high power fields. Melanin content was graded on a scale of 0–3, with 0 or 1 considered weak (W), and 2 or 3 considered strong (S).

Immunostaining

Immunohistochemistry for leptin and iNOS was performed as previously described.³ Briefly, tissue sections were deparaffinized in xylenes, and rehydrated in graded concentrations (100% to 85%) of ethanol. Sections were placed in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) and microwaved intermittently for a total of 10 minutes, to maintain boiling temperature. After cooling, the slides were placed in 3% H₂O₂ in cold methanol for 15 minutes to block endogenous peroxidase activity. This step was followed by permeabilization with 0.05% Triton X-100 (Sigma) in phosphate-buffered

saline for 15 minutes. An avidin-biotin-peroxidase complex (ABC) kit (Vectastain, Vector Laboratories) was then used for antigen detection. After a 30-minute incubation with blocking serum, the primary antibody was applied and incubated for two hours at room temperature. The slides were then washed, incubated for 30 minutes with secondary biotinylated antibody, followed by a 30-minute incubation with the ABC reagent. The immunolabeling was developed with the chromogen 3-amino-9-ethylcarbazole (AEC). Hematoxylin was applied as a counter stain.

Immunolabeling of COM was scored separately for two variables; first, for the percentage of positive cells; second, for the overall intensity of immunoreactivity of the positive cells. Scoring for the percentage of positive tumor cells was defined as follows: “Low (L)”, 0–25% positive cells; “Medium (M)”, 26–75% positive cells; and “High (H)”, 76–100% positive cells. Intensity scoring consisted of: “Low (L)”, absent or weak staining; “M (M)” moderate staining; and “High (H)”, strong staining. The slides were interpreted independently by two readers (VRG and JAE) and differences subsequently reconciled. The scoring system used here was the same as that applied to our previously published human data.³

Statistical Analysis

Differences in the mean mitotic index of high, medium, and low-staining cases in each category were analyzed by ANOVA using Minitab software. The exception was leptin percentage for which there was only one low staining sample. In this case the mean mitotic index was compared between high and medium cases by an unpaired t-test.

Results

Dogs

The study population consisted of twenty dogs. Breeds were diverse and consisted of two Golden Retrievers, two Rottweilers, two Cocker Spaniels, two Labrador Retrievers, one Australian Shepherd, one Dachshund, one Chow Chow, one Great Dane cross, one Rhodesian Ridgeback, one Yorkshire Terrier, and six mixed breeds. OM sites included six cases of hard or soft palate, five of tongue, five of gingiva, three of lip, and one of larynx. The study population consisted primarily of older dogs. Other than the two year old Great Dane, all dogs were eight years or older. Seven dogs are known to have died as a direct consequence of their malignancy and the outcomes of the remainder are unknown.

Expression of Leptin and iNOS

The objective of this study was to determine if tumor iNOS or leptin correlated with survival in dogs with OM. Long term follow-up data, particularly survival data, were not available for the majority of the study population. Instead, mitotic index, a predictor of outcome, was utilized as a surrogate for death from disease.⁶

The antibody to leptin yielded positive immunostaining of dog adipose tissue which served as a positive control (Fig. 2a). Leptin was widely expressed throughout the OM tumors and, in eleven the cases, could be found in 75% of the tumor cells (Fig. 3a and b). In only one tumor was leptin expressed in 25% of the cells. The intensity of staining was variable. Six, nine, and five cases exhibited low, moderate, and high intensity staining, respectively. Neither leptin percentage nor intensity demonstrated a statistically significant association with mitotic index.

Dog macrophages within the tumor served as a positive control for iNOS (Fig. 2b). The antibody utilized is widely cross-reactive with iNOS from multiple species including dogs,

goats, and humans.⁷ A low percentage of iNOS expression was detected in five cases and was associated with a high mitotic index (mean 31.20; $p = 0.006$) (Fig. 3c and d). The mean mitotic indices for high and medium percentage cases were 12.13 and 4.43, respectively. In fifteen of the twenty tumors, iNOS staining intensity was low or medium.

Production of melanin by OM is an indicator of differentiation.⁸ Each case was examined for high or low melanin content. There was an inverse correlation between OM mitotic index and degree of melanin pigmentation.

Discussion

We examined the expression of two inflammatory mediators, leptin and iNOS, in OM in dogs. Due to incomplete follow-up data on all subjects, we utilized mitotic index as a surrogate for survival. Weak or strong expression of melanin, a positive indicator of tumor differentiation, was also determined to support the use of mitotic index in this role. Findings revealed ubiquitous and generally robust expression of leptin, reflecting the pattern seen in human cutaneous melanoma. iNOS expression was more variable and, in contrast to human cutaneous tumors, showed a significant inverse correlation between percentage expression and mitotic index ($p = 0.006$).

Research into the role of leptin in insulin resistance, cardiac disease, and inflammatory disorders has been ongoing in dogs for some time. The dominant, if not only, source of leptin in these conditions is thought to be adipose tissue. More recently, it has been discovered that cutaneous melanoma tumors in humans and cultured human melanoma cells produce cytoplasmic leptin, as well as the membrane leptin receptor. In the presence of leptin, human-derived cutaneous melanoma cell lines activate the p44/42 MAPK pathway (Ras-Raf-MEK-ERK) and proliferate. Although it is unknown if these findings hold true in human mucosal melanoma tumors, it is intriguing to speculate that the same leptin-driven tumorigenic processes occur in OM. Identifying leptin as a promoter of OM growth could lead to targeted therapies such as anti-receptor antibodies or free receptor directed to leptin in the circulation. The present study lacked a statistically significant correlation of immunostaining scores with mitotic indices, likely due in part to the small number of subjects. Hence, it could not be determined whether leptin actually impacted tumor behavior in OM or was simply an incidental finding unrelated to tumorigenesis. Future studies will require a larger number of subjects to fully determine the impact of leptin on the clinical behavior of this malignancy. *In vitro* molecular pathway studies with OM cell lines, immunostaining for the leptin receptor, and comparison of circulating leptin levels in sick and healthy dogs would contribute to our understanding of the role of this hormone in OM.

The iNOS data were more difficult to interpret and the small number of samples made it difficult to draw conclusions about the role of iNOS in OM. There was a significant inverse relationship between tumor mitotic index and iNOS percentage expression in OM suggesting that OM which produce less iNOS are less aggressive tumors. This contrasts with findings in humans where high iNOS expression predicts poor survival. In retrospect, there are a number of reasons to anticipate that mucosal and cutaneous melanoma might differ in their dependence on iNOS activity. The major signaling pathways that lead to iNOS expression in mucosal melanoma in dogs or humans are unknown. However, it is safe to assume that, unlike human cutaneous melanoma, OM is not UV induced and its growth might be independent of UV-linked pathways such as p38 MAPK that ultimately lead to iNOS expression. Other undefined stimulatory networks might dominate proliferation in OM such that high or low iNOS levels might not be relevant to the mitotic rate. The potential contribution of iNOS-inducing cytokines and growth factors secreted into the

microenvironment by keratinizing epithelial cells and epidermal appendage structures, which are absent in OM, is another point of consideration.

Other intrinsic differences in molecular and genetic features between cutaneous and mucosal melanomas have previously been noted. For example, *BRAF* mutations are common in cutaneous melanoma in humans but found infrequently in OM in dogs and mucosal melanomas in humans.⁹ In contrast *KIT* mutations are found at low but consistent rates in OM in dogs and mucosal melanomas in humans.¹⁰ Such similarities suggest that OM in dogs might share more biologic properties with mucosal melanomas in humans, and studying the two in tandem would be a rational approach.

While the data presented here are limited by the small number of subjects, the findings represent a contribution to the growing trend of collaboration between veterinary and human oncologists, and a much needed movement toward the inclusion of spontaneous animal tumors in clinical trials.

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Abbreviations

iNOS	inducible nitric oxide synthase
COM	canine oral melanoma
NO	nitric oxide
ABC	avidin-biotin-peroxidase
AEC	3-amino-9-ethylcarbazole
MAPK	mitogen activated protein kinase

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Figure 1. Primary melanomas. Shown are H&E stained sections of melanomas taken from (a) human skin (20 $\times$, Bar = 20 μ m) and (b) from the oral cavity of a dog (40 $\times$, Bar = 10 μ m)

Figure 2.

Immunohistochemical controls for canine anti-leptin and anti-iNOS. (a) Canine adipose tissue is immunoreactive with the anti-leptin antibody used in this study. (b) A lymph node photographed in the region of a tumor deposit (not shown) contains multiple macrophages that express iNOS (red granules, arrow). The macrophages also take up melanin which appears as brown granules (arrowhead). (40 $\times$, AEC, Bar = 10 μ m)

Figure 3. Detection of leptin and iNOS in COM. Leptin expression is diffuse and intense (a), whereas iNOS immunostaining is light to moderate (c). IgG controls are shown in (b) and (d). (40 $\times$, AEC, Bar = 10 μ m).