Blood Vessel Density in Canine Osteosarcoma

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ABSTRACT

Canine osteosarcoma is a prevalent bone neoplasm which has similarities to the human disease. We used a retrospective study to investigate the possibility that tumor vascularity may provide useful prognostic information, indicative of the role of this parameter in progression of this cancer. We quantified microvessel density in 52 histological specimens of primary tumor, immunostained for von Willebrand's Factor to identify vascular endothelium. For the 20 cases not euthanized at presentation or lost to follow-up, we found significantly higher tumor microvascular densities in animals presenting with detectable pulmonary metastases (5 of 20), and significantly lower densities in animals without metastatic disease at presentation, but later surviving to develop pulmonary metastases (7 of 20; $P < 0.05$). Animals with no evidence of pulmonary metastases at time of death (8 of 20) had intermediate vascular densities in their tumors. The results of this preliminary study suggest that vascularity of the primary tumor may be an indication of tumor progression. Future studies with a larger number of cases should establish whether vascular density can be a useful prognostic parameter for canine osteosarcoma.

RÉSUMÉ

L'ostéosarcome canin est un néoplasme osseux prévalent qui possède des similarités avec son pendant humain. Une étude rétrospective fut entreprise afin de déterminer si l'évaluation de l'état vasculaire de la tumeur pouvait fournir une information prédictive et indicative du rôle de ce paramètre dans la progression de ce type de cancer. La densité de la microvascularisation de 52 spécimens de tumeur primaire fut quantifiée à l'aide d'une technique d'immunocoloration pour le facteur de von Willebrand présent dans l'endothélium vasculaire. Pour les 20 cas qui ne furent pas euthanasiés lors de l'examen initial ni perdus lors des suivis, la densité de la microvascularisation était significativement plus élevée chez les animaux présentés ayant des métastases pulmonaires détectables (5/20), et une densité significativement plus faible chez les animaux présentés qui n'avaient pas de métastase à l'examen initial mais qui survécurent et développèrent éventuellement des métastases pulmonaires (7/20; $P < 0,05$). Les animaux sans évidence de métastase pulmonaire au moment de leur décès (8/20) avaient une densité de vascularisation intermédiaire dans leurs tumeurs. Les résultats de cette étude suggère que la vascularisation des tumeurs primaires serait un indicateur de la capacité de progression de la tumeur. Des études subséquentes avec un plus grand nombre de cas devrait permettre d'établir si la densité de vascularisation peut être un paramètre utile de pronostic des ostéosarcomes canins.

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INTRODUCTION

Studies of several types of companion animal cancer suggest that most malignant tumors respond poorly to radiation or chemotherapy, and that surgically removed tumors tend to recur (1–5). Osteosarcoma cancer in dogs consists of aggressive, invasive and highly cellular tumors, accounting for more than 85% of canine skeletal tumors. This tends to be a highly variable lesion which may be misdiagnosed by histopathology (6,7). These tumors form most commonly in the appendages of long bones (appendicular lesions), but may also develop in flat or cuboidal bones (axial lesions). Appendicular tumors are reported to be the most common, with 75% of all canine osteosarcomas falling into this category (6). Limb amputation or other surgical reduction is the method of choice for controlling the primary tumor, improving the quality of life, but this generally has little effect on long term survival. In the absence of adjunct therapy (e.g. chemotherapy) over 90% of dogs with osteosarcoma will develop metastasis within 1 y of diagnosis, with greater than half showing metastatic disease within 5 mo (6,8). Mean survival time may be only 3–6 mo after surgery, and animals which develop metastases early have greatly reduced survival times (7).

Thus, canine osteosarcoma has a variable natural history, and although the outlook is universally poor, there are important differences in tumor biology which may lead to advancements in our understanding of this cancer. It has long been known that tumor growth and spread is severely restricted until a blood supply develops, and that the expression of angiogenic molecules to induce new vessel formation is a characteristic of highly aggressive tumors (9). Recent studies of several types of human cancers have documented useful prognostic indicators between the vascular

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TABLE I. Case characteristics of 52 osteosarcoma tumors grouped by sex and location

	Male	Female	Axial	Appendicular
% of all cases	58%	42%	38%	62%[a]
Mean age (mo)	84.7 (39.9)	89.8 (29.9)	104.4 (48.5)	83.4 (37.8)
Mean weight (kg)	37.6 (13.1)	33.9 (17.7)	28.1 (9.4)	41.2 (15.8)[b]
Mean vessel density (number/field at 200 × magnification)	44.5 (21.2)	43.8 (18.5)	45.2 (21.4)	41.2 (18.0)

Values are Mean (Standard Deviation)
[a] Ratio between Axial and Appendicular cases significantly different from 1:1 ($P < 0.05$)
[b] Weight of Appendicular cases significantly different from weight of Axial cases ($P < 0.05$)

density of a tumor and its apparent metastatic potential (10–14). Useful prognostic indicators have not yet been developed for canine osteosarcoma; the general assumption is that all tumors will behave in a very aggressive manner. However, for those dogs that present with no apparent detectable metastatic disease at initial diagnosis, there are no parameters available to predict the rate of development of metastases or local regrowth (6). Clinical trials of therapeutic strategies can only be critically analyzed and evaluated once the biologic behavior of this tumor is described (2).

The goals of this retrospective study of canine osteosarcoma were to characterize and quantify vascular density in canine osteosarcoma primary tumors, and to evaluate the possible contribution of vascularity to differences in disease progression and metastatic behaviour.

MATERIALS AND METHODS

CASE SELECTION

The Ontario Veterinary College, (OVC) patient base was examined, and 76 cases of canine osteosarcoma from 1985–1995 were identified based on initial diagnosis and availability of specimens. Of these cases, 27 were located in the axial skeleton and 49 in the appendicular skeleton. Slides from available blocks were examined and those showing a generous cross-section of the tumor were selected for further analysis (15). Hematoxylin and eosin stained slides of these cases were re-evaluated by an anatomic pathologist, and immunostained for von Willebrand Factor as described below. Some specimens were removed from this study due to incorrect diagnosis, or unsuitable specimen quality (only fragments of tumor available in block; poor immunostaining). The remaining 52 cases, consisting of 20 axial and 32 appendicular tumors, were analyzed as described below. Followup information was obtained by examining the records and contacting the referring veterinarians where necessary.

IMMUNOSTAINING

Specimens consisted of decalcified tissue embedded in paraffin, and were sectioned at 8 μm. Slides were deparaffinized and hydrated, then treated with 0.05% trypsin in phosphate-buffered solution for 10 min at room temperature, to expose antigen epitopes. Endogenous peroxidase activity was depleted by incubation of slides with 3% H_2O_2 for 5 min at room temperature. After blocking non-specific binding sites with 5% bovine serum albumin (BSA), sections were incubated with polyclonal antibody raised in rabbits against human von Willebrand Factor (Dako Corp., Carpinteria, California, USA), diluted 1:200 in 0.1% BSA, followed by goat anti-rabbit IgG conjugated with biotin (Sigma-Aldrich Canada Ltd., Oakville, Ontario), diluted 1:20 in 0.1% BSA. Sections were then incubated with EXTRA-Avidin-Peroxidase solution (Sigma-Aldrich), diluted 1:20 in 0.1% BSA, and colour developed with AEC (3-amino-9-ethylcarbazole; Sigma-Aldrich) to produce a brick red reaction product. Slides were then counterstained with Meyer's haematoxylin and mounted with Aquapolymount (Polysciences, Warrington, Pennsylvania, USA).

SPECIMEN EVALUATION

Vascular density was determined according to the method of Weidner (15). Sections were scanned at a microscope magnification of 20 × or 80 × to identify heavily stained vascular "hot spots" within the tumor or immediate peritumoral area (15). Counts of the 3 most vascular areas for each specimen were made at microscope magnification of 200 × (field area 0.74 mm^2), and the maximum count obtained from each specimen was used for further analysis. Positively stained endothelial cells in microvessels were clearly seen in all specimens. A visible vascular lumen was not required for a vessel to be included in the count. Very tortuous microvessels were seen especially in highly vascularized areas. In such cases, each profile was counted as an independent vessel, although they may be considered sections of the same microvessel as it travels through the plane of section. This approach is valid, as the tortuosity of newly formed microvessels is related to the strength of the angiogenic response in many other tumors (15). Larger vessels, consisting of small arteries and veins, were very rare in these vascular hot spots, and were not included in the counts.

All assessments were made "blind"; details of tumor location, patient characteristics, and outcome were not revealed until all slides had been examined. Some specimens were re-evaluated at a later date, and counts obtained agreed within 10%. Patient records, radiographs, and contact with referring veterinarians where appropriate, were used to obtain data on patient characteristics, gross pulmonary metastatic status, and eventual outcome. These data and vessel density data were analyzed using simple linear regression, ANOVA, and student's *t*-test.

RESULTS

IMMUNOSTAINING

We obtained intense staining for von Willebrand Factor in both tumor and peritumoral tissue in these canine osteosarcoma specimens (Figure 1). The pattern of staining was indicative of endothelial location within easily identified larger vessels, as well as microvessels. Non-specific staining by secondary antibody alone was negligible, and endogenous peroxidase activity was completely eliminated by pre-treatment of sections with hydrogen peroxide.

CASE CHARACTERISTICS

Of the 52 cases examined in this study, approximately equal numbers were male and female (Table I). There were significantly more appendicular tumors than axial ($P < 0.05$; Table I). There were no significant differences in body weight at diagnosis between male and female cases, but appendicular cases were significantly heavier than were axial cases ($P < 0.05$; Table I). There were no significant differences in age at diagnosis between axial and appendicular cases, nor between males and females (Table I).

Cases were divided into groups based on the metastatic status of the animal. Group 1 consisted of dogs that had gross pulmonary metastases at the time of diagnosis, $n = 5$. Dogs in Group 2 were apparently metastasis free at diagnosis, but later developed gross pulmonary metastases, $n = 7$. The earliest development of metastatic disease in this group occurred within 2 mo of surgery. Group 3 was the largest consisting of 40 cases which did not have detectable gross pulmonary metastatic disease at the time of diagnosis. This group could be further subdivided (12 cases euthanized at presentation, 16 cases lost to follow-up, and 12 cases with known survival, apparently metastasis free at death). Of the 12 dogs that were free of gross pulmonary metastasis at death, 4 had died less than 2 mo post diagnosis and were considered lost to follow-up. Therefore, of this group of 40 dogs there were 8 animals with sufficient follow-up information for further analysis. In total 20 dogs (Group 1, $n = 5$; Group 2, $n = 7$; Group 3, $n = 8$) were used for detailed analysis of survival rates. There were no significant differences in age at onset for these 3 groups ($P < 0.05$; Table II), while cases presenting with gross metastases weighed significantly less than cases that developed metastases later ($P < 0.05$). Of these 20 cases, 5 were axial and 15 were appendicular; approximately 25% of cases at each tumor location presented with metastases (1 of 5 for axial, 4 of 15 for appendicular). Approximately 50% of animals not presenting with metastases later developed detectable metastatic disease, again equally distributed by tumor location (2 of 4 for

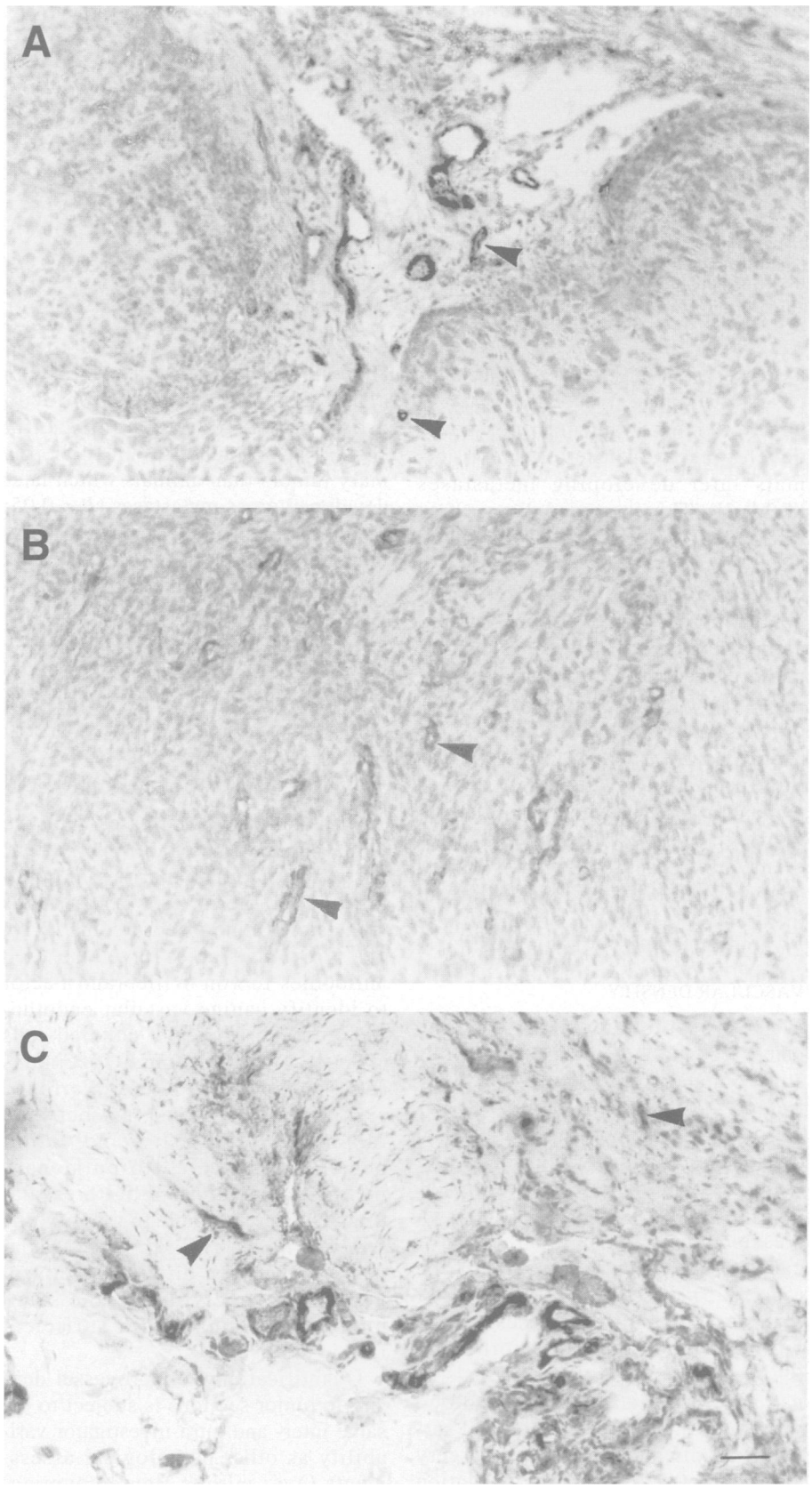

Figure 1. Photomicrographs showing vascular "hot spots" in osteosarcoma specimens after immunohistochemical staining for von Willebrand Factor and counterstaining with hematoxylin. Microvessels can been seen as dark staining structures (arrowheads) embedded in the tumor parenchyma. A: Tumor with low vascular density (12/field). B: Tumor with intermediate vascular density (33/field). C: Tumor with high vascular density (65/field). Scale Bar = 100 μm.

TABLE II. Case characteristics of osteosarcoma specimens with known outcomes grouped by metastatic status

	Number of cases	Age at onset (mo)	Vessel density	Weight at onset (kg)	Mean disease free interval (mo)
Gross pulmonary metastases at presentation (Group 1)	5	64 (33.4)	74 (20.0)[a]	26 (4.9)[b]	—
Later development of gross pulmonary metastases (Group 2)	7	88 (31.5)	33 (15.5)	44 (12.7)	12 (9)
No gross pulmonary metastases at death (Group 3)	8	95 (36.7)	42 (18.8)	31 (10.5)	20 (6)[c]

Data are shown as Mean (Standard Deviation)
[a] Significanlty higher vessel densities than animals with gross metastases at presentation ($P < 0.025$), and later development of metastases ($P < 0.05$)
[b] Significantly different weight at onset than for animals developing metastases ($P < 0.05$)
[c] Signfiicantly longer disease free interval than animals later developing metastases ($P < 0.05$)

axial cases, 5 of 11 for appendicular cases). Disease free interval was nearly twice as long for dogs never developing metastases than for animals later developing metastases (Table II). This increased disease-free interval was statistically significant ($P < 0.05$). These 20 cases were also divided into groups based on therapeutic approach: biopsy only, surgery alone (amputation or local excision), palliative radiation of primary lesions, surgery plus chemotherapy plus adenovirus-mediated interleukin 2 gene therapy, and surgery plus chemotherapy. Only "surgery alone" (7 cases) and "surgery plus chemotherapy" (9 cases) groups had sufficient cases for meaningful survival analysis, and we found no significant differences in survival between these 2 groups (results not shown).

VASCULAR DENSITY

The vascular density of canine osteosarcoma "hot spots" seen in this study ranged from 9 to 104 profiles/field at 200 × magnification. No significant differences in mean vessel density were found when comparing male versus female cases, or appendicular versus axial cases (Table I). Vessel density at presentation for cases treated with surgery alone (57 ± 25) was significantly higher than vessel density for cases treated with surgery and chemotherapy (37 ± 20; $P < 0.05$). There was also a negative correlation between blood vessel density at presentation and survival in animals treated with surgery alone ($r = 0.777$), but no correlation between vessel density and survival in animals receiving surgery and chemotherapy (results not shown).

When cases were divided based on metastatic status, significant differences in vascular density were seen (Table II). Animals presenting with gross metastases had significantly higher vessel densities in their primary tumors than animals which later developed gross metastases ($P < 0.05$; Table II). Animals without gross metastasis at death had intermediate vascular densities. These were significantly different from animals presenting with gross metastases ($P < 0.025$), but not from animals later developing gross metastases ($P = 0.16$; Table II).

DISCUSSION

This study involved cases from the OVC canine osteosarcoma patient base, and shared similarities in population characteristics with previous studies of canine osteosarcoma (3,16, 17). Previous studies have also used antibodies to von Willebrand Factor to identify canine vascular endothelium, both in studies of cultured cells (18,19) and histological specimens (20,21) as described here. We found significant relationships between tumor vessel density and tumor behavior, as related to pulmonary metastatic status on initial presentation. We were not able to identify differences in survival rates or vascular densities between different treatment modalities, likely due to the small number of animals with sufficient follow-up.

Quantification of microvessel density in tumor sections is subject to the same inter- and intra-investigator variability as other pathological assessments (e.g., mitotic figure counting) (15). None the less, microvascular density has proven to be a powerful predictor of tumor progression and outcome in virtually all studies performed to date. The original work with human breast carcinoma established the techniques (especially the importance of microscope field size and magnification, and the necessity of scanning sections for "hot spots") and found that microvessel density was the best independent predictor of both disease free interval and survival, especially in node negative patients (10). Since this study, the value of tumor microvascular density in breast cancer prognosis has been confirmed by other groups (22), and established for many other solid human tumors, including carcinomas of the lung, digestive and urinary tract, skin tumors, including melanoma, tumors of the nervous system, and head and neck cancers (23–25). However, to date there are no published studies of microvascular density in sarcomas. This could be due to the relative rarity of these tumors in human beings leading to a paucity of cases to compare, or to the fact that human sarcomas have a general overall poor prognosis, with little stratification of outcome (15). Both of these factors make sarcomas less attractive for such analysis, relative to other solid tumors (15).

Canine osteosarcoma occurs at approximately twice the rate as this cancer in humans. This, coupled with the large companion animal population in North America makes canine osteosarcoma a relatively common bone lesion, with numerous cases available for study (16). Although prognosis for long term survival is guarded, some therapies improve the disease free interval and survival times and most studies report a small proportion of dogs with very good post surgical survival (e.g. 1 y or longer) (8,26,27). Currently, there are no useful biologic indicators to identify the population of animals which

will have the longest disease-free interval, nor is there any way to assess which of the many therapy modalities might be most appropriate for each case. This report of blood vessel density in canine osteosarcoma may provide direction for further study.

Our results suggest that for animals treated by surgical removal of the tumor alone, in the absence of any adjunct therapy, blood vessel density may be an important prognostic indicator, with very high vessel density suggesting a short survival, likely due to the prior existence of metastatic disease. There is a positive correlation between tumor grade and microvascular density, and metastatic disease and microvascular density in prostate carcinoma (28), gastric carcinoma (29) and non-small-cell lung carcinoma (11). There is also evidence from lung carcinoma to suggest that metastases have higher microvascular densities than their primary tumors (30). These studies of human cancers suggest that a well vascularized canine osteosarcoma may represent a metastatically competent form (31). In addition to provision of a route for metastases, recent evidence suggests that tumor vasculature is negatively correlated with cancer cell apoptosis. Thus, enhanced cancer cell survival due to presence of blood vessels will contribute to tumor progression (32).

Some studies of experimental tumor systems also suggest that there are important interactions between primary tumors and distant micrometastases which affect tumor progression. Primary tumors may produce angiogenesis inhibitors (such as angiostatin, endostatin; 33,34). Removal of such primary tumors leads to a decrease in circulating levels of these inhibitors, and subsequent explosive growth of dormant micrometastases. Such a mechanism might explain the significantly shorter survival times seen in our cases which present metastases free, but rapidly succumb to metastatic growth after primary tumor removal.

Since up to 90% of canine osteosarcoma cases have undetectable micrometastases at the time of diagnosis (3,17), identifying those cases vulnerable to aggressive metastatic disease based on tumor microvessel density may allow for directed anti-angiogenic therapies in addition to cytotoxic approaches. A recent study reported that 32% of dogs undergoing amputation for treatment of osteosarcoma developed overt lung metastases less than 4 mo after surgery, despite being treated with cisplatin chemotherapy (26). For those animals presenting without detectable metastases, we would like to have parameters which could distinguish those that are especially vulnerable to rapid development of gross metastatic disease. Our results indicate that vascular density is not able to distinguish these 2 groups in our study. What is not yet known is whether primary tumor vascular density is high prior to or after shedding of cells which form micrometastases. Since we do not have markers for microscopic metastatic disease, we are as yet unable to differentiate this state from "no metastatic disease" in canine osteosarcoma. However, animals in our study which later developed metastases did have the lowest blood vessels densities, although not statistically significant from animals that never developed gross metastases. We consider this a promising trend, and it may be that a larger study with more cases will clarify the issue. Given that dogs presenting with established metastatic disease prior to removal of the primary tumor had significantly higher blood vessel densities than any other group in our study, concomitant anti-angiogenic therapy would seem to be a prudent and effective course for these dogs. It is also likely that animals presenting without detectable pulmonary metastases would also benefit from such adjuvant therapy, but further study is required.

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