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Vascular Endothelial Growth Factor Expression in Canine Intracranial Meningiomas and Association with Patient Survival

Simon R. Platt, Timothy J. Scase, Vicki Adams, Lara Wieczorek, Jodi Miller, Filippo Adamo, and Sam Long

Background: Vascular endothelial growth factor (VEGF) is a regulator of angiogenesis and vascular permeability. In human patients with meningiomas, increased VEGF expression is predictive of postsurgical recurrence. The objectives of this study were to evaluate VEGF expression in canine intracranial meningiomas and to determine whether an association between VEGF expression and patient survival existed.

Methodology: Tumor tissue from 17 dogs with histologically confirmed intracranial meningiomas was obtained surgically. All dogs then were treated with radiotherapy. Immunohistochemistry was performed on 5-μm sections of paraffin-embedded tumor tissue with rabbit anti-human VEGF polyclonal antibody. The extent, intensity, and distribution of VEGF staining for each section were assessed with light microscopy by means of a semiquantitative scale. Survival was analyzed by the Kaplan-Meier procedure. Survival rates among groups were compared by log-rank tests with the significance set at $P \leq .05$.

Findings: VEGF expression was detected in all tumors, with >50% of cells staining positively in tissues from 15/17 dogs. Shorter survival times were associated with greater VEGF expression ($P = .01$).

Conclusions: VEGF expression can be measured in canine intracranial meningiomas and may be associated with poor outcome.

Significance: The extent of VEGF expression in canine intracranial meningiomas may be used as a prognostic marker and suggests a potential future target for therapy.

Key words: Dog; Malignancy; Neoplasia; Vascularity.

Angiogenesis, the process leading to the formation of new vessels from an existing vascular network, is essential for the growth, invasion, and metastasis of solid tumors. Several studies indicate that the growth of cerebral neoplasms in humans depends on angiogenesis.[1,2] Tumors release angiogenic factors, such as vascular endothelial growth factor (VEGF), which induce the growth of a capillary network.[3] The degree of VEGF-induced tumor angiogenesis seems to be an important predictor of tumor progression and recurrence in a variety of cancers.[2]

VEGF is a relatively specific mitogen and chemotactic factor for endothelial cells and is one of several survival factors necessary for sustaining new blood vessels.[4,5] VEGF stimulates endothelial nitric oxide synthase, resulting in the generation of nitric oxide and activation of the angiogenic cascade.[6,7] In addition, VEGF induces endothelial cell production of proteases that are necessary for degradation of the basement membrane during angiogenesis and that serve as a survival factor for endothelial cells. VEGF promotes microvascular permeability to plasma proteins at the level of small capillaries and venules, and its effect is 50,000 times more potent than that of histamine.[8] Increased vessel permeability results from the direct effects of VEGF on endothelial cells, mobilization of endothelial cytosolic calcium, and increases in fenestrae and pinocytic vesicles.[9] The permeability effects of VEGF can be modulated by other angiogenic factors in vivo.

In humans, up-regulation of VEGF has been observed in intracranial meningiomas.[10] VEGF messenger RNA (mRNA) and protein expression are correlated with meningioma vascularity, and increased VEGF protein expression predicts meningioma recurrence after resection in humans.[2,11] The extent of VEGF expression does not seem to be associated with the size of the meningioma or the degree of malignancy in humans,[10,12] but some studies contradict this conclusion.[13]

In veterinary oncology, the prognostic significance of angiogenesis has been investigated for canine cutaneous mast cell tumors,[14] canine cutaneous squamous cell carcinomas,[15] canine seminomas,[16] canine mammary tumors,[17,18] and feline invasive mammary carcinomas.[19] Additionally, plasma VEGF has been investigated as a potential predictor of response to treatments such as radiation, but VEGF expression has not yet been proven to be a prognostic factor.[20]

The objectives of this study were to evaluate VEGF expression in intracranial meningiomas of dogs and to determine whether an association between expression and patient survival was present.

Materials and Methods

Formalin-fixed, paraffin-embedded biopsy specimens of intracranial meningiomas from dogs were retrieved from the archives of the Pathology Unit, Centre for Preventive Medicine at the Animal Health Trust (2000–2004). The excisional samples had been surgically obtained by rostrotentorial craniectomy in all dogs, after diagnosis by magnetic resonance imaging. All the patients had undergone postsurgical hypofractionated radiotherapy, modified from that previously described.[21] In brief, each patient had received 5 once-weekly fractions (5, 8, 8, 8, and 8 Gy) for a total dose of 37 Gy by means of a 4-MeV linear accelerator at the Queen's

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Reprint requests: Simon R. Platt, Lanwades Pk., Kentford, Newmarket, Suffolk CB7UU, UK; e-mail: Simon.platt@aht.org.uk.

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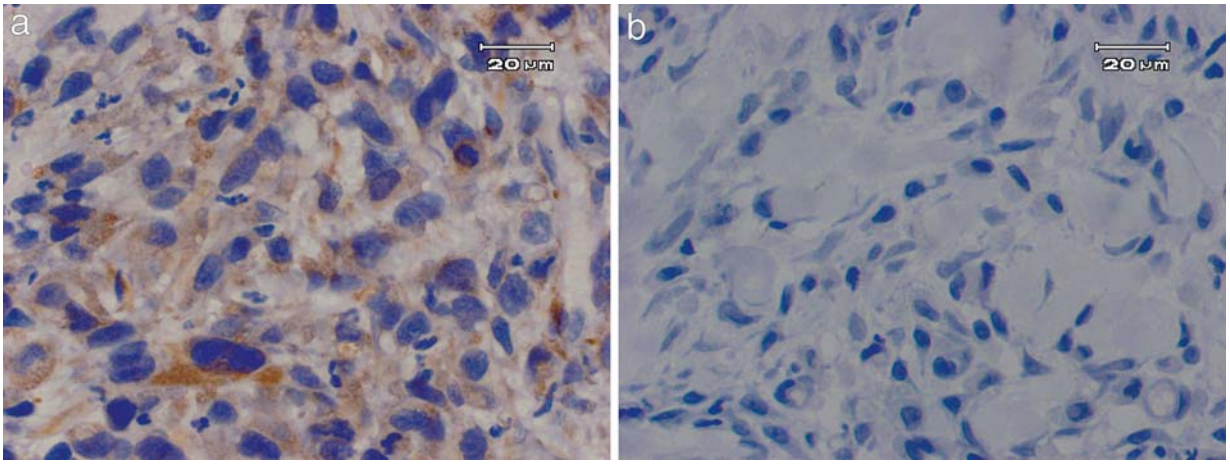

Fig 1. **(a)** Positive control for immunohistochemical detection of vascular endothelial growth factor (VEGF) expression. Reactive endothelial cells in a section of canine granulation tissue exhibit moderate positive cytoplasmic staining for VEGF. **(b)** Negative control for immunohistochemical detection of VEGF expression. No positive immunohistochemical staining is evident by reactive endothelial cells in this section of canine granulation tissue when anti-VEGF antibody is replaced with antibody diluent.

Veterinary Hospital, University of Cambridge. The protocol, the only one available in the UK at the present time, used a 4 × 4-cm field with 3 portals with the isocenter over the tumor's center. All patients had been treated presurgically with corticosteroids at anti-inflammatory dosages. Dogs received follow-up until the time of death.

Overall survival time was defined as the time from the day of diagnosis until the time of death.

The 5-µm tumor sections taken from each paraffin block were stained with hematoxylin and eosin for histologic evaluation. All tumors were confirmed to be benign meningiomas.[a]

The 5-µm sections were mounted onto positively charged, capillary gap glass slides. Immunohistochemical staining for VEGF immunoreactivity was performed with an automated staining system.[b] The positive control tissues for VEGF expression were formalin-fixed, paraffin-embedded sections of canine cutaneous granulation tissue (Fig 1a). For negative control tissues, the primary anti-VEGF antibody in the canine cutaneous granulation tissue was replaced with antibody diluent[c] (Fig 1b). Antigen retrieval was performed by heating the mounted sections in a microwave oven for a total of 10 minutes in a citrate buffer, pH 6.0.[d] Endogenous peroxidase activity within the tissue sections was blocked with hydrogen peroxide,[e] and then sections were incubated for 30 minutes with a rabbit anti-human VEGF polyclonal antibody[f] diluted 1 : 150 with antibody diluent.[c] Detection of primary antibody binding was performed by a 2-layer method.[g] First, chromagen was developed with diaminobenzidine, and then slides were counterstained with hematoxylin.

VEGF expression in each slide was assessed with light microscopy as follows. After the entire stained section was scanned at 40× magnification, 5 representative nonadjacent and non-overlapping fields from each tumor were selected. At 400× magnification, the percentage of positive cells in each field (counting at least 100 cells) was evaluated. The extent, intensity, and distribution of VEGF staining for each section were assessed by means of a semiquantitative scale. The extent of VEGF staining was assessed by estimating the percentage of neoplastic cells exhibiting positive staining for VEGF (Fig 2a,b). The extent of staining was arbitrarily grouped as <25%, 25–50%, 51–75%, and >75%. On the basis of the low number of cases in the first few groups, results were expressed as ≤75% or >75%. The intensity of immunohistochemical staining was subjectively assessed on a scale of 0 to 3. The distribution of positive immunohistochemical staining was defined as diffuse, patchy, or multifocal.

Survival was analyzed by the Kaplan-Meier procedure. Survival rates among groups were compared by log-rank tests with the significance set at $P \leq .05$.

Results

The mean age of the 17 dogs with benign meningiomas included in this study was 9.6 ± 2.3 (range 5.4–12.8 years). Nine (53%) dogs were neutered females, 3 (17%) were neutered males, 3 (17%) were intact males, and 2 (12%) were intact females. At the time of follow-up, 3 (17%) of the dogs were still alive and 14 (83%) had died. The mean and median survival times of the dogs were 537 and 434 days, respectively (range 88–1,298 days). VEGF expression was detected in all 17 tumors, with >50% of cells in 15 (88%) and >75% of cells in 10 (59%) of the tumors exhibiting positive immunohistochemical staining. Fourteen (83%) tumors exhibited diffuse staining distribution, and 3 (17%) tumors exhibited patchy distribution.

Shorter survival times were associated with greater VEGF expression (Table 1). The extent of staining was associated with survival ($P = .01$, Fig 3), with the median survival time of 748 days for tumors with ≤75% of cells staining compared with 442.5 days for tumors with >75% of cells staining. The intensity of staining also was associated with survival ($P = .03$), with more intense-staining tumors having a mean survival of 425 days as compared with 728 days for less intense-staining tumors (Fig 4). The distribution of staining was not significantly associated with survival ($P = .4$).

Discussion

In this study, we qualitatively examined VEGF expression in intracranial meningiomas of dogs and its relationship with survival after surgical resection and radiation therapy. VEGF was strongly expressed in the majority of canine meningiomas, and shorter survival was associated with increased VEGF expression within the tumors.

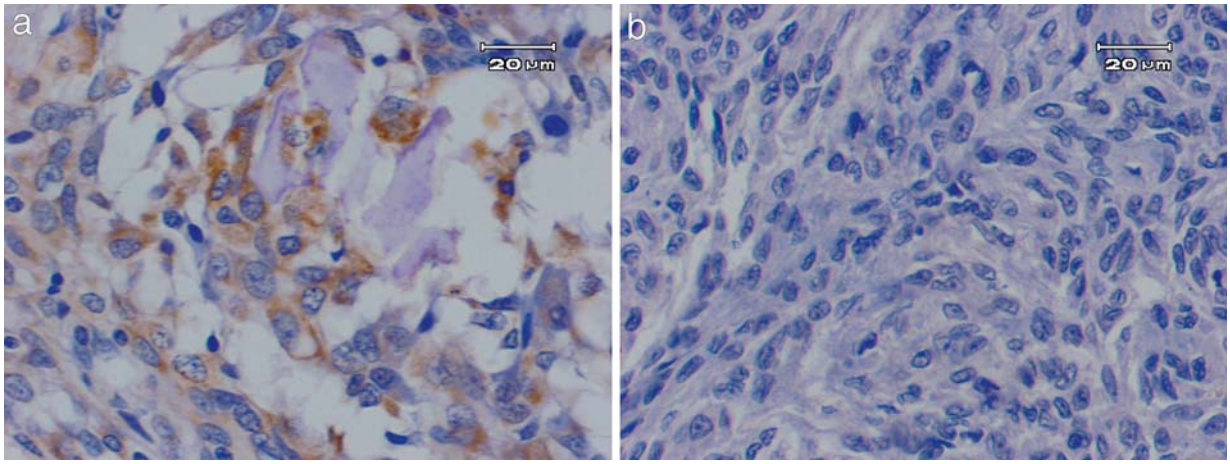

Fig 2. **(a)** The majority of neoplastic cells within a meningioma exhibit moderate positive cytoplasmic immunohistochemical staining for VEGF. **(b)** No positive immunohistochemical staining is evident in neoplastic cells within a meningioma when the anti-VEGF antibody is replaced with antibody diluent.

Meningiomas are common central nervous system tumors in dogs, with the majority being intracranial and arising from arachnoid cells. Histologically, these tumors are diverse because of their ability to undergo both mesenchymal and epithelial differentiation. In humans, 15 distinct meningeal variants with 3 grades of malignancy have been classified by the World Health Organization (WHO).[a] In domestic animals, 9 histologic patterns have been described, with 7 of the patterns similar to those of the variants included in the classification system for human meningiomas.[22] Of the 9 animal patterns, most are classed as benign meningiomas, similar to those in the classification scheme for human meningiomas. Benign meningiomas were evaluated as a group in this study because of the low numbers of the different subtypes of benign meningiomas, such as meningothelial and fibrous. This approach could introduce an error when interpreting the results because VEGF expression has been found to differ by a factor of 10 among subtypes in humans, possibly demonstrating varying dependence on angiogenesis.[13]

Increased VEGF synthesis is considered one of the first expressions of the ''angiogenic switch'' in tumors and contributes to the acquisition of malignant characteristics, such as rapid growth and metastasis.[23] Expression of VEGF is regulated by numerous factors.[1] Potent stimulators of VEGF expression include insulin, growth factors and cytokines including platelet-derived

Table 1. Survival times associated with vascular endothelial growth factor (VEGF) expression.

				Survival Time		
VEGF Expression	Dogs	Censored	Events	Median	95% CL	*P* Value[a]
Extent[b]						.03
<25%	1	0	1	[780][c]	NA[c]	
25–50%	1	0	1	[315][c]	NA[c]	
51–75%	5	3	2	612[d]	536, –[e]	
>75%	10	1	9	442.5	185, 478	
Extent[b]						.01
≤75%	7	3	4	748	536, –[e]	
>75%	10	1	9	442.5	185, 478	
Intensity						.03
+/1	8	3	5	748	536, –[e]	
++/2	9	1	8	425	185, 478	
Distribution						.4
Patchy	3	1	2	792	324, –[e]	
Diffuse	14	3	11	474	217, 748	
Total	17	4	13	434	324, 748	

CL, confidence level; NA, not applicable.

[a] *P* value for log-rank test with 3 *df* for extent with four categories and 1 *df* for all other variables.

[b] Extent is the percent of cells staining, grouped as original 4-category variable and as 2-category variable.

[c] Not applicable because survival time is only for 1 case.

[d] Mean survival time (SE = 24) because median survival time was not reached.

[e] Upper confidence limit is unbounded because of small sample size.

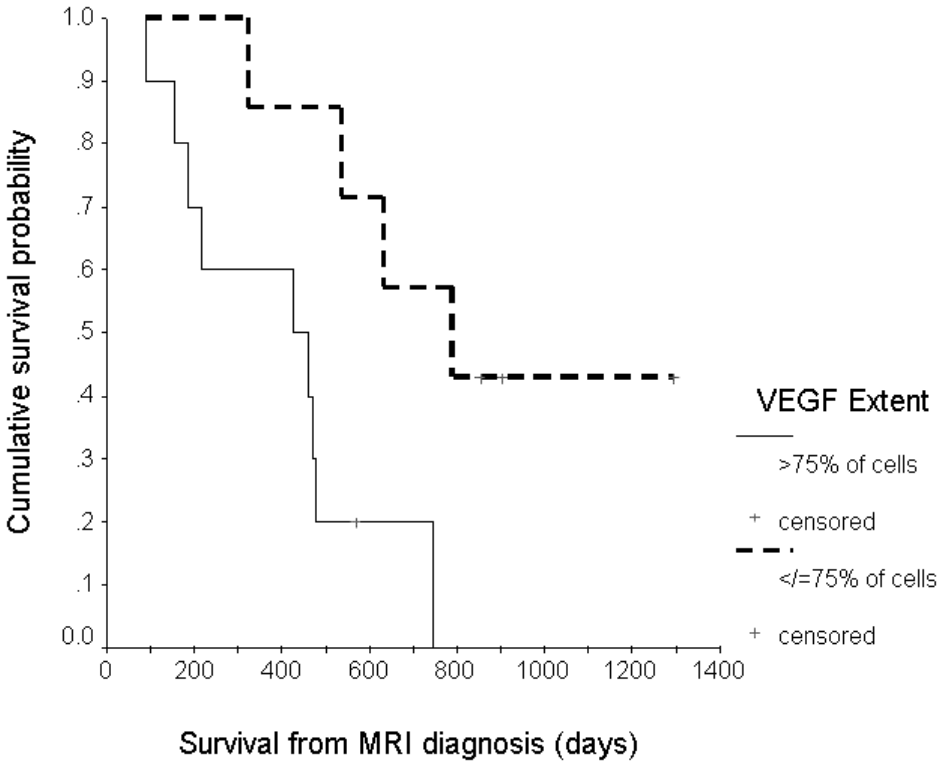

Fig 3. Kaplan-Meier survival curves for the extent of vascular endothelial growth factor staining (17 dogs).

growth factor, epidermal growth factor, tumor necrosis factor-α, interleukin-1β, interleukin-6, and nitric oxide.[1] Induction of VEGF expression by hypoxia probably is the most important of these factors.[1] Overexpression of VEGF can enhance the invasive potential of tumors and is associated with poor prognosis for several solid tumors.[24]

Meningiomas characteristically are highly vascular tumors, but regional heterogeneity in the degree of vascularity exists even within individual meningiomas.[12]

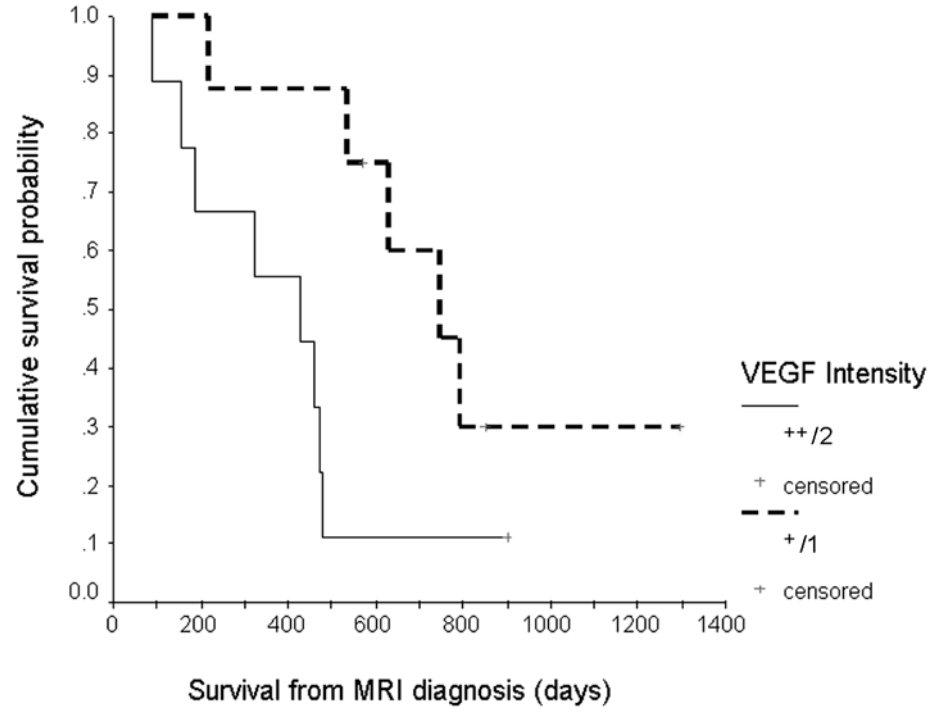

Fig 4. Kaplan-Meier survival curves for the intensity of vascular endothelial growth factor staining (17 dogs).

Up-regulation of VEGF and increased expression of VEGF-*B* have been observed in human meningiomas.[10] VEGF mRNA and protein concentrations are correlated with meningioma vascularity, and high VEGF expression can predict meningioma recurrence after resection in humans.[2]

The prognostic value of VEGF expression in human meningiomas, however, is controversial.[11–13] In our study, despite its relatively small sample size, a significant difference in survival was noted among dogs with different tumoral VEGF expression. This apparent species difference in the prognostic usefulness of VEGF staining in meningiomas may reflect the different treatment modalities used for humans and dogs.

The link between VEGF up-regulation to hypoxia in the most central or necrotic components of the tumor is suggested by the fact that VEGF expression is highest in tumors with widespread necrosis.[25] Hypoxia can increase transcription of the VEGF-encoding gene and can stabilize its mRNA product, which may be responsible for the presence of strong VEGF immunolabeling near necrotic regions of malignant tumors.[26] The hypoxic response could also act synergistically with other tumor-related factors, including cytokines such as tumor necrosis factor-α produced by macrophages and other inflammatory cells, to up-regulate VEGF in neoplasms.[16] We did not control for the presence or absence of necrosis, and no information was available on where within the tumor the specimen was collected. Therefore, additional study is necessary to prospectively evaluate the density of VEGF expression in canine meningiomas in the tumoral center compared with its periphery before we can conclude that the VEGF expression at any given site is representative of the tumor as a whole.

The growth of meningiomas seems less dependent on up-regulation of VEGF by hypoxia and tumor necrosis and may be related to stimulation by the estrogen and progesterone system.[1] Estrogen and progesterone receptor expression has been detected in human and canine meningiomas.[27,28] Progesterone receptor expression was found to be lowest in canine meningiomas with a high proliferation index, as determined by immunohistochemical detection of proliferating cell nuclear antigen, demonstrating a significant indirect relationship.[27,28] Progesterone receptor expression also was found to be predictive of survival in dogs with meningiomas after surgical resection and radiotherapy.[28]

All patients in this study received corticosteroids as part of their treatment regimen. Corticosteroid use has been shown to reduce VEGF expression, and steroids may reduce peritumoral edema in humans by this effect.[12] Biopsy specimens collected from veterinary patients before they receive steroids may have higher VEGF expression, but this hypothesis remains to be investigated.

Recent investigations demonstrated that the molecular pathways and function of the VEGF signaling system are virtually identical in humans and dogs, both in health and in disease.[29] On the basis of this knowledge and our study, we plan to continue investigating the diagnostic and prognostic value of VEGF in intracranial tumors of dogs.

Footnotes

[a] World Health Organization Classification of Tumors: Pathology and Genetics: Tumors of the nervous system. Kleihues P, Cavenee WB, eds. Lyon, France: IARC Press, 2000

[b] Techmate DakoCytomation, Ely, UK

[c] ChemMate Antibody Diluent, DakoCytomation, Ely, UK

[d] ChemMate Target Retrieval Solution, DakoCytomation, Ely, UK

[e] ChemMate Peroxidase Peroxidase Blocking Solution, DakoCytomation, Ely, UK

[f] sc-152, Santa Cruz Biotechnology, Inc, Santa Cruz, CA

[g] Envision system, DakoCytomation, Ely, UK

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