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Plasma Vascular Endothelial Growth Factor (VEGF) Measured in Seventy Dogs with Spontaneously Occurring Tumours

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Abstract. *Background: Vascular endothelial growth factor (VEGF) acts specifically on endothelial cells mediating tumour neovascularisation and initiating tumour growth and metastasis. In humans, high VEGF levels are correlated with poorer prognosis but in dogs minimal information on plasma VEGF is available. Therefore, we analysed plasma VEGF in a variety of spontaneous canine tumours. Materials and Methods: Plasma from seventy dogs with various spontaneous tumours was taken prior to radiation therapy. A human VEGF ELISA was used for analysis. Results: Mean plasma VEGF was 7.2±7.8 pg/ml. Mean plasma VEGF level varied among different tumour types with the highest level in oral melanomas (12.4 pg/ml). In patients with sarcomas of soft tissue or bone origin, plasma VEGF levels increased significantly with decreasing haemoglobin concentration (p=0.013). Conclusion: Canine plasma VEGF levels depend on tumour histology, with higher levels found in more aggressive tumours. The negative correlation between plasma VEGF and haemoglobin (hb) is most probably due to tissue hypoxia seen in anaemic animals.*

Under normal physiological conditions, angiogenesis is a tightly controlled process, whereas dysregulation of vascular endothelial cells is a key feature in tumour development (1). Angiogenesis is necessary for tumour growth and tumour invasion. Vascular endothelial growth factor (VEGF) represents one of the most potent agents promoting tumour angiogenesis. VEGF has angiogenic, mitogenic and vascular permeability enhancing activity specific for endothelial cells (2). It promotes proliferation of endothelial cells, resulting in sprouting of vessels from pre-existing microvessels. These effects are mediated through the high affinity receptors VEGFR-1/Flt-1, VEGFR-2/Flk/KDR and VEGFR-3/Flt-4 (3,4). VEGF is involved in development and growth of a wide variety of different tumours (5,6) and plays a role in the formation of distant metastasis (7-9).

VEGF has been measured in serum and plasma of tumour patients. VEGF is actively secreted from tumour tissue and its soluble form ($VEGF_{165}$) is detectable in the blood compartment. VEGF is stored in platelets and it is released during blood clotting, resulting in increased VEGF concentration in serum (10,11).

Plasma VEGF correlates to stage of disease in human cancer patients and high plasma VEGF levels did correlate with unfavourable prognosis (5,12). In carcinoma and sarcoma patients serum/plasma VEGF levels from high stage disease are significantly higher than from benign or pre-cancerous lesions (6,13-15). Consequently, serum/plasma VEGF was highest in patients with distant metastasis. Therefore, elevation of VEGF in the blood compartment seems to be correlated with disease progression.

VEGF has been measured in different tumour types. A mean plasma VEGF level of 49.2±85 pg/ml was found in patients suffering from different carcinomas (16). In hepatocellular carcinoma a mean plasma VEGF level of 34.4±27.0 pg/ml was seen (12). In sarcoma patients only serum VEGF was analysed and a mean serum VEGF level of 580.0 pg/ml was found. Specifically in osteosarcoma patients, a mean serum VEGF level of 232.0 pg/ml was detected (17).

One major stimulus for increased VEGF expression is hypoxia (18). Recently, an association between tumour hypoxia and increased systemic levels of VEGF has been demonstrated in head and neck cancers (19). Anaemia, even if it is mild, can worsen tumour oxygenation and can lead to elevated tumour hypoxia (20). An association between anaemia and elevated levels of plasma VEGF was found in human patients (21).

Hypoxia can lead to up-regulation of VEGF through the hypoxia inducible factor-1 (HIF-1). In hypoxic cells the heterodimer HIF-1 can bind to DNA at specific regions. When HIF-1 binds to these hypoxia-responsive elements (HREs) the transcription of several genes occurs (18). Liu *et al.* identified a

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Table I. *Tumour histology, tumour stage, volume of primary tumour and number of animals.*

Tumor histology	Stage of disease (N)				Tumor	N°
	I	II	III	IV	volume (cm^3)	Total
Sarcoma	7	6	14	2	68.3±87.1	42
Fibrosarcoma	4	3	4	--	29.5±20.1	11
Osteosarcoma	1	1	5	--	67.5±77.4	15
Sarcoma, other	2	2	5	2	95.6±113.7	16
Histiocytosis, malig.	--	--	--	--	89.3±57.3	5
Carcinoma	4	3	2	1	65.5±106.6	14
Melanoma	1	--	4	1	28.7±49.1	6
Epulis	2	1	--	--	25.9±42.5	3

hypoxia-responsive enhancer in the promotor region of the VEGF gene where HIF-1 can bind. This is sufficient to mediate up-regulation of VEGF transcription (22).

Spontaneous canine tumours have been proposed to be a good model for cancer research (23) especially in the field of tumour angiogenesis. Interestingly, canine VEGF is 95% identical to human isoforms. In the loop regions, responsible for receptor binding, the sequences are completely identical (24). Spontaneous tumours are common in dogs. Forty-five percent of all dogs older than ten years are expected to die from cancer. On average, dogs develop cancer twice as frequently as men (25).

This study was to investigate plasma VEGF levels in dogs with spontaneous neoplasm.

Table II. *Mean plasma VEGF in various tumour histologies.*

Tumor histology	Plasma VEGF (pg/ml) Mean
Sarcoma	6.2±6.8
Fibrosarcoma	4.1±5.6
Osteosarcoma	9.5±7.9
Sarcoma, other	4.7±5.6
Histiocytosis, malig.	0.8±1.4
Carcinoma	10.8±8.1
Melanoma	12.4±12.6
Epulis	0.9±1.3

Materials and Methods

Patient selection. Seventy tumour-bearing dogs were included in this study. The mean age of patients was 9.1 years (range 3-16 years). The mean weight was 30.6 kg (range 5.1-66 kg). There were 43 male dogs and 27 female dogs (14 neutered males and 9 neutered female dogs). All patients were staged based on the World Health Organization (WHO) system. The staging included a complete physical examination, thoracic radiographs, biopsies of the primary tumour, fine-needle aspiration of enlarged lymph nodes and, if indicated, further diagnostic work up was done. Tumour diagnosis and grading was determined by routine histopathology. The length, width and depth of tumours were measured and the volume was calculated by using the rotation ellipsoid formula ($\pi abc/6$). Patients with sarcoma of soft tissue or bone origin (n=42), carcinomas (n=14) or oral melanomas (n=6) as well as three dogs with an epulis were included in the study. Patients entered in the study were either stage I (n=14), stage II (n=10), stage III (n=22) or stage IV (n=5). For 19 patients no staging system was applicable. The mean tumor volume was 63.6 cm^3 (range 0.1-392.5 cm^3). Detailed data are given in Table I.

Haematologic parameters of all patients were recorded. The mean red blood cell count (RBC) was $6.5x10^6/\mu l$ (range 3.7-$12.4x10^6/\mu l$). The mean packed cell volume (PCV) was 42.6% (range 26-55%). The mean haemoglobin (hb) level was 14.7 g/dl (range 6.1-20.1 g/dl). There was no difference in RBC, PCV or hb levels in different tumour groups.

Sample preparation. Blood samples were taken prior to treatment. Three millilitres of blood were collected into sterile CTAD tubes (Beckton and Dickinson Vacutainer System, France) and placed on ice immediately. CTAD tubes contained sodium citrate, theophyllin, adenosine and dipyridamine allowing maximal platelet stabilisation. The tubes were centrifuged within 15 min at 2500 x g for 30 min at 4°C. The resulting plasma was separated and stored immediately at -80°C.

VEGF-assay. VEGF concentration was determined by using the Human VEGF enzyme linked immunosorbent assay (ELISA, R&D System Inc., Abingdon, United Kingdom) designed for detection of $VEGF_{165}$. This ELISA has already been proven reliable for plasma VEGF evaluations in dogs in recent studies (26,27). After thawing, every aliquot was assayed twice and the mean value was taken for statistical analysis. The VEGF ELISA was accomplished using the protocol from the manufacturer.

Statistical analysis. Statistical analysis (StatView Version 4.0 statistical software application, Abacus concept) was performed using the Mann-Withney *U*-test, the ANOVA test and the Pearson correlation. Plasma VEGF levels showed a skewed distribution, therefore they were transformed logarithmically before analysis (plasma ln VEGF). The independent variables; tumour volume, stage of disease, tumour histology and hb were correlated to plasma VEGF. *P* values lower than 0.05 were considered significant.

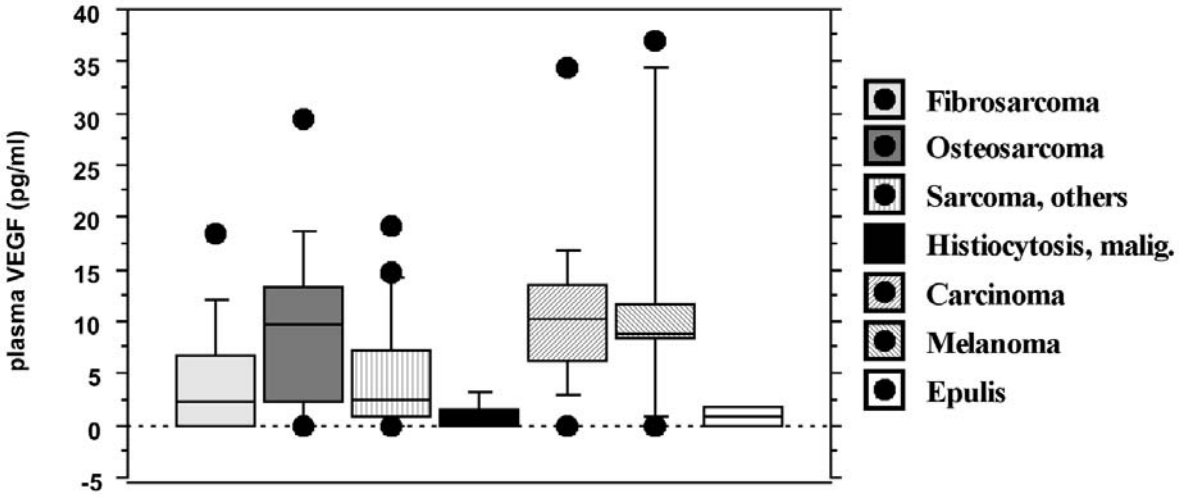

Figure 1. *Plasma VEGF was measured from 70 dogs. The box plots show plasma VEGF levels separated according to tumour histology. Plasma VEGF values were significantly different in the analysed tumour groups ($p=0.02$, ANOVA test). The lowest mean plasma VEGF level was found in epulis (0.9 pg/ml) and the highest level in oral melanoma (12.4 pg/ml).*

Results

Plasma from seventy tumour patients with various tumour types were analysed for plasma VEGF. The mean plasma VEGF level of all tumour patients was 7.2 pg/ml±7.8 pg/ml (mean±SD) with a 95% confidence interval from 5.3-9.0 pg/ml (Table II). Comparison of age, gender and weight to plasma VEGF were not significant.

Plasma VEGF levels in different tumour types differed significantly ($p=0.02$), with the lowest mean plasma VEGF level in epulis (0.9 pg/ml) and the highest level in oral melanoma (12.4 pg/ml) (Figure 1, Table II). The correlation between plasma VEGF and tumour volume was not significant ($p=0.62$). There was also no association between plasma VEGF levels and stage of disease ($p=0.29$). Analysing stage of disease or tumour volume separately for tumour groups did not reveal a statistically significant result either.

The analysis, however, showed that sarcoma patients with low haemoglobin had significantly higher plasma VEGF levels compared to normemic patients ($p=0.013$) (Figure 2). This was not seen in dogs with carcinoma or melanoma. For further analysis, patients were separated in three groups according to their hb level. The first group had marked anaemia (hb: < 12 mg/dl, n=9), the second group had mild anaemia (hb: 12-14mg/dl, n=21) and the third group had a normal to elevated hb concentration (>14mg/dl, n=40). Mean plasma VEGF for these groups were not statistically different (Table III).

Discussion

Spontaneously arising canine tumours have comparable histologies and the biological behaviour is similar to their human counterpart (23). Thus, it is likely that VEGF also plays a role in the development and growth of canine tumours.

VEGF is not only expressed by tumour tissue. Another major source of VEGF are platelets (28). VEGF stored in platelets is released during blood clotting. Several studies have proposed the use of plasma VEGF to minimize artifactual increase of VEGF due to sample handling and blood clotting. CTAD tubes are proposed to stabilize platelets and to minimize VEGF release. Plasma VEGF is thought to mostly reflect tumour-released VEGF (28,29). Due to this fact, plasma VEGF concentrations measured in this study are lower than published in other studies, where no CTAD tubes were used. In a previously published study we found increased plasma VEGF levels in dogs with spontaneous tumours compared to healthy dogs (30).

In people, high VEGF levels have been associated with poor outcome and higher incidence of metastasis. These results strongly suggest that VEGF may be involved in growth and invasion of the primary tumour as well as in the growth of metastasis (1). Significantly higher VEGF levels in stage III and IV compared to pre-cancerous lesions and stage I disease were found (12,14). However, in these studies a difference between stage II and stage III/IV was not detectable. In our study, the plasma VEGF level from patients with stage I-IV was compared and there was no significant increase of plasma VEGF with advancement in stage of disease. Plasma VEGF varied widely in our rather small study group and therefore the difference in mean plasma VEGF between stages was probably too small to reach statistical significance.

Since histologically different types of tumours also differ in their biological and clinical behaviour, we separated patients into different groups according to tumour type.

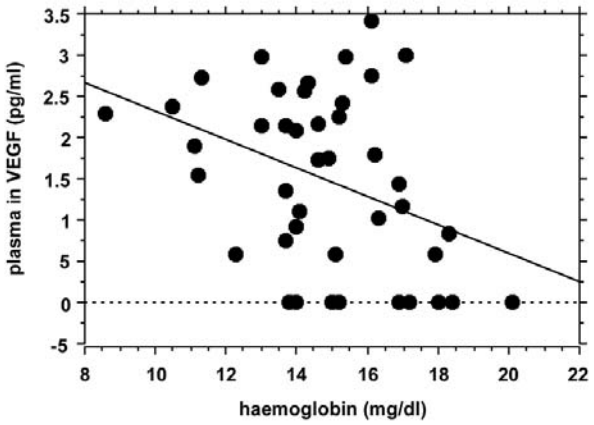

Figure 2. *This graph shows plasma VEGF (pg/ml) and haemoglobin levels (g/dl) from dogs with different types of sarcomas. Plasma VEGF and haemoglobin levels showed a negative correlation. Dogs with a low haemoglobin level had significantly higher plasma VEGF levels compared to normemic patients (p=0.013).*

Plasma VEGF levels were analysed separately for tumour histology. Low levels of plasma VEGF were found in dogs with epulis, a benign tumour with a minimal metastatic potential. In contrast, high plasma VEGF levels were found in more aggressive tumours, such as oral melanoma, carcinoma and osteosarcoma. Oral melanomas are known to behave aggressively in dogs and metastasise at an early stage of disease (31,32). The same is true for osteosarcoma (33). Our data of plasma VEGF indicate a close relationship between plasma VEGF and aggressiveness of tumour. These data might be useful since a comparison of oral melanoma, carcinoma and sarcoma has not been performed yet. It has been shown that plasma VEGF in carcinoma with different loco-regional appearance did not differ according to the location of the tumour (21). This might be due to the fact that the biological behaviour of carcinomas is similar. Serum VEGF in childhood soft tissue sarcoma differed according to tumour histology e.g. ostesarcomas had mildly elevated serum VEGF levels.

In our study, the analysis of hb and plasma VEGF in sarcoma patients revealed an increase of plasma VEGF when haemoglobin decreased. Anaemia had the potential to increase tumour hypoxia in an animal model (35,36). Tumour hypoxia in patients with low haemoglobin levels resulted in increased VEGF expression (21). Tumour hypoxia occurs frequently in human solid tumours (16,18) and tumour hypoxia was also found in spontaneous canine tumours (37). In conclusion, data showed that anaemia has an impact on tumour VEGF expression. This is important since tumour hypoxia is correlated with poor treatment outcome (38). The prognostic relevance of plasma VEGF in spontaneous canine tumours should be further analysed.

Table III. *Patients grouped according to haemoglobin concentration and corresponding mean plasma VEGF.*

Haemoglobin (g/dl)	Plasma VEGF (pg/ml) mean	N
< 12 mg/dl	7.2±4.0	9
12-14 mg/dl	8.0±9.3	21
> 14 mg/dl	6.9±7.9	40

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