Vascular endothelial growth factor in prognosis of splenic malignant tumours in dogs

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Abstract

The aim of the study was to determine the levels of the vascular endothelial growth factor (VEGF) in the serum of dogs suffering from splenic malignant tumours, prior to splenectomy, as well as three and six months after the surgery. Tumours and blood samples were collected from 10 dogs of various breeds, aged between 7 and 13 years, and from 10 control animals. Tumour sections were fixed in 10% buffered formalin for 24 h. The type of tumour was determined according to the WHO classification. Blood samples were centrifuged and the obtained sera were subjected to immunoenzymatic assays to determine the VEGF levels. The median of VEGF levels in the serum of dogs suffering from splenic malignant tumours was 37.85 pg/mL (15.40–107.18 pg/mL). The highest values were observed in dogs with confirmed metastases (107.18 pg/mL and 65.43 pg/mL). The VEGF values in control group were between 0.1 pg/mL and 13.04 pg/mL. A comparative analysis of the VEGF levels against the animals' survival time indicated that VEGF overexpression may serve as a prognostic factor in cases of malignant tumours of the spleen.

Key words: dog, vascular endothelial growth factor, splenic malignant tumour.

Introduction

Nowadays, splenic malignant tumours in dogs are relatively common. They can develop as primary tumours or through metastases. The most often diagnosed splenic malignant tumours in carnivores include: haemangiosarcoma, lymphoma, and sarcoma, whereas the less common benign tumours are: haemagioma, lipoma, and myoma (13).

Haemangiosarcomas are tumours originating from vascular endothelial cells. They belong to the group of slowly developing tumours and can typically reach the size between several to over a dozen millimetres before diagnosis. Often internal haemorrhages or necrosis spots are observed within the tumour mass. Haemangiosarcoma metastases usually occur in the liver and lungs (12).

Splenic lymphomas typically occur in the form of multifocal lymphomas, in which lesions may extend beyond the spleen to lymph nodes, liver, and bone marrow (14). Fibrosarcoma and anaplastic sarcoma belong to other types of tumours found in splenic parenchyma. They usually occur in the form of single protuberances appearing on the organ's surface. The clinical symptoms of spleen tumours depend on their particular location and the size of the lesion itself. Non-specific symptoms are common and may include fatigue, abdominal expansion, vomiting, pallor of the mucous membrane, extensive emaciation, circulatory and respiratory disorders, and haematological changes such as leukocytosis, anaemia or thrombocytopenia (15).

Tumour rupture may result in the animal's sudden death among symptoms of shock. Clinical diagnosis is performed on the basis of a clinical, ultrasonographic, and/or radiological examinations, but final diagnosis is based on histopathological examination. The recommended course of treatment in cases of splenic malignant tumours usually involves splenectomy (13).

Splenic tumours are well vascularised. Numerous studies have described the formation of tumour own vascular network in the process known as angiogenesis, which conditions the development of the tumour and indicates the occurrence of symptoms. In tumours lacking their own network of blood vessels, an equilibrium is maintained between cell apoptosis and
proliferation. However, the balance can be disturbed due to overproduction of factors stimulating angiogenesis, i.e. VEGF, FGF, etc.

VEGF - vascular endothelial growth factor - is an endothelial cell mitogen and factor increasing blood vessel permeability. Its activity is not only angiogenic, it also acts as an autocrine factor directly stimulating the proliferation of primary cancer cells, thus initiating the process of metastasis or relapse (2, 17). Increased VEGF expression related to negative prognoses was observed in the cases of various malignant tumours in humans and animals (10, 16, 18). Elevated VEGF levels were noted in relation to myeloid leukaemia, where they correlated with lower patient survivability and lower incidence of full remission (10). VEGF levels were also studied in dogs suffering from haemangiosarcoma. Elevated levels were reported in dogs diagnosed with tumours, although no correlation was observed in terms of advancement of the disease or tumour size (4). Similar results were reported by Wergin and Kaser-Holtz (23), who demonstrated that the level of VEGF were undetectable in healthy dogs, while in dogs suffering from tumours, the obtained results were statistically significant (P = 0.008) and were within the range of 7.43 ± 11.2 pg/mL. Aresu et al. (1) found increased VEGF levels in the serum of dogs suffering from lymphoma in comparison to healthy animals. Furthermore, the researchers reported that the levels decreased during chemotherapy in dogs with B-cell lymphoma, but remained unchanged in the case of T-cell lymphomas. VEGF expression was also reported by Wolfsberger et al. (24) in 60% of the analysed cases of canine lymphomas.

The aim of this study was to determine the levels of VEGF in the serum of dogs suffering from splenic malignant tumours prior to splenectomy, as well as one and six months after the surgery, and to compare them with values obtained in a control group of healthy dogs brought to the clinic for sterilisation.

Material and Methods

The research material comprised tumour and blood samples collected from 10 dogs of various breeds, aged between 7 and 13 years, undergoing splenectomy in the course of treatment for splenic malignant tumours (Figs 1, 2).

The dominant clinical symptom in most of the dogs was obstinate vomiting. USG examinations revealed foci of proliferative disorders in the spleen. All patients underwent X-ray examinations to exclude or confirm the presence of cancerous metastases to the lungs.

After each splenectomy, the sampled material was delivered to the Department of Pathological Anatomy at the Faculty of Veterinary Medicine, University of Life Sciences in Lublin. Tumour samples were preserved in 10% buffered formalin for 24 h.

The tissue samples stained with haematoxylin and eosin were examined to determine the type of tumour according to the applicable WHO classification (8, 20). Control group comprised 10 healthy dogs, aged between 2 and 7 years, brought to the clinic for sterilisation.

Blood was collected from all dogs in order to obtain serum for immunoenzymatic assays.

In order to monitor the VEGF levels in dogs suffering from splenic tumours, blood was collected three times: before surgery (VEGF "0"), one month after surgery (VEGF "I"), and six months after surgery (VEGF "II"). VEGF level was determined with the use of the ELISA (Quantikine Canine Immunoassay, R&D Systems). On every stage of VEGF determination, the assay was performed in accordance with the manufacturer's guidelines.

Statistical analysis was conducted to verify any significant correlations between the level of VEGF of sick animals and the control group, and to determine whether VEGF values may be useful in monitoring the health of an animal in the post-surgery period at least for six months. The Mann-Whitney test was used to verify the significance of observed discrepancies between the two groups when the variables were not normally distributed.
**Results**

Histopathological examinations confirmed the malignant character of all studied tumours. Seven cases of haemangiosarcoma and three cases of malignant lymphoma were diagnosed. In two cases, X-ray examinations revealed the presence of proliferative lesions in the lungs and liver.

Detailed results of histopathological and immunohistochemical examinations are presented in Table 1. The median of VEGF concentration in dogs suffering from splenic tumours was 37.85 pg/mL (15.40–107.18 pg/mL). The highest values were observed in dogs with confirmed proliferative lesions present in other organs (107.18 pg/mL and 65.43 pg/mL) and in animals suffering from a relapse of the disease.

The VEGF values recorded in control group were between 0.1 pg/mL and 13.04 pg/mL. The median was 11.14 pg/mL (Fig. 3).

Comparison of VEGF values in the sera of affected dogs and control group indicated that the level of the growth factor was statistically significantly higher in the sera of dogs at VEGF "0" - i.e. collected prior to the surgery (Me = 37.85). A statistically significant result was also obtained when the comparison was made between the VEGF values in control group and VEGF "I" - i.e. in the blood serum tested one month after the surgery (Me = 39.45).

Tests performed on animals, which survived for six months after the surgery revealed no statistically significant differences compared to the control group (Me = 10.37 - sick dogs and Me = 11.14 - control group). The obtained results may indicate successful treatment or regression of the disease and the resulting extension of the animals' life expectancy. Detailed results are presented in Fig. 3.

A comparative analysis of the VEGF levels against animals' survival period was also performed. Half of the animals, whose health was monitored in the post-splenectomy period, died within six months after the surgery. Significant differences between the VEGF level in the group of dogs that died within six months from the surgery and the group of animals surviving that period were observed already on the day of the procedure, with different medians in the particular group (Me = 64.50 and Me = 26.52 respectively). The results may indicate that high VEGF levels (over 45 pg/mL) observed on the day of the diagnosis may be a negative diagnostic prognostic factor (Table 2 and Fig. 4).

<table>
<thead>
<tr>
<th>Dog</th>
<th>Histopathological diagnosis</th>
<th>Metastases</th>
<th>VEGF 0 (pg/mL)</th>
<th>VEGF I (pg/mL)</th>
<th>VEGF II (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rottweiler. ♂, age 10</td>
<td>haemangiosarcoma</td>
<td>none</td>
<td>63.80</td>
<td>87.51</td>
<td>died</td>
</tr>
<tr>
<td>Maltese ♂, age 12</td>
<td>haemangiosarcoma</td>
<td>none</td>
<td>15.40</td>
<td>24.12</td>
<td>30.60</td>
</tr>
<tr>
<td>Cocer spaniel. ♀, age 9</td>
<td>haemangiosarcoma</td>
<td>none</td>
<td>29.08</td>
<td>19.02</td>
<td>10.37</td>
</tr>
<tr>
<td>German shepherd ♂, age 9</td>
<td>haemangiosarcoma</td>
<td>none</td>
<td>65.21</td>
<td>307.18</td>
<td>died</td>
</tr>
<tr>
<td>Mixed breed. ♀, age 13</td>
<td>haemangiosarcoma</td>
<td>none</td>
<td>26.52</td>
<td>15.4</td>
<td>11.26</td>
</tr>
<tr>
<td>Dachshund. ♂, age 11</td>
<td>haemangiosarcoma</td>
<td>none</td>
<td>21.18</td>
<td>17.50</td>
<td>4.23</td>
</tr>
<tr>
<td>German Mastiff ♀, age 12</td>
<td>haemangiosarcoma</td>
<td>none</td>
<td>46.62</td>
<td>54.77</td>
<td>died</td>
</tr>
<tr>
<td>Mixed breed. ♂, age 11</td>
<td>lymphoma multicentric</td>
<td>proliferation in the lungs</td>
<td>65.43</td>
<td>68.59</td>
<td>died</td>
</tr>
<tr>
<td>Collie rought. ♂, age 12</td>
<td>lymphoma</td>
<td>none</td>
<td>28.30</td>
<td>15.71</td>
<td>9.49</td>
</tr>
<tr>
<td>Mixed breed. ♂, age 7</td>
<td>lymphoma multicentric</td>
<td>proliferation in the liver</td>
<td>107.18</td>
<td>128.41</td>
<td>died</td>
</tr>
</tbody>
</table>
VEGF values breakdown by group of animals

Control group

<table>
<thead>
<tr>
<th>VEGF Values</th>
<th>Median</th>
<th>25% - 75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF 0</td>
<td>-4.73</td>
<td>0</td>
</tr>
<tr>
<td>VEGF 1</td>
<td>12.45</td>
<td>5.83 - 19.08</td>
</tr>
<tr>
<td>VEGF 2</td>
<td>20.39</td>
<td>10.68 - 30.63</td>
</tr>
</tbody>
</table>

**Fig. 3.** VEGF values in healthy dogs and animals suffering from splenic malignant tumours

**Table 2.** VEGF values in dogs with different survival time after surgery

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival time &gt; six months</th>
<th>No survivors</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>VEGF 0</td>
<td>5</td>
<td>15.40</td>
<td>29.08</td>
</tr>
<tr>
<td>VEGF I</td>
<td>5</td>
<td>15.40</td>
<td>24.12</td>
</tr>
<tr>
<td>VEGF II</td>
<td>5</td>
<td>4.23</td>
<td>30.60</td>
</tr>
</tbody>
</table>

**Fig. 4.** VEGF values in dogs with different survival time after surgery (VEGF 0)
Discussion

It has been considered that VEGF is a mitogen of endothelial cells and that its overexpression in the serum, plasma, or tissues correlates with the malignant character of neoplastic lesions diagnosed in humans and animals (16). VEGF increases the permeability of blood vessels and consequently facilitates the circulatory propagation of cancerous cells and stimulates tumour growth by increasing the availability of nutrients. VEGF binds with at least three different types of receptors: VEGFR-1 (Flt-1 - fms-like tyrosine kinase-1), VEGFR-2 (KDR-kinase domain region in humans and Flk-1- foetal liver kinase-1 in mice), as well as VEGFR-3 (Flt-4 – foetal liver kinase 4), all of which belong to the receptor group containing a tyrosine kinase domain (9).

The expression of VEGF and its receptors in normal canine tissue was studied by Uchide et al. (19). The authors observed that only small fractions of smooth muscle cells in the spleen show expression of VEGF and its receptors. However, all haemangioma and haemangiosarcoma (HSA) endothelial cells showed cytoplasmic reactivity to VEGF, as indicated by the large number of positively stained cells. Similar results were obtained by Yonemaru et al. (21). They demonstrated that the percentage of cells showing VEGF expression was significantly higher in haemangiosarcomas than in haemangiomas (P < 0.05), and the expression of VEGF and Flk-1 receptor may be related to the proliferation of haemangiosarcoma cells.

Sabattini and Bettini (11) tested the presence of VEGF-C in samples of HSA and haemangiomas of the internal organs and the skin of dogs. They observed a weak growth factor expression in the case of both types of the tumour. The obtained results may provide confirmation of the affinity of the receptors for particular VEGF isoforms.

In other studies, high levels of VEGF and the VEGFR-1 expression were observed in dogs with lymphoma, while most of the studied samples displayed negative immunoreactivity towards the VEGFR-2 receptor (24). The changeability in terms of receptor expression reflects the fact that lymphoma is a heterogeneous lymphoproliferative tumour. The same researchers noted that dogs suffering from lymphoma, when treated with chemotherapy, survived for up to 266 d, but the survivability did not correlate with VEGF expression.

VEGF expression was also studied in the serum and plasma of dogs suffering from haemangiosarcoma. Significantly elevated levels of the growth factor were observed in malignant tumours, although they were not correlated with the stage of the disease progression (3, 4).

In the present research, statistically significant increase in the levels of VEGF was observed in the serum of animals with malignant spleen tumours prior to splenectomy, as compared with the results obtained in control group (P < 0.001). It can be assumed that overexpression of VEGF observed during diagnosis constitutes a prognostic factor relative to the animal’s life expectancy. The observed lack of correlation between tumour size and VEGF level may indicate dependence of the growth factor on the secretory activity of tumour cells.

Other researchers tried to determine the relationship between vascular density and VEGF expression in soft tissue sarcomas. Elevated VEGF expression was observed not only in tumour cells but also in the peritumour region. It was demonstrated that measurements of the VEGF level may provide valuable information concerning the biological qualities of soft tissue sarcomas, but its actual prognostic value requires further investigation (5). The same author reported that the VEGF was observed to decrease following sarcoma resection (6).

Similar results were obtained in the present study. When malignant tumours were diagnosed early enough in animals whose VEGF levels were elevated in comparison to the control group, but remained within the range of 21.18 to 29.08 pg/mL, the levels were observed to decrease following spleen resection and the animals' life expectancy was statistically significantly increased.

Elevated VEGF levels were also observed in haematological hyperplasia. In the case of myeloid leukaemia, the elevated VEGF level correlated with a shorter life expectancy and a lower chance of full remission (10, 22).

In human and animal lymphoma, VEGF overexpression was shown to have a prognostic value (1, 25). It was observed that patients suffering from malignant lymphoma and showing elevated VEGF levels had only a 49% chance of survival for the next five years. It was also demonstrated that the vascular growth factor and FGF, apart from influencing vascularisation, also affect haematopoiesis and the cells of the myeloid parenchyma (25).

VEGF regulation is also related to the incidence of hypoxia in neoplastic cells, therefore, VEGF expression is often particularly high in tumours showing high levels of necrosis - as is the case of splenic tumours. Hypoxia increases VEGF gene transcription rate and mRNA stability and may explain the higher expression of VEGF in neoplastic cells in the vicinity of necrosis areas (3).

Nowadays, research is focused on the role of VEGF in neoplastic growth and the possibility of treatment with antibodies against VEGF receptors located in tumour cells (7). Currently, the emphasis is put on hybrid treatments where antiangiogenic drugs are combined with chemotherapeutics and radiation (9).
It can be concluded that the discrepancies in terms of VEGF levels were observed between healthy dogs and dogs with malignant tumours. It can be also assumed that an increase in the VEGF level observed after splenectomy may, in the case of splenic haemangiosarcomas, be a negative prognostic factor in dogs.

References