Vascular endothelial growth factor A (VEGF-A) concentrations in canine malignant mammary tumours with and without metastases to regional lymph nodes

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SUMMARY

In the present study the concentrations of vascular endothelial growth factor A (VEGF-A) in canine malignant mammary gland tumours and normal mammary gland tissues were examined. The homogenates of 36 malignant mammary gland tumours (26 tumours without metastases to regional lymph nodes and 10 tumours with metastases to regional lymph nodes) and 10 samples of normal canine mammary gland tissue were used in the study. The concentrations of VEGF-A in the homogenates were determined using specific canine ELISA assay. The results obtained showed that the mean concentration of VEGF-A in the malignant mammary tumours, both with and without metastases to regional lymph nodes (65.85 ± 28.24 pg/mg protein and 23.09 ± 10.86 pg/mg protein, respectively), was significantly higher (p < 0.05) than in the normal mammary glands (11.45 ± 6.47 pg/mg protein). Moreover, the mean concentration of VEGF-A was significantly higher (p < 0.05) in the malignant mammary tumors with metastases to regional lymph nodes compared to that in the non-metastatic malignant mammary tumours. In the homogenates of mammary carcinomas, the mean VEGF-A concentration was higher in grade 3 tumours (61.96 ± 38.60 pg/mg protein) compared to that in grade 1 and grade 2 tumours (20.34 ± 7.53 pg/mg protein and 29.75 ± 17.65 pg/mg protein, respectively). A significant difference (p < 0.05) in VEGF-A concentration between grade 3 and grade 1 tumours was found. These results indicate that increasing the malignancy of canine mammary tumours and their potential for metastasis is accompanied by an increased production of VEGF-A in the tumour. This may suggest that an increased production of VEGF-A plays a role in malignant transformation and facilitates spreading of tumor cells and formatting of metastases in canine malignant mammary gland tumours.

KEYWORDS: canine mammary gland tumours, VEGF-A, female dogs, metastases
regarding these tumours remain unclear. Therefore, evaluation and understanding the pathophysiology and molecular mechanisms involved in progression and metastasis of canine malignant mammary tumours is essential, among others, to develop novel therapeutic methods for controlling this disease.

It is known that tumour cells require oxygen and nutrients to survive and proliferate (21). The supply of nutrients and oxygen takes place through the blood vessels (21, 33). One of the proteins that plays a role in the formation of new blood vessels is vascular endothelial growth factor (VEGF) (6, 17, 34). The VEGF family consists of five secreted proteins: VEGF-A (also referred to as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGH) (6, 34). VEGF-A is one of the most potent inducers of angiogenesis (21, 34). VEGF-A realizes biological functions, involving blood vessels formation, mainly via activation of VEGF receptor 2 (VEGFR-2) expressed by endothelial cells (21, 34). During tumour angiogenesis, VEGF-A secreted by tumour cells and surrounding stroma, induces endothelial cell proliferation, survival and migration. In addition, it increases microvascular permeability, which allows tumour cells to escape from the blood vessels and form distant metastases (17, 21, 40). Tumour-derived VEGF-A also has global impacts on healthy vasculatures in multiple organs and tissues (40).

The role of VEGF-A in various malignant tumours have been intensively studied in humans. Among others things, numerous studies have focused on breast cancer (7, 14, 30, 41), which shares many clinical and molecular similarities with canine mammary malignant tumours (1). Several studies on mouse models of breast cancer and breast cancer cell lines have demonstrated that VEGF-A can stimulate tumour cell proliferation, migration, invasion and survival (4). Immunohistochemical studies have found that above 70% of breast cancers are positive for VEGF-A (20). Other studies have shown VEGF-A over expression in breast cancer but low expression of VEGF-A in non-neoplastic breast tissues (4, 8). The increased expression of VEGF-A has been closely correlated with disease progression and shorter survival rate (8). A significant correlation between a high expression of VEGF mRNA and the presence of metastases in axillary lymph nodes has been observed (4). It has been found significantly higher intra-tumoral VEGF-A concentrations in breast cancer tissues than in fibromas or normal breast tissues (4). Also blood VEGF-A concentrations have been found to be higher in the women with breast cancer than in the healthy women (7, 11, 30, 41), as well as in the patient with metastatic breast cancer compared to those with non-metastatic form of this cancer (30). The metastatic breast cancer patients with elevated levels of serum VEGF-A have had significantly worse clinical outcome (7). Considering the importance of VEGF-A for carcinogenesis, VEGF-A and its receptor have become one of the most important therapeutic targets for treating various cancers in human, including breast cancer (40).

Few studies have addressed VEGF-A in female dogs with malignant mammary gland tumours. Some previous immunohistochemical studies have shown that VEGF-A expression in canine malignant mammary tumours is closely related to the aggressive characteristics of the tumour and poor prognosis (22, 25). The significantly higher level of VEGF mRNA has been demonstrated in the metastatic canine mammary tumours than that in the non-metastatic ones (19). Moreover, some authors have reported significantly higher serum VEGF-A concentrations in the dogs with neoplastic lesions, including malignant mammary gland tumours, compared to the healthy dogs (15, 18, 36). On the other hand, some authors have not found the relationship between expression or circulating level of VEGF-A and canine mammary tumour malignancy (27, 31). So far, very little is known about the tissue levels of VEGF-A in canine malignant mammary tumours. Therefore, the aim of this study was to evaluate the VEGF-A concentration in homogenates of malignant canine tumours with and without metastases to regional lymph nodes.

Material and methods

The study was performed in accordance with animal protection regulations (Animal Experimentation Act dated 15th January 2015).

Tissue samples. Thirty six malignant mammary gland tumours and 10 samples of normal mammary gland tissue were used in this study. Material for the study obtained from female dogs that underwent surgery because of spontaneously occurring mammary tumours in the Department and Clinic of Animal Reproduction, University of Life Sciences in Lublin. The samples of normal mammary gland tissue collected from healthy glands which were removed together with tumours according to regional mastectomy procedure. All tumours were 4.6 to 9.1 cm in diameter. Only primary tumours, located in the 4th (caudal abdominal) or 5th (inguinal) mammary gland, without inflammatory reaction and/or ulceration, and distant metastasis were included in the study. To rule out other diseases, all the operated female dogs were thoroughly clinically examined before surgery and routine hematological and biochemical blood tests as well as urine determinations were performed. Moreover, three-view thoracic radiographs and abdominal ultrasound examinations were performed. The mammary tumours were excised by regional mastectomy (the 4th and 5th mammary glands were removed together with the superficial inguinal lymph node). Immediately after removal the tumour was divided in half. One half of the tumour was collected and then frozen and stored at −67°C until used for VEGF-A determination. The other half of the tumour and removed lymph nodes were placed in 10% neutral buffered formalin solution and submitted to histological analysis. When no metastatic cells were detected in regional lymph nodes by haematoxylin and eosin staining, immunohistochemical techniques were performed to better determine the nodal status.

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Histological examination. Representative tissue pieces of tumour for histological examination and lymph nodes for histological/immunohistochemical evaluation were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin blocks and sliced into 4 µm sections. The microscopic preparations, stained with haematoxylin and eosin, were evaluated histologically. Tumours were classified according to the Goldschmidt et al. (13) classification. Malignant epithelial neoplasms (carcinomas) were graded according to the Nottingham method for human breast cancers (12), adopted for canine mammary tumours (23).

Immunohistochemistry. Immunohistochemical examinations using mouse monoclonal primary antibody against cytokeratin clone AE1/AE3 (M3515, Dako, Glostrup, Denmark), diluted 1:100, were carried out to detect neoplastic cells in regional lymph nodes. For immunohistochemical examinations, a system of detection of antigen-antibodies complexes was used based on secondary antibodies combined with biotin directed against mouse monoclonal primary antibodies LSAB plus, peroxidase (HRP, K0690, Dako). The enzyme labelling the reaction site was HRP conjugated with streptavidin; tetrahydrochloride-3,3-diaminobenzidine (DAB) was used as a chromogen (SK-4100; Vector Laboratories, Peterborough, UK). The sections were counterstained with Mayers’ haematoxylin.

Tumour tissue homogenization. Fragments of normal mammary gland tissues and tumour samples, measuring 1.5 cm × 1.5 cm × 1.5 cm and including intratumor and peripheral tumour area were taken for homogenate preparation. Tissue samples were washed in cold 0.9% NaCl and homogenized (4°C, 5 min, at the speed 10,000 rev/min) in phosphate buffer (0.05 M at pH 7.2) with addition of TRITON X-100 and in the presence of protease inhibitor cocktail (P2714 SIGMA; containing: AEBSF Aprotinin Bestatin E-64 EDTA, Leupeptin) using Ultra Turrax T 25 (Ikawerk Janke and Kunkel Inc., Staufen, Germany). After centrifugation (4°C, 20 min, 6000 × g) supernatants were collected and analyzed for total protein and ELISA.

Total protein determination. The total protein concentration in homogenates was measured based on biuret reaction using commercial available kit (Cormay, Lublin, Poland). The measurement of the absorbance of the samples was done in duplicate at a wavelength of 546 nm.

VEGF-A determination. The concentration of VEGF-A in the homogenates was analysed using a specific canine ELISA assay (RayBiotech, Peachtree Corners, USA) according to manufacturer’s instructions. Each sample was analysed in duplicate. The absorbance was measured using a microplate reader (LabSystems Multiskan RC) at 450 nm. Standard curve and calculated data values were performed using Genesis software (GENESIS LITE Version 3.03, Life Sciences, UK). The detection limit of canine VEGF-A was 6 pg/ml. The results were expressed as pg of VEGF-A per mg of total protein (pg/mg protein).

Statistical analysis. Statistical analysis was performed using the computer program STATISTICA version 10.0 (Statsoft, USA). The Kruskal-Wallis test followed by Mann-Whitney test with the Bonferroni correction was applied to determine significant differences in the concentrations of VEGF-A between the study groups. All values are expressed as the mean ± standard deviation (SD). Differences at p < 0.05 were considered statistically significant.

Results and discussion

Histopathological data. Among the 36 canine malignant mammary gland tumours involved in the study, predominated tubulopapillary carcinomas and complex carcinomas – 13 and 10, respectively. Seven tumours were histologically diagnosed as solid carcinomas, three as anaplastic carcinomas, and three as carcinosarcomas. Ten malignant mammary gland tumours (3 tubulopapillary carcinomas, 3 carcinosarcomas, 2 anaplastic carcinomas, 1 complex carcinoma and 1 solid carcinoma) metastasized to regional lymph nodes (Fig. 1) and 26 tumours did not metastasize to regional lymph nodes. Among 33 malignant epithelial mammary gland tumours (carcinomas), 12 were grade 1 (G1) tumours, 14 grade 2 (G2) tumours and 7 grade 3 (G3) tumours.

VEGF-A concentration. The mean concentrations of VEGF-A in the homogenates of examined mammary

**Fig. 1. Inguinal lymph node metastases:** a – small clusters of epithelial neoplastic cells (AE1/AE3 positive) in the subcapsular sinuses; b – disseminated nest-like lymph node metastasis within the parenchyma of the lymph node. Immunohistochemistry, cytokeratin AE1/AE3, Mayer’s haematoxylin counterstain. Bar = 50 µm
gland tumours and normal mammary gland tissues are presented in Table 1. The concentration of VEGF-A in the malignant mammary gland tumours, both with and without metastases to regional lymph nodes, was significantly higher (p < 0.05) than in the normal mammary gland tissues. In the malignant mammary tumours with metastases to regional lymph nodes, the concentration of VEGF-A was significantly increased (p < 0.05) compared to that in the malignant mammary tumours without metastases. In the mammary carcinomas, the concentration of VEGF-A was the highest in grade 3 tumours and differed statistically significantly from those in grade 1 (Tab. 2). There was no a significant difference in the concentration of VEGF-A between grade 3 and grade 2 carcinomas as well as between grade 2 and grade 1 carcinomas, although, the VEGF-A concentration value was markedly higher in grade 2 tumours than in the grade 1 tumours.

In dogs, studies on VEGF-A levels in malignant mammary gland tumours are very limited. To the authors knowledge, there is only one report regarding the concentration of VEGF-A in normal and neoplastic canine mammary gland tissues (39). The authors of this report, unlike our findings, have not found significant differences between the VEGF-A concentrations in the homogenates of malignant mammary tumours and healthy mammary gland tissues. Our results correspond to the results of studies on the concentration of VEGF-A in the serum of dogs with mammary tumours (18, 38). These studies have shown significantly increased serum VEGF-A concentrations in the dogs with malignant mammary tumours compared to the dogs with benign mammary tumours and the healthy dogs. The immunohistochemical studies have demonstrated a high expression of VEGF-A both in breast cancer and malignant canine mammary tumours, and low in benign tumours and normal mammary gland (4, 5, 26, 28). According to the study by Queiroga et al. (26) an increased expression of VEGF-A has been significantly associated with an infiltrative growth and the presence of necrosis. In addition, in malignant canine mammary tumours microvessel density was significantly higher in VEGF-A positive tumours compared to VEGF-A negative tumours (26). This indicates that VEGF-A plays an important role in canine mammary cancer angiogenesis.

Malignant mammary gland tumours in female dogs metastasize mainly through the lymphatic vessels and therefore metastases to the regional lymph node is an early step in metastasis of these tumours (35). Due to the lymph flow from the 4th and 5th pairs of mammary gland to the superficial inguinal lymph nodes these lymph nodes are exposed to the metastatic spread of malignant tumours located in the 4th and 5th pairs of mammary gland (24). Our findings demonstrated that the VEGF-A concentration in the tissues of tumours with metastasis to the superficial inguinal lymph nodes was significantly higher compared to the non-metastatic tumours. In agreement with our results Moschetta et al. (22), similarly as previously Kato et al. (18), have found a significantly higher serum level of VEGF-A in the dogs with malignant mammary tumours that metastasized to lymph nodes than in the dogs with these tumours without metastases. Moreover, the plasma and serum VEGF-A levels have been significantly higher in the dogs with postoperative pulmonary metastasis compared to those with no such metastasis (18). Significantly higher serum/plasma VEGF-A concentrations in the women with metastatic breast cancer compared to the women without metastasis have also been reported (2, 9). The metastatic breast cancer patients with elevated concentrations of serum VEGF-A have significantly worse clinical outcome (7). Kim et al. (19) have found a significantly higher level of VEGF mRNA in the metastatic canine mammary tumours than that in the non-metastatic mammary tumours. The study by Qui et al. (25) has shown a close correlation between overexpression of VEGF-A in canine malignant mammary tumours and lymph node metastases. Studies on mammary carcinoma models have demonstrated acceleration of metastatic development associated with VEGF-A overexpression (32). These findings may indicate that metastatic malignant mammary tumours are associated with increased production of VEGF-A in the microenvironment of a tumour. It is known that VEGF-A can be produced by both the tumour cells and stromal cells (21). It can be assumed that the increased level VEGF-A facilitate spreading of tumor cells and formatting of metastases. Studies on mammary carcinoma models have demonstrated acceleration of metastatic development associated with VEGF-A overexpression (32). VEGF-A may

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of samples</th>
<th>VEGF-A concentration (pg/mg of protein)</th>
<th>Kruskal-Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic tumours</td>
<td>10</td>
<td>65.85 ± 28.24</td>
<td>H = 28.701</td>
</tr>
<tr>
<td>Non-metastatic tumours</td>
<td>26</td>
<td>23.09 ± 10.86</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Normal mammary gland</td>
<td>10</td>
<td>11.45 ± 6.47</td>
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Explanations: a – the some superscripts indicate statistically significant differences at p < 0.05

<table>
<thead>
<tr>
<th>Grade</th>
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</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>12</td>
<td>20.34 ± 7.53</td>
<td>H = 8.027</td>
</tr>
<tr>
<td>G2</td>
<td>14</td>
<td>29.75 ± 17.65</td>
<td>p = 0.018</td>
</tr>
<tr>
<td>G3</td>
<td>7</td>
<td>61.96 ± 38.60</td>
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facilitate the metastatic cascade in the different ways. The data from studies on transgenic mice have shown that VEGF-A stimulates mammary tumour growth through increased neovascularization and inhibition of apoptosis (32). It is believed, that in tumour-associated angiogenesis VEGF-A plays a pivotal role (34). It stimulates endothelial cell proliferation and migration, and prevents regression of newly formed vessels (26). The presence of new blood vessel growth will provide an opportunity to form a subpopulation of tumour cells with high metastatic potential (33). VEGF-A induces vascular permeability that in case of malignant tumours may facilitate the escape of tumour cells into the blood stream, promoting the establishment of distant metastases (21). VEGF-A also mediates cell invasion by expression matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9), which degrade the basal membrane and extracellular matrix, allowing migration of endothelial cells but also tumour cells (21). Yet another mechanism facilitating the metastatic spread of tumour cells by VEGF-A is increased survival of tumour cells in the circulation and metastatic cells at the site of metastasis (32).

In the present study the concentrations of VEGF-A in mammary carcinomas increased with the histological grade, reaching the highest values in the grade 3 tumours. This may suggest that increased level VEGF-A is responsible for transforming tumour cells into a more malignant phenotype or that more malignant tumours acquire the ability to produce more VEGF-A. Our results are consistent with the results of the studies by Restucci et al. (28) and Anadol et al. (5). These authors have found the highest levels of VEGF-A protein expression and VEGF mRNA expression in the grade 3 malignant canine mammary tumours. Al-Dissi et al. (3) have observed a moderate correlation between VEGF-A expression and the histological grade of canine mammary adenocarcinomas. In many human malignant tumours the increased expression of VEGF-A has been correlated with the histological grade of malignancy (10). However, some studies in humans and dogs have not confirmed significant differences in VEGF-A levels among different grade canine mammary carcinomas (27, 39) and breast cancers (14).

In conclusion, the results of our study demonstrate that concentrations of VEGF-A are significantly increased in the canine malignant mammary gland tumours and they are significantly higher in the tumours with metastases in regional lymph nodes than in those without metastases. In addition, a high concentration of VEGF-A in the homogenates of mammary carcinomas is associated with the high tumour grade. These results indicate that increasing the malignancy of canine mammary tumours and their potential for metastasis is accompanied by an increased production of VEGF-A in the tumour. This may suggest that an increased production of VEGF-A plays a role in malignant transformation and facilitates spreading of tumor cells and formatting of metastases in canine malignant mammary gland tumours.

References

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