Immunohistochemical evaluation of neoangiogenesis in canine mast cell tumours

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Abstract

The aim of the study was to assess the microvessel density based on the analysis of the expression of the CD31, VEGF, and LIMS-1 proteins in canine mast cell tumours. The study was conducted on 60 mastocytomas; 16 cases were classified as the grade I, 26 as the grade II, and 18 as the grade III. Statistical analysis showed a positive correlation only between the grade of the tumour and the expression of LIMS-1. In conclusion, LIMS-1 could be successfully used as a prognostic endothelial cell marker in mast cell tumour. CD31 may be a useful marker, but further examinations are necessary. VEGF is not recommended.

Keywords: dogs, mast cell tumour, neoangiogenesis, LIMS-1, CD31, VEGF.

Introduction

Mastocytoma (MCT), or a mast cell tumour, is one of the most frequently occurring mesenchymal skin tumours in dogs. It occurs in dogs of all ages. There is no clear gender predisposition, but it has been found in some breeds more frequently than in others, i.e. in Boxers, Golden Retrievers, Labrador Retrievers, Bullterriers, and Shar-pei (1, 7, 19). The tumour cells have numerous cytoplasmic granules. These contain a number of biologically active substances, or mediators, that lead to the degradation of the extracellular matrix (ECM), facilitating the formation of metastases and neoangiogenesis (3, 8, 17). MCTs are commonly assessed using the scale created by Patnaik et al. (20), which distinguishes three malignancy grades. The survival rate of dogs with grade I is high and this lesion is described as a benign tumour. Grade II is characterised by heterogeneous tumours. Within this group, predicting the prognosis is a challenging task. Grade III tumours are the most malignant, and associated with poor prognosis. The survival rates in dogs with grades II and III, in comparison with grade I, is 2 and 16 times lower, respectively.

During the early stages of the growth, the tumour acquires nutrients and oxygen from the blood via diffusion. Since hypoxia initiates apoptosis through the activation of the TP53 gene, the growing tumour, which progresses to 1-2 mm (roughly 10^5 cells), cannot survive without vascularisation. As a result, the neoplastic tissue is subjected to hypoxia and acidification, and, consequently, necrosis. Tumour cells attempt to prevent this by stimulating the formation of their own blood vessels (neoangiogenesis) (13).

It has been proven that an increased blood vessel density in the tumour is associated with its increased size, smaller cell differentiation, increased invasiveness, an infiltration of surrounding lymph vessels as well as metastases to lymph nodes and other organs (25).

One of the methods of determining the metastatic potential of a tumour is the assessment of its neoangiogenesis. This can be done using a panel of antibodies aimed at various structural proteins found in blood vessels, such as CD31, and those which show the expression of proangiogenic factors, such as LIMS-1, VEGF, or interleukin -12 (24, 25, 28).

CD31, known as PECAM-1 (platelet/endothelial
### Material and Methods

The study was conducted on 60 MCTs. The lesions were detected in animals of various ages (ranging from 6 months to 14 years; 42 animals were between 6 and 9 years old) and both sexes (33 male and 27 female). Most MCTs were excised from 14 mixed-breed dogs, 12 Boxers, 8 Labrador Retrievers, 6 American Staffordshire Terriers, 3 Bull Terriers and 2 Weimaraners. The remaining dogs were each of a different breed.

Fragments of tumours were fixed in 7% buffered formalin for 24 h and embedded in paraffin blocks. Paraffin sections (4 μm-thick) were stained with haematoxylin and eosin. Immunohistochemical studies were performed with sections placed on silanised slides. Subsequently, the sections were cleared in xylene and passed to water through a row of alcohols of a decreasing concentration. Antigen retrieval was achieved by cooking the slides in citrate buffer (pH 6.0) at 97°C for 20 min. The slides were double-washed in TBS. Then they were blocked in 10% normal serum with 1% BSA in TBS. Next, the sections were overlayed with primary antibodies specific for VEGF Clone VG1 (Dako®) (diluted 1:100), CD31 Clone JC70A (Dako®) (diluted 1:100), and LIMS-1 Product No. SAB2701201 (Sigma®) (diluted 1:200), and incubated overnight at 4°C. The following day, the slides were washed in TBS. After rinsing, the endogenous peroxidase was blocked in 3% solution of hydrogen peroxide for 10 min. The immunohistochemical reactions were developed using a 3,3-diaminobenzidine tetrahydrochloride (DAB) solution. Finally, the sections were rinsed in distilled water, cell nuclei were counterstained with haematoxylin, and the specimens were dehydrated in a row of alcohols. For each marker, a positive and a negative control was included. The specificity of the immunolabelling was verified by incubating the sections with PBS instead of the specific primary antibody. For a positive control, sections of canine skin were selected and all the tested antibodies as well as reagents were used.

The CD31, VEGF, and LIMS-1 immunostained blood vessel endothelial cells were examined. Hot spots containing the largest density of blood vessels were selected at low magnification (100×). Each slide was evaluated based on 5 hot spot areas, where the average number of positively labelled blood vessels was counted at a magnification of 200×. The vessels with a clearly defined lumen, but no single endothelial cells, were included for microvessel count.

Microphotographs of all the examined neoplastic lesions were taken using the Olympus BX53 optical microscope (Olympus, Japan), coupled with a ColorView IIIu digital camera (Olympus, Japan). The obtained results were analysed statistically, using the Spearman’s rank correlation, Friedman ANOVA and the Kruskal-Wallis non-parametric ANOVA using Statistica PL software (StatSoft Polska). The significance was set at P < 0.05.

### Results

Based on the H&E staining, the tumours were divided into groups according to the scale by Patnaik et al. (20). Sixteen tumours were classified as MCT I, 26 as MCT II, and 18 as MCT III. A graph with
detailed results is shown in Fig. 1.

There was a lack of CD31 reaction in 6 tumours. A maximum average of 14 vessels was observed. In 36 cases, 3 - 6 vessels were found. An average of 2 - 4 vessels were found in MCT I (n = 9). In MCT II (n = 17) and III (n = 11), 4 - 6 vessels were found (Fig. 5). A larger number of vessels were observed only in individual cases. The results are shown in Fig. 1. Stained blood vessels are presented in Fig. 2.

The analysis of the expression of LIMS-1 revealed that there was no reaction in 10 samples, whereas a maximum of eight vessels were observed. Two to three vessels were seen in 27 cases. The results presented in Fig. 1 show that 0 - 2 vessels were most often found in MCT I, 2 - 3 vessels (n = 15) were seen in MCT II, and 2 - 4 vessels (n = 10) were observed in MCT III (Fig. 5). A colour reaction was also visible in mast cells, but only stained blood vessels were taken into account. labelled blood vessels and mast cells are visible in Fig. 3.

Immunostaining of VEGF protein showed no labelled blood vessels in 13 lesions; a maximum of 6 VEGF-positive vessels were observed. Two to three vessels were seen in the majority of the samples (n = 33). In MCT I, an average of 0 - 2 vessels were found (n = 12), while 2 or 3 vessels were stained in MCT II and III (n = 15 in MCT II and n = 12 in MCT III, Fig. 5). A colour reaction was also found in mast cells although it was not taken into consideration when assessing the samples (Fig. 4).

The statistical analysis, using the Spearman’s rank correlation (where P < 0.05), showed a positive correlation only between the grade of the tumour and the expression of LIMS-1 (r = 0.34). There was no correlation between the grade of the tumour and the expression of CD31 (P ≥ 0.05) or VEGF (P ≥ 0.05). However, a statistically significant correlation (P < 0.05) was observed between the examined proteins themselves. The correlation coefficient (r) equalled 0.69 between CD31 and LIMS1, r = 0.33 between CD31 and VEGF, and r = 0.48 between VEGF and LIMS1 (Fig. 5).

The results of the Friedman ANOVA test showed a significantly greater expression of CD31 than VEGF and LIMS-1 (P < 0.05). There was no statistically significant difference in the number of vessels stained when using VEGF and LIMS-1.

Using the Kruskal-Wallis ANOVA by ranks, it was shown that, despite no statistically significant correlations between the grade of the tumour and the CD31 expression, there was a statistically significant difference in the expression of this protein between MCT II and III groups. Similar results were obtained with LIMS-1, where more blood vessels were stained in grade II and III tumours. VEGF did not show a statistically significant correlation with the grade of the tumour. no statistically significant differences in the expression of this protein were also found between different grades of the tumours.

Fig. 1. The number of all cases (shown as a percentage), where 0-2, 3-6, or 7-14 vessels were found in mast cell tumours of grades I, II, and III.
Fig. 2. CD31 expression pattern with labelled blood vessels. 200×

Fig. 3. Immunolabelled blood vessels in LIMS-1 examination. In mast cells colour reaction was also visible, but it was not taken into account during analysis. 400×

Fig. 4. VEGF expression pattern. Labelled blood vessels and mast cells are visible. Immunostained mast cells were not assessed. 400×
Discussion

The process of new blood vessels formation in a tumour is an important indicator of its malignancy (6, 10). Many authors have shown that high blood vessel density is present in aggressive tumours, which have a tendency to metastasise and which are associated with a poor patient prognosis (10, 15, 26). This is related to a better nutrient and oxygen supply to the tumour cells (9). In the mastocytoma, tumour cells secrete a number of mediators that stimulate neoangiogenesis (such as the above-mentioned VEGF or the fibroblast growth factor-2 - FGF-2); hence the suspicion that as the number of blood vessels increases, so may the tumour malignancy. Mast cells can either activate the content of their cytoplasmic granules or selectively activate mediators, which can also affect the inhibition or stimulation of blood vessel formation (27).

In this study, the expression of all endothelial cell markers was found in blood vessels. A statistical analysis using the Spearman rank correlation showed that only the expression of LIMS-1 positively correlated with the grade of the tumour. Such a relationship was not observed in the case of CD31 and VEGF. Therefore, the results of the study indicate that with the progression of the tumour, the expression of LIMS-1 increases. In humans, a relationship was found between the expression of this protein and the malignancy of the gastric adenocarcinoma and leukaemia (25, 29). Unfortunately, there have been no reports of the role of this protein in canine cells.

The density of the blood vessels varied depending on the antibody used. The results of the Friedmann ANOVA test showed a stronger expression of the CD31 protein than the remaining endothelial cell markers. This means that more reactive blood vessels were found using this marker. This is particularly noticeable in MCT II and III. The difference in the number of positively reacting blood vessels is most likely due to the fact that, as an endothelial cell marker, CD31 marks very small blood vessels, which are formed by immature endothelial cells (28).

As previously mentioned, a larger number of blood vessels visualised using CD31 and LIMS-1 can be found in MCT II and III. This finding was confirmed statistically using the Kruskal-Wallis rank ANOVA. The blood vessel density correlated with high grade tumours. This is supported by the findings of Sharma et al (23). Additionally, when analysing the expression of CD31, a higher density of vessels was

Fig. 5. The number of vessels in the MCT I, II, and III is presented statistically. SD – standard deviation, SE – standard error.
found in MCT II, and not in MCT III. The intensity of the MCT III growth may be greater than the rate of new blood vessel formation (13). The H&E staining of grade III tumours showed larger areas of necrosis, probably resulting from insufficient blood supply to the tissue. The results suggest that the blood vessel density in MCT is not an indicator that is strictly dependent on the malignancy and progression of the tumour. Clinical utility of the markers of angiogenesis remains debatable since many studies show no relationship between the tumour vascularisation and effects of the therapy used (4, 14).

The analysis of the expression of VEGF shows no connection with the tumour grade, which has been confirmed by Amorim et al. (2) and Rebuzzi et al. (21). These studies evaluated the expression of proteins using various methods. Amorim et al. (2) determined the number of blood vessels in the field of view, while Rebuzzi et al. (21) explored the reactions of mast cells. The authors of these studies highlighted significant differences in the expression of VEGF in different tumour grades (2, 21). Giantin et al. (12) carried out a similar study, where they assessed the staining of mast cells and found a strong correlation between the expression of VEGF and the MCT grade. In addition, in this study, in none of the 6 groups examined there were more than 6 blood vessels in one field of view were observed. When using the remaining two antibodies, such areas were numerous. Due to considerable differences in the results and no correlation between the expression of VEGF and the tumour grade, and on the basis of literature, the authors believe that VEGF cannot be considered as a good marker of neoangiogenesis in the mastocytoma.

Blood vessel density can be a good prognostic marker for the mastocytoma. However, this study has demonstrated that only LIMS-1 correlated with the tumour grade, and can thus be effectively used in the diagnosis of canine MCT. The obtained results and statistical analysis suggest that CD31 may be a useful marker, but further studies are necessary.

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**References**


