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Angiogenesis in Canine Mammary Tumours: A Morphometric and Prognostic Study

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Summary

Angiogenesis in canine mammary tumours (CMTs) has been described previously; however, only the intratumoural (IT) region has been studied and information on peritumoural (PT) angiogenesis is lacking. In this study, the blood vessel density (BVD), blood vessel perimeter (BVP) and blood vessel area (BVA) in IT and PT regions of 56 benign CMTs, 55 malignant CMTs and 13 samples of normal mammary gland tissue were analyzed. In addition, the blood endothelial cell proliferation (BECP) as an indicator of ongoing angiogenesis was investigated. The prognostic value of each parameter was also examined. Blood vessels and proliferating blood endothelial cells were present in IT and PT regions of both benign and malignant tumours. The vessels in the PT region had a significantly higher area and perimeter compared with those in the IT region. Malignant tumours showed significantly more vessels with a smaller total BVA and a higher BECP compared with benign tumours and control tissue. In the PT regions there was a significantly higher BVD, BVA and BVP compared with the vessels in control tissue. Only the IT and PT BVD and PT BECP in benign tumours allowed prediction of survival. The morphology of blood vessels in CMTs shows similarities with those in human breast cancer, which strengthens the case for the use of dogs with CMTs in comparative oncology trials.

Keywords: blood vessels; dog; immunohistochemistry; mammary tumour

Introduction

Mammary tumours represent the most common form of neoplasia in women and bitches. During their growth, both angiogenesis and lymphangiogenesis are induced, since the formation of these new blood and lymphatic vessels originating from the pre-existing vascular network is essential for tumour growth, invasion and metastasis (Folkman, 1986; Fox *et al.*, 1996; Saaristo *et al.*, 2000; Al-Rawi *et al.*, 2005; Sleeman and Thiele, 2009; Holopainen *et al.*, 2011). The characteristics of angiogenesis in human breast tumours and their correlation with metastasis and prognosis have been extensively studied (Weidner *et al.*, 1991; Uzzan *et al.*, 2004). In veterinary medicine the presence of angiogenesis in canine tumours, including canine mammary tumours (CMTs), has been a subject of interest. However, most of these studies have only focused on the blood vessel density (BVD) in the intratumoural (IT) region (Griffey *et al.*, 1998; Graham and Myers, 1999). Moreover, not all of the studies took into account the area, perimeter and proliferation status of these blood vessels and none of them evaluated peritumoural (PT) regions. As the PT region is also exposed to angiogenic stimulation, investigation of

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the different blood vessel characteristics in this region can provide interesting additional information. Different blood vessel markers have been used in the past; however, a previous study compared different immunohistochemical blood and lymphatic vessel markers in normal and neoplastic canine mammary tissue. CD31 was found to be the most suitable blood vessel marker (Sleeckx *et al.*, 2013a), so it was used to investigate the blood vessels in CMTs. The combination of CD31 and the proliferation marker Ki67 was used to assess ongoing angiogenesis. Labelling of lymphatic vessels with prox-1 was performed to discriminate between lymphatic and blood vessels (Van den Eynden *et al.*, 2006).

Since CMTs show similarities to human breast cancer (Khanna and Hunter, 2005; Porrello *et al.*, 2006; Lavalle *et al.*, 2009; Uva *et al.*, 2009; Klopfleisch *et al.*, 2011; Queiroga *et al.*, 2011b; Casteleyn *et al.*, 2013), pet dogs with naturally occurring CMTs represent a valuable animal model in comparative oncology trials. Data from these studies may support the development of anti-angiogenic therapeutics (Rusk *et al.*, 2006; Paoloni *et al.*, 2009). Acquiring more information on blood vessel characteristics in CMTs and comparing these data with the findings in human breast cancer is required for the establishment of a comparative oncology model for anti-angiogenic therapy.

The aims of the present study were (1) to investigate in detail the blood vessel characteristics in IT and PT regions, both in benign and malignant CMTs, (2) to examine the proliferation status of tumour-associated blood vessels as an indicator of ongoing angiogenesis and (3) to assess the prognostic value of these blood vessel characteristics.

Material and Methods

Samples

Samples of healthy canine mammary glands ($n = 13$) were collected during necropsy examination of bitches with normal non-neoplastic mammary glands. These dogs varied in age from 6 to 16 years (mean age 10.75 years) and different breeds were represented. The CMTs ($n = 111$) were removed surgically from female dogs with a mean age of 10 years (age range 5–17 years) and were submitted to the Laboratory of Applied Veterinary Morphology of the University of Antwerp, Belgium. The CMTs were classified according to the World Health Organization criteria (Misdorp *et al.*, 1999). Grading of the malignant tumours was performed according to the Elston and Ellis method adapted to CMTs (Clemente *et al.*, 2010; Pena *et al.*, 2013). Clinical follow-up data (i.e. recurrence, metastases and survival) were recorded based on assessment of case records and client and veterinarian follow-up for a minimum of 12 months.

Immunohistochemistry

Samples were fixed in 4% neutral buffered formalin, processed routinely and embedded in paraffin wax. Serial sections were stained with haematoxylin and eosin (HE) and for immunohistochemistry (IHC) they were labelled with antibodies specific for CD31 (Dako, Glostrup, Denmark), Ki67 (Dako) and prox-1 (RELIATech, Wolfenbüttel, Germany) (Table 1). Three washes with Dako wash buffer were performed between each step of the procedure. Reactions were 'visualized' using 3,3′-diaminobenzidine (DAB, Dako) and counterstaining with haematoxylin was performed. Positive controls included a section of canine haemangioma for CD31 and a section of canine lymph node for prox-1. Blood and lymphatic vessels in normal mammary tissue and in non-neoplastic areas of the tumour tissue samples served as additional internal controls. Epidermis in the sections was used as an internal positive control for Ki67 because of the physiological presence of proliferating keratinocytes in the stratum basale. For negative controls, the primary antibody was replaced with 0.05 M Tris buffered saline (TBS) containing 0.3% Triton X-100 (Sigma Aldrich, St Louis, Missouri, USA) and 1% bovine serum albumin (Sigma Aldrich).

Assessment of Morphological Characteristics

All slides (four from a total of 124 cases, $n = 496$) were evaluated using an Olympus BX61 microscope (Olympus, Aartselaar, Belgium) equipped with an Olympus DP50 digital camera connected to a computer system running the Olympus software programme Analysis Pro™. Image analysis was performed without knowledge of clinicopathological details. Microvessel 'hotspots' were identified in HE-stained sections as areas containing numerous microvessels at low magnification (×40 and ×100) (Vermeulen *et al.*, 2002). Two non-overlapping hotspots were chosen for IT and PT regions of the tissue. Three non-overlapping hotspots were selected in sections of normal mammary tissue. Digital images of these hotspots were taken at a magnification of ×200. In each hotspot, the number of blood vessels per mm^2 (BVD), the total and average blood vessel perimeter (BVP) and the total and relative area occupied by blood vessels (BVA) were analyzed. Evaluation of the microvessels was performed according to