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Correlation of quantified contrast-enhanced power Doppler ultrasonography with immunofluorescent analysis of microvessel density in spontaneous canine tumours

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ABSTRACT

Conventionally, tumour vascularity is assessed invasively by immunofluorescent analysis. Quantified contrast-enhanced power Doppler ultrasound has been used to measure tumour angiogenesis non-invasively in humans and experimental animals. The purpose of this study was to correlate quantified contrast-enhanced power Doppler ultrasound with immunofluorescent results in 45 spontaneous canine tumours. With power Doppler, mean vascularity was high in squamous cell carcinomas, moderate in malignant oral melanomas and low in sarcomas. There was high mean vascularity in squamous cell carcinomas and low mean vascularity in sarcomas and malignant oral melanomas. Although Doppler parameters correlated moderately with microvascular density for all tumours ($P = 0.004$, $r = 0.4$), they did not correlate within histology groups. These analyses show that vascularity differs among canine tumour histology groups. However, dependent on the method used, measurement of tumour vascularity can provide different biological information.

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Introduction

Tumour vasculature plays an important role in cancer research. Formation of new blood vessels, called angiogenesis, allows a tumour to grow beyond the size of the prevascular phase (Folkman, 1995). It has been shown that highly vascularised tumours have a higher potential to metastasise than tumours with poor vascularity (Luong et al., 2006). Further, increased vascularity is indicative of poorer survival in a variety of human (Zetter, 1998) and canine (Preziosi et al., 2004; Mukaratirwa et al., 2006) neoplasms. Vascularity has also been used as a valuable criterion to differentiate between benign and malignant human tumours, with higher densities of vessels in human breast cancer (Yang et al., 2002).

Since angiogenesis is important for tumour growth, anti-angiogenic drugs have been developed as treatments that inhibit new blood vessel formation, either directly by inhibiting specific molecules, or indirectly by influencing endothelial cell function (Rosen, 2000). Conventional radiotherapy has been shown not only to target tumour cells, but also vascular endothelial cells (Folkman and Camphausen, 2001). Tumour angiogenesis is an important predictor of response to chemotherapy in cancer patients (Chen et al., 2004). Consequently, the need to evaluate treatment efficacy has resulted in an increased interest in assessment of tumour vasculature.

The standard method for quantifying tumour angiogenesis is by immunofluorescent analysis of intratumoral microvessel density (MVD), which quantifies the number of vessels per unit volume (Weidner, 1995). However, to determine MVD, tissue samples have to be obtained via invasive biopsy procedures, represent only a certain area within the tumour and results are not immediately available for the clinician. To monitor changes in tumour vascularity in response to ionising radiation, serial histological analysis is impractical in most patients, since tissue must be obtained repeatedly by invasive procedures.

Colour and power Doppler ultrasonography are non-invasive techniques for assessment of vascularity and blood volume in tumours (Bodner et al., 2002; Eriksson et al., 1991). The entire tumour can be surveyed without affecting the tumour itself. Ultrasonography also allows serial measurements of the same tumour over time during a treatment regimen. Power Doppler ultrasonography has been shown to be more sensitive than colour

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Doppler for detecting low velocities and for depicting small vessels (Lencioni et al., 1996; Tschammler et al., 2002). Contrast-enhanced Doppler ultrasonography further improves the visualisation of vascularisation in tumours. The intravenous administration of a microbubble-based contrast agent can increase the Doppler signal intensity up to 25 dB. This increases the sensitivity for low-flow situations and provides a better depiction of vascularity than non-contrast-enhanced Doppler ultrasonography (Schroeder et al., 2003).

The purpose of the present study was to correlate the non-invasive quantification of vascularity and blood volume via contrast-enhanced power Doppler ultrasonography with the immunofluorescent analysis of MVD in spontaneous canine tumours.

Materials and methods

Patient selection

The study was performed using 45 dogs with 45 spontaneous tumours presented to the Section of Diagnostic Imaging and Radio-Oncology at the Vetsuisse Faculty of the University of Zürich. Dogs were included in the study if: (a) the tumour was superficial and accessible for ultrasound; (b) tissue core biopsy samples were available for histological examination; (c) tumour size allowed tissue core biopsy samples with a length of 22 mm to be taken, and (d) dogs were in good clinical condition and able to undergo general anaesthesia.

After owner consent was obtained, each patient underwent a physical examination and thoracic radiography. Breed, sex, weight and age of dogs were recorded. If indicated, fine needle aspiration of regional lymph nodes, abdominal ultrasound and computed tomography of the primary tumour were done. For tumour diagnosis, routine histological evaluation of tumour specimens was performed.

Anaesthesia was initiated by use of midazolam (2 mg/kg, IV; Dormicum; Roche Pharma SA) or diazepam (0.2 mg/kg, IV; Valium; Roche Pharma SA), followed by propofol (Propofol; Fresenius Kabi AG) given to effect; in addition butorphanol (0.1–0.3 mg/kg, IV; Morphasol, Gräub AG) was administered to some dogs. Anaesthesia was maintained with isofluran (Isoflo; Abbott AG) delivered in oxygen through an endotracheal tube. Heart rate and oxygen saturation, as measured by pulse oximetry (SpO2), were monitored continuously during anaesthesia to assess the status of each dog. At the time of Doppler ultrasound examination, the heart rate of each dog was recorded. All patients received IV lactated Ringer's solution at the rate of 10 mL/kg/h.

The length (a), width (b) and depth (c) of the tumours were determined from caliper measurements during anaesthesia. Tumour volume was calculated using the rotation ellipsoid formula ($\pi abc/6$).

Ultrasonographic imaging of tumours

Whenever necessary, the hair overlying the tumour was clipped. For each ultrasonographic examination, acoustic gel (Aquasonic 100, Parker) was applied to the skin and imaging was performed by use of a 5–12 MHz linear transducer (ATL 5000; Philips AG). For power Doppler ultrasonography, settings were constant for all examinations (81% colour gain, medium wall filter, 500 Hz pulse repetition frequency, frame rate 10–12 Hz). The maximal image depth was 25 mm. The ultrasound contrast agent used was a first-generation microbubble suspension (400 mg/mL Levovist; Schering) that was administered at a dose rate of 80 mg/kg.

Initial ultrasound scanning of the entire tumour was performed in B-mode to define its boundaries and morphological features. A rectangular sample volume was then placed over a selected region of the tumour and the surrounding tissue, denoting the region in which power Doppler ultrasonographic data would be acquired. A sliding scan of the entire tumour was performed to subjectively assess overall tumour vascularity. Finally, an area that subjectively represented the tumour's typical vessel density was chosen. The probe remained in the same location for ultrasonographic examination after administration of contrast medium. A bolus injection of the microbubble contrast agent was administered by hand via a catheter placed in a peripheral vein.

After blooming artefacts had degraded, a minimum of five power Doppler images were captured digitally and analysed (Schärz et al., 2005). Two parameters were computed for post-contrast power Doppler by calculating the median of the five images. Fractional area (FA) represents a vascularity index and indicates the percentage area of the tumour occupied by large blood vessels. Colour-weighted fractional area (CWFA) determines the mean blood volume within the tissue.

Immunofluorescence staining of microvessels

After the ultrasound procedure, a tissue core biopsy was taken with a 14 G a needle using an automated device (Magnum Biopsy gun; Bard). Samples always were obtained in the centre of the area of the power Doppler analysis under ultrasound guidance at an insertion angle of 45–60° to the image plane of the linear transducer. The sample depth was always 22 mm and therefore consistent with the ultrasound image depth of 25 mm. Tumours with a bone interface were only sampled in the soft tissue part. The specimens were immediately frozen with isopentane and liquid nitrogen and then stored at −80 °C. Tissue samples were cut into 7 μm thick sections with a cryostat microtome (Leica; CM1850), applied to slides and air dried for 20 min at room temperature before staining.

Slides were fixed in 4% paraformaldehyde for 30 min and blocked with normal goat serum for 1 h before incubating overnight at 4 °C with a commercially available rabbit–anti-human F8RA antibody (1:200; Dako Polyclonal; Dako) tested in canine tissues (von Beust et al., 1988). This step was followed by incubation with a secondary goat–anti-rabbit antibody labelled with fluorescein isothiocyanate (FITC; 1:2000; Sc3839; Santa Cruz Biotechnology) for 1 h at room temperature. Slides were mounted with Dako Fluorescent Media and examined with an inverse confocal laser scan microscope (400× total magnification, LSM 410; Carl Zeiss).

Computerised image analysis (Qwin; Leica Microsystems AG) was performed on five digital images from randomly chosen visual fields (high power fields). The count of microvessels per high power field was recorded and the mean of five fields was calculated. The programme was optimised for quantitative microscopy, producing precise results with megapixel accuracy (Sims et al., 2002; Law et al., 2003). The software was adapted to the project and the accuracy of the vessel count was tested before use. Statistical analyses were performed with computer software programmes (StatView 5.0.1, SAS Institute Inc. and SPSS 10.0, SPSS Schweiz AG). Values of $P < 0.05$ were considered to be significant.

Results

Twenty-five sarcomas, 10 oral squamous cell carcinomas and 10 malignant oral melanomas were included in the study (Table 1). The age of the dogs ranged from 2 to 15 years (mean 9.4 years). Twenty different breeds, along with mixed breed dogs, were included. The bodyweight ranged from 2.8 to 75 kg (mean 29.6 kg). Twenty-eight dogs were male and 17 female.

Thirty-six dogs were anaesthetised with midazolam and four dogs with diazepam; five dogs were given butorphanol additionally; subsequently, every dog received propofol. At the time of Doppler ultrasonographic examination, the mean heart rate was 115 beats per minute (bpm) (SD 23 bpm; reference range 84–110 bpm) (Haskins, 1996). The mean tumour volume was 83.5 cm^3 (SD 170.3 cm^3).

Descriptive statistics for post-contrast power Doppler parameters and MVD are shown in Table 2. Post-contrast power Doppler FA and CWFA were significantly correlated with MVD for all tumours ($P = 0.006$, $r = 0.4$, and $P = 0.005$, $r = 0.41$, respectively). If correlations were calculated for tumour histology groups, no significant associations were found ($P = 0.47$–0.96) (see Figs. 1 and 2). Highly significant differences were found between sarcomas, oral squamous cell carcinomas and malignant oral melanomas for the mean values of post-contrast power Doppler FA and CWFA ($P = 0.0001$, 1-way ANOVA and post-hoc Bonferroni–Dunn test).

Table 1
Location and histological classification of 45 spontaneous canine tumours examined by contrast-enhanced power Doppler ultrasonography

Histological classification	Location	Number of tumours
Fibrosarcoma	Oral cavity	5
	Head	2
	Limb	1
Osteosarcoma	Oral cavity	4
Histiocytic sarcoma	Oral cavity	2
Spindle cell sarcoma	Oral cavity	2
	Trunk	1
Myxosarcoma	Oral cavity	2
Haemangiopericytoma	Limb	4
	Trunk	1
Undifferentiated sarcoma	Oral cavity	1
Squamous cell carcinoma	Rostral oral cavity	10
Malignant melanoma	Oral cavity	10

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