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Immunohistochemical analysis of urokinase plasminogen activator and its prognostic value in canine mammary tumours

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ABSTRACT

Urokinase plasminogen activator (uPA) has been associated with aggressive behaviour and poor prognosis in human breast cancer, but there is no information on its expression in canine mammary tumours (CMT). uPA immunohistochemical expression was studied in 119 CMT (24 benign, 95 malignant) to investigate its relationship with clinical and histopathological parameters. Dogs with malignant mammary tumours (MMT) underwent a 2-year follow-up evaluation.

MMT expressed significantly more uPA than benign tumours. In MMT, high uPA stromal expression was significantly associated with larger tumour size, high Ki-67 expression, invasive growth, high histological grade, regional lymph node metastases, development of distant metastases, and lower overall survival (OS) and disease-free survival (DFS). On the basis of these results, uPA could be considered a useful prognostic factor in dogs with MMT.

Introduction

Tumour invasion and metastasis are the leading causes of morbidity and mortality in humans with breast cancer (Walker et al., 1997) and dogs with malignant mammary tumours (MMT) (Lana et al., 2007). These events involve multiple processes, such as degradation of extracellular matrix (ECM) and basement membrane (BM) (Ulisse et al., 2009). One of the major proteolytic systems involved in the ECM and BM degradation is the urokinase plasminogen activator (uPA) system, comprising uPA, its cell surface receptor (uPAR) and its inhibitors – plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2) (Han et al., 2006).

uPA is a member of the serine protease family that is important to convert circulating plasminogen into the active serine protease, plasmin (Andreasen et al., 1997). Upon binding to its receptor, uPA induces direct plasmin-mediated proteolysis or indirect activation of other proteases, such as metalloproteinases (MMPs) (Han et al., 2006), contributing to the degradation of the ECM during tissue remodelling processes such as wound healing and post-lactational mammary gland involution. ECM degradation also allows tumour cells to access the systemic circulation and colonize different organs (Rabbani and Xing, 1998; Nielsen et al., 2001). Moreover uPA stimulates the release and activation of various growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and transforming growth factor (TGF)-β (Guo et al., 2000), events that are considered determinant features of malignancy (Andreasen et al., 1997). The net result of this proteolytic flux, combined with uPA-dependent intracellular signalling, is acceleration of tumour cell invasion and tumour-associated angiogenesis (Guo et al., 2000; Bajou et al., 2002). In addition, uPA is associated with cell proliferation, chemotaxis (Han et al., 2006) and apoptosis (Hildenbrand et al., 2009).

The four major components of this system (uPA, uPAR, PAI-1 and PAI-2) have been established as prognostic factors in primary breast cancer (Dublin et al., 2000; Konecny et al., 2001; Harbeck et al., 2002; Han et al., 2006; Hildenbrand et al., 2009). Several studies assessing both mRNA and protein levels have found that elevated levels of uPA are associated with both aggressive tumour characteristics and poor prognosis (Ulisse et al., 2009).

Canine mammary tumours (CMT), like human breast cancer, are a heterogeneous group of neoplasms showing great variability in behaviour. To the best of our knowledge, uPA has not been studied in CMT. Therefore, the main purpose of the present study was to investigate the immunohistochemical distribution pattern of uPA in CMT and its role in tumour behaviour through its association with clinicopathological parameters with recognized prognostic value.

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Materials and methods

Specimens

Tumours ($n = 119$) were surgically removed from 119 female dogs, aged 5–16 years. Animals with MMT were enrolled in a 2-year post-operative follow-up study with no adjuvant therapy. The largest cross-sectional diameter of each tumour was recorded and categorized as either <3 cm or ⩾3 cm. All specimens were fixed in 10% neutral buffered formalin for 48 h. Tumours ⩽1 cm were paraffin embedded in one block, while larger tumours were cut sequentially at 5 mm intervals to provide a series of tissue blocks representative of the entire lesion.

After dehydration and embedding in paraffin wax, 3 μm sections were cut from each block. One section was stained with haematoxylin and eosin (HE) for diagnostic purposes and two representative blocks from tumours >1 cm were selected for immunohistochemical studies. When available, local and regional lymph nodes were processed and examined as previously described (Matos et al., 2006a).

Tumours and lymph nodes were evaluated independently by two observers (F.G. and I.A.) according to the criteria of the World Health Organization (WHO) for the histological classification of mammary tumours of domestic animals (Misdorp et al., 1999). Tumour histological grading was performed as previously described, according to the Nottingham method for human breast tumours (Matos et al., 2006a,b). The mode of growth of each tumour was assessed and classified as expansive (cohesive and well delimited growth of the tumour mass pushing normal surrounding tissue) or invasive (when there was an infiltrative growth or when lymphatic or blood vessels invasion was registered).

uPA immunohistochemistry (IHC)

Selected tumour sections adjacent to those used for HE staining were analysed by IHC using the modified avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981). Sections were dewaxed in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked by treating with 3% hydrogen peroxide in methanol for 10 min. Slides were then incubated with normal rabbit serum (Dako) diluted 1:5 in TBS containing 10% bovine serum albumin (BSA) for 20 min at room temperature. Excess serum was drained and slides were then incubated overnight at 4 °C in a wet chamber with the anti-uPA (M-20) goat polyclonal antibody (Santa Cruz Biotechnology; dilution 1:40 in TBS with 5% BSA). Slides were then incubated with biotinylated rabbit anti-goat antibody (Santa Cruz Biotechnology; dilution 1:100 in TBS with 5% BSA) for 30 min and then with the ABC (Vector) for further 30 min. Colour was developed with a solution of 3,3′-diaminobenzidine and the sections were then counterstained with haematoxylin, dehydrated, and mounted. To confirm the specificity of the IHC staining, the primary antibody was replaced with non-immune goat immunoglobulin. Positive controls consisted of sections from human breast cancer tissue known to express uPA and canine normal renal tissue (Bailey et al., 2006).

uPA expression evaluation was semi-quantitative and based on the percentage of neoplastic and stromal cells (fibroblasts) with cytoplasmic staining according to the scoring method (cut-off 10%) used in human breast oncology (Hildenbrand et al., 2009). In carcinosarcomas, uPA expression was evaluated in both malignant components (carcinomatous and sarcomatous) and in normal stromal fibroblasts. The slides were examined independently by two observers (A.S. and A.M.) and, when there was disagreement (<5% of the slides), a consensus was obtained using a multi-head microscope.

MIB-1 immunohistochemistry

Selected tumour sections adjacent to those used for HE staining were immunostained and evaluated for Mindbomb homolog 1 (MIB-1) labelling index (Ki-67 expression), as previously described (Matos et al., 2006b).

uPA SDS–PAGE and western blot

In order to confirm the specificity of the antibody for canine uPA, canine tissues samples (normal renal tissue and mammary carcinoma) were evaluated by Western blot. Cytosolic fractions of both samples were obtained by homogenization and incubation in a lysis buffer (Protein extraction reagent kit, Thermo Scientific) containing protease inhibitors (Complete Mini Protease inhibitor cocktail tablets, Roche Diagnostics) for 10 min at room temperature. Lysates were centrifuged at 10,000 *g* for 5 min at 4 °C. Supernatants were then collected and stored at −80 °C.

For Western blot analysis, cytosolic fractions (10 mg of total protein/well) contained on 10% sodium dodecyl sulfate (SDS) gel were blotted for 3.5 h (40 v, 300 mA) into a PVDF membrane (Amersham Biosciences). Membranes were cut into strips and endogenous peroxidase was blocked by treatment with 0.6% hydrogen peroxide in PBST (PBS, 0.05% Tween-20, pH 7.5) for 10 min. After washing in PBST, strips were incubated for 3 h in PBST buffer, containing 3% BSA to block non-specific binding, and further incubated overnight at 4 °C with an anti-uPA (M-20) goat polyclonal IgG (Santa Cruz Biotechnology) diluted 1:100 in PBST. After washing in PBST (3 × 5 min; 2 × 10 min), strips were incubated with horseradish peroxidase-coupled polyclonal donkey anti-goat IgG (Santa Cruz Biotechnology) diluted 1:5000 in PBST for 1 h. Bound antibodies were detected using a 3,3′,5,5′ tetramethylbenzidine (TMB) liquid substrate system for membranes (Sigma).

Follow-up study

All animals were examined prior to surgery, 3 weeks after surgery and every 3 months thereafter for a 2-year period. Each examination consisted of a complete physical examination, thoracic radiographs (three views) and complete abdominal ultrasound. Whenever necessary, additional examinations (e.g. aspiration cytology, excisional biopsy, skeletal radiography) were performed with the owner's consent. Complete necropsies were performed in all dogs that died spontaneously or were euthanased. Suspected metastases were confirmed histologically.

Statistical analysis

For statistical purposes, tumours were grouped according to a cut-off value of 10% of uPA positive cells (tumour and stromal). Fisher's exact test was used to evaluate the relationship between uPA expression in MMT and the following clinicopathological parameters: tumour size, mode of growth, histological grade, MIB-1 labelling index, regional lymph node metastases and distant metastases.

Overall survival (OS) was calculated from the date of tumour removal to the date of animal death/euthanasia due to tumour metastasis. Disease-free survival (DFS) was calculated from the date of surgery to the date of detection of the first local recurrence or development of distant metastases. The Kaplan–Meier method was used to compute OS and DFS times and to construct the survival curves. The log-rank test was used to analyse the significance of the differences between the groups. In the OS study, the dogs were censored if and when they died for causes unrelated to mammary tumours, were lost to follow-up, or were alive 2 years after surgery. In the DFS study, the dogs were censored if and when they were lost during follow-up, died for causes unrelated to mammary tumours before developing signs of metastatic disease, or were free of distant metastases 24 months post-surgery. The significance level was $P < 0.05$. Statistical analysis was performed using the statistical package SPSS 16.0 (SPSS Inc.).

Results

Benign tumours consisted of 6 simple adenomas, 11 complex adenomas and 7 benign mixed tumours. The group of MMT included 31 solid carcinomas, 21 complex carcinomas, 20 tubulopapillary carcinomas, 2 micropapillary carcinomas, 2 mucinous carcinomas, 2 anaplastic carcinomas, 1 spindle cell carcinoma, 14 carcinosarcomas and 2 carcinomas in benign tumours.

Positive controls showed diffuse granular cytoplasmic labelling with strong intensity. Negative controls did not show stained cells. Western blot analysis (Fig. 1) demonstrated the presence of canine high and low molecular weight uPA protein isoforms (50 and 30 kDa) in the studied samples confirming that the antibody used in the IHC analysis was able to specifically identify canine uPA.

In neoplastic tissues, anti-uPA immunoreactions were found in epithelial and myoepithelial cells (in adenomas and carcinomas – Figs. 2 and 3) and in sarcomatous cells (in carcinosarcomas); reactive fibroblasts (Fig. 4a and b) and macrophages. uPA expression was significantly higher in stromal ($P < 0.001$) and neoplastic cells

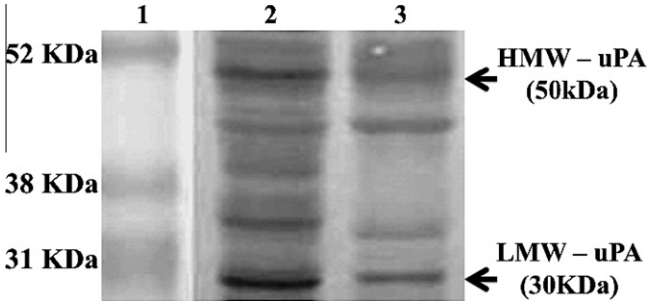

Fig. 1. Western blot analysis demonstrating urokinase plasminogen activator (uPA) expression. Lane 1, molecular weight marker; lane 2, normal canine renal tissue; lane 3, mammary carcinoma. High (HMW-uPA) and low (LMW-uPA) molecular weight uPA isoforms in the expected molecular masses (50 and 30 kDa).