Urokinase plasminogen activator expression in canine malignant mammary tumours by immunohistochemical study

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Abstract

Immunohistochemical expression of urokinase plasminogen activator (uPA) was studied in 37 canine malignant mammary tumours to define the relationship between their histopathological type and grade. In 29 (78.4%) cases, expression of uPA by neoplastic cells was more than 10% and in 34 samples (91.9%) uPA expression by stromal cells (fibroblasts) was more than 10%. The uPA was expressed in epithelial and myoepithelial cells of carcinomas and carcinosarcomas and mesenchymal population of carcinosarcoma, chondrosarcoma, and carcinomas arising in benign tumours. The intensity and percentage of expression of uPA by stromal cells was associated with their histological grade (P < 0.05). However, no significant relationship was detected between uPA expression by neoplastic cells (epithelial, myoepithelial, and mesenchymal cell) and histological grade. Increased expression of uPA by tumour stroma was associated with poor prognostic factors. Stromal expression of uPA could be a prognostic indicator for canine mammary tumours.

Key words: bitches, mammary gland tumours, urokinase plasminogen activator, immunohistochemistry.

Introduction

The ability of tumour to influence the regulation of extracellular matrix (ECM) in part determines its capability to invade adjacent tissue and eventually to metastasise. Thus, all systems regulating the ECM can be of interest in the quest of prognostic markers (2).

During cancer invasion and metastasis, proteases facilitate the degradation of extracellular matrix (19). The plasmin/plasminogen system contains two types of activators: tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), the uPA receptor (uPAR) and two inhibitors of plasminogen activator: inhibitor-1 (PAI-1) and inhibitor-2 (PAI-2) (14). The uPA, as a member of the serine protease family, is a key regulator of biologic processes implicated in tumour cell migration, invasion, and metastasis (30). The uPA is produced and secreted into ECM as an inactive proenzyme (pro-uPA) that becomes activated upon binding to its receptor (5). When bound to uPAR, uPA efficiently converts the inactive zymogene, plasminogen, into the active serine protease, plasmin, which then directly or indirectly cleaves ECM components including laminin, fibronectin, fibrin, vitronectin, and collagen (27). Plasmin can also activate latent elastase and matrix metalloproteinase (MMPs) potent enzymes that can also digest a variety of ECM components. The ability of the uPA system to mediate endothelial cell migration and differentiation further contributes to its role in tumour progression through induction of angiogenesis (7). Furthermore, evidences demonstrate that the cell surface associated uPA/uPAR complex is causatively involved in tumour invasion and metastasis of many types of cancers by exerting multifaceted functions via either direct or indirect
interactions with integrins, endocytosis receptors, and growth factors (1). The uPA is most probably a facilitator of cancer invasion by stimulating extracellular matrix degradation. However, the exact role of uPA in regulating tumour growth is not completely understood. The uPA can stimulate 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 (13). These processes are considered as determinant features of malignancy (1). The net result of this proteolytic flux, combined with uPA-dependent intracellular signalling, is the acceleration of tumour cell invasion and tumour associated angiogenesis (13). Moreover, uPA has been directly associated with cell proliferation, migration, and chemotaxis (14). All these events could have a significant impact on tumour cell dissemination (8).

Despite important predictive roles of uPA in breast cancer, their precise tissue localisation in cancer is still discussed. Some authors have found uPA by immunostaining method not only in stromal cells but also in cells of breast cancer (10). However, other authors found that uPA is exclusively or mainly localised in tumour (9) or stromal (23) cells.

Although extensive studies have established the prognostic potential for uPA expression in different human tumours, there are few studies concerning the expression of this protein in canine tumours. Only Santos et al. (26) studied the role of uPA in canine mammary tumours.

The aim of the study was to evaluate the expression of uPA in canine mammary tumours and its relationship with their histological type and grade.

Material and Methods

Malignant tumours (n = 37) of the mammary glands from various pure or mixed breed bitches aged from 4 to 15 years (mean, 8.5 years) were obtained between September 2009 and September 2011. They were selected from surgically treated cases or archive of the Department of Pathology.

The tissues were fixed in 10% neutral buffered formalin and processed routinely for histological examination. Histological classification was performed independently by two authors of this study, according to the diagnostic criteria of the WHO (21). The same sections were used to determine the histological grade (grades I, II or III) based on the Elston and Ellis (12) classification for human breast tumours, on the basis of the assessment of three morphological features: tubule formation, nuclear pleomorphism, and mitotic counts. Both classification and grading were assessed using a multi-head microscope.

For immunohistochemical study, tissue sections were deparaffinised by heat at 60°C for 10 min, followed by three washes in xylene. After gradual hydration through graded alcohols, the slides were incubated in citrate buffer (pH 6.0) for two cycles of 5 min in microwave oven for antigen retrieval. The sections were allowed to cool for 20 min, and then rinsed with Tris-buffered saline (TBS). The sections were treated with 3% H₂O₂ in distilled water for 5 min to inhibit endogenous peroxidase activity. The sections were incubated with anti-uPA (M-20) goat polyclonal antibody, diluted 1:40 for 1 h. Slides were then incubated with rabbit anti-goat antibody (Santa Cruz Biotechnology; Germany) for 30 min. The immune precipitate was visualised by treatment with 3, 3'-diaminobenzidine and counterstained with haematoxylin. For negative controls, the primary antibody was replaced with TBS. Positive controls consisted of human breast carcinomas known to express uPA (3). Immunohistochemical studies were performed independently by the two authors.

The evaluation of uPA expression was semi-quantitative and was based on percentage of both neoplastic and stromal cells (fibroblasts) with cytoplasmic staining according to scoring method (cutoff 10%) used in human breast oncology (17, 26). Staining of less than 10% of neoplastic cells or fibroblast was interpreted as negative uPA expression, and more than 10% as positive uPA expression. Furthermore, the intensity was graded as 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive). Statistical analysis was performed using SPSS 16.0 statistical software. The relationship between uPA expression and histological grade was analysed using the Chi-square test and Fisher’s exact test. P values of less than 0.05 were considered significant.

Results

Tumours included 32 carcinomas (Table 1), four carcinosarcomas and one sarcoma (chondrosarcoma).

In 29 (78.4%) cases, the expression of uPA by neoplastic cells was >10% and in 34 (91.9%) samples uPA expression by stromal cell was >10% (Table 2).

The uPA was expressed in epithelial, myoepithelial, and mesenchymal cell populations. Reactive fibroblast from the tumour-associated stroma also demonstrated uPA expression (Figs 1 and 2).

The intensity of expression of uPA within the stromal cell was associated with histological grade (P < 0.05). However, no significant relationship was detected between uPA expression in neoplastic cells (epithelial, myoepithelial, and mesenchymal cell) and histological grade (Fig. 3).
Table 1. Relationship between histological grading and tumour type in 32 dogs with mammary carcinoma

<table>
<thead>
<tr>
<th>Histopathological type</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple carcinoma</td>
<td>10 (47.6%)</td>
<td>9 (42.9%)</td>
<td>2 (9.5%)</td>
<td>21 (65.6%)</td>
</tr>
<tr>
<td>Complex carcinoma</td>
<td>-</td>
<td>5 (100%)</td>
<td>-</td>
<td>5 (15.6%)</td>
</tr>
<tr>
<td>Carcinoma arising in benign tumour</td>
<td>2 (50%)</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>4 (6.7%)</td>
</tr>
<tr>
<td>Special type of carcinoma</td>
<td>-</td>
<td>-</td>
<td>2 (100%)</td>
<td>2 (6.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>12 (37.5%)</td>
<td>15 (46.9%)</td>
<td>5 (15.6%)</td>
<td>32 (100%)</td>
</tr>
</tbody>
</table>

Fig. 1. Tubular carcinoma (grade 3). Strong expression (intensity 3+) of uPA in stromal cells (black arrows) and no expression in neoplastic cells (asterisks) (IHC 100 ×)

Fig. 2. Carcinoma arising in benign tumour (grade 2). Stromal cells show uPA expression with intensity 2+ (black arrows) with no expression in neoplastic cells (asterisks) (IHC 100 ×)

Fig. 3. Carcinosarcoma (poorly differentiated). Note strong expression (intensity 3+) of uPA in stromal cells (black arrows) and moderate expression (intensity 2+) in neoplastic cells (asterisks) (IHC × 400)

Table 2. The uPA expression in neoplastic and stromal cells of 37 canine malignant mammary tumours

<table>
<thead>
<tr>
<th>37 canine malignant mammary tumours</th>
<th>Number/ (%) of tumours exhibiting neoplastic cells with uPA expression</th>
<th>Number/ (%) of tumours exhibiting stromal cells with uPA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10%</td>
<td>&gt;10%</td>
</tr>
<tr>
<td></td>
<td>8 (22.6%)</td>
<td>29 (78.4%)</td>
</tr>
</tbody>
</table>

Discussion

The prognostic relevance of the activation of the urokinase system has been extensively studied in human tumours (15, 24). It has been related to an advanced stage of progression in breast, colorectal, bladder, ovarian, gastric, and pancreatic cancers. The tumour expression of uPA was reported to be positively related with loss of histologic differentiation, and also tended to increase in advanced stages of ovarian cancer (4). In colon cancer, high levels of uPA and uPAR were associated with poor differentiation (8). In breast cancer, high levels of uPA have been shown to predict poor prognosis (16, 17, 28).

The study describes the expression of uPA in canine malignant mammary tumours, and its relationship with histological type and grade. Ulisse et al. (29) demonstrated that uPA levels were significantly higher in malignant tumours when compared to benign human breast tumours. Santos et al. (26) demonstrated that canine malignant mammary tumours expressed significantly more uPA than benign tumours. Our study detected an association between uPA expression and higher histological grade, which is in agreement with the above research.

The higher expression of uPA in canine malignant tumours suggests its possible involvement in malignant neoplasia (26). According to the function of uPA, the plasminogen activator system is believed to influence progress of tumour dissemination and metastasis. In contrast to other extracellular matrix degrading proteases, uPA exhibits a substrate specificity for proteolysis of plasminogen to activate plasmin. Plasmin, in turn, represents a broad spectrum of proteases cleaving extracellular matrix proteins such as fibrin, fibronectin, laminin, and proteoglycans, either directly or via activation of other matrix-degrading enzymes or procollagenases like matrix metalloproteinases (11). Thus, this tumour-associated protease facilitates cell-mediated dissolution of the
extracellular matrix, which is necessary for tumour dissemination and possibly recurrence (18).

To establish whether plasmin-directed proteolysis is likely to be involved in early invasion, Nielsen et al. (22) studied, using in situ hybridisation, the expression of uPA in human ductal carcinoma in situ lesions with and without micro invasion. The uPA mRNA was detected in periductal stromal cells in all of nine ductal carcinoma in situ with micro invasion, and in two out of nine ductal carcinoma in situ without micro invasion.

The uPA mRNA signal was observed in numerous stromal cells in micro invasive areas. Double immunofluorescence analyses, using emission finger printing, indicated that the uPA expressing stromal cells included both myofibroblasts and macrophages. The early invasive carcinoma cells were negative for uPA. It was evident that uPA and uPAR co-localised in both fibroblast-like and macrophage-like cells. They concluded that periductal macrophages and myofibroblasts were strongly involved in the initial steps of breast cancer invasion by focally up regulating the expression of the plasminogen activation system.

Dublin et al. (10) used immunohistochemistry to examine uPA, uPAR, and PAI-1 in a pilot study on 142 cases of breast carcinoma. The aim of the study was to determine whether there were any relationships between the expression of the proteins in either tumour cells or fibroblasts, and clinical and pathological features. Strong uPA expression in both cell types was significantly related to high tumour grade. Fibroblastic expression of uPAR was only related to the presence of invasion. Strong PAI-1 expression in both cell types was seen in high-grade tumours (tumour cells and fibroblasts) but only fibroblastic expression was related to the presence of invasion. Fibroblastic expression of uPA showed a tendency toward a shorter time to relapse, none of the plasminogen activator proteins was significantly associated with relapse-free survival. These results suggested that strong expression of uPA, uPAR, and PAI-1 in fibroblasts has a bigger impact on the clinical behaviour of breast cancer than their expression by tumour cells.

For the first time in veterinary oncology research, Santos et al. (26) have studied immunohistochemical expression of uPA in 119 canine mammary tumours (24 benign, 95 malignant) to investigate its relationship with clinical and histopathological parameters. In malignant mammary tumours, high uPAstromal expression was significantly associated with larger tumour size, higher Ki-67 expression, invasive growth, high histological grade, regional lymph node metastases, development of distant metastases, lower overall survival (OS), and disease-free survival (DFS).

The study showed that the intensity and percentage of expression of uPA by stromal cells was associated with histological grade (P < 0.05) but no significant relationship was detected between uPA expression by neoplastic cells (epithelial, myoepithelial and mesenchymal cells) and histological grade (P < 0.05). The results are in agreement with other researches (10, 26). Santos et al. (25) indicated that the high stromal expressions of uPA and MMP-9 in aggressive tumours are potential therapeutic targets in the post-operative treatment of canine mammary cancer.

In conclusion, the over-expression of uPA by tumour stroma was associated with poor prognostic factors and poor outcome in dogs affected with malignant mammary tumours. Additional studies comprising larger groups of animals are warranted in order to confirm these findings and to investigate the usefulness of uPA immunoreactivity as a prognostic factor in canine mammary tumours.

References


