Matrix metalloproteinase-9 expression in mammary gland tumors in dogs and its relationship with prognostic factors and patient outcome

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Objective—To immunohistochemically evaluate matrix metalloproteinase (MMP)-9 expression in benign and malignant mammary gland tumors (MMTs) in dogs and relate expression to prognostic factors and patient outcome.

Animals—118 female dogs with naturally occurring mammary gland tumors and 8 dogs without mammary gland tumors.

Procedures—24 benign mammary gland tumors and 94 MMTs (1/affected dog) were obtained during surgical treatment; control mammary gland tissue samples were collected from unaffected dogs after euthanasia for reasons unrelated to the study. Tumors were evaluated for proliferation, invasive growth, histologic grade, and metastatic capacity; expression of MMP-9 was determined immunohistochemically, and its relationship with clinical and histologic findings was investigated. For dogs with MMTs, follow-up continued for 2 years; data were used to compute overall survival time and disease-free interval and construct survival curves.

Results—MMTs had significantly higher MMP-9 expression in stromal cells and in neoplastic cells than did the benign neoplasms. Stromal MMP-9 expression was also higher in highly proliferative tumors and in tumors with invasive growth, high histologic grade, and metastatic capacity. Furthermore, tumors from patients with shorter overall survival times and disease-free intervals had higher expression of MMP-9 in stromal cells.

Conclusions and Clinical Relevance—In dogs with MMTs, level of MMP-9 expression by stromal cells was related to factors of poor prognosis and shorter overall survival times and disease-free intervals. These results suggested that MMP-9 produced by tumor-adjacent stromal cells contributed to MMT progression in female dogs and that assessment of MMP-9 expression may be a valuable prognostic factor. (Am J Vet Res 2012;73:689–697)
proteases, such as plasmin and other MMPs. Urokinase-type plasminogen activator is one of the most frequently implicated proteases in the activation of several pro-MMPs, including pro-MMP-9.

The multiple effects of MMPs on neoplastic diseases are attributable to the MMPs’ ability to regulate the tumor microenvironment by cleaving various substrates, including structural components of the ECM, growth factor–binding proteins and growth factor precursors, receptor tyrosine kinases, cell adhesion molecules, and other proteases. Thus, MMPs are instrumental in the molecular crosstalk between tumor and stromal cells.

The exact location of the production of these enzymes in tumors is still debated. There is a growing body of evidence that, in humans, MMP-9 originates essentially from tumor-adjacent stromal components of many cancers, including breast carcinoma. In patients with breast carcinoma, high stromal MMP-9 expression is associated with poor prognosis.

To date, immunohistochemical investigations of MMP-9 in MTs in dogs have focused on its expression by neoplastic cells, with no evaluation of stromal cell expression. Hence, the influence of stromal cell–derived MMP-9 on the biological behavior of MTs in dogs is still unknown. Furthermore, to our knowledge, there are no reports of survival studies of dogs with MTs in which tumor expression of MMP-9 was investigated. Therefore, the main purpose of the study reported here was to evaluate the expression of MMP-9 in BMTs and MMTs in dogs and relate MMP-9 expression to prognostic factors and patient outcome.

Materials and Methods

Dogs and specimen collection—One hundred eighteen client-owned sexually intact or spayed female dogs with naturally occurring MTs were included in the study. For inclusion in the study, each dog had to have ≥ 1 MTs with no evidence of distant metastasis at the time of diagnosis, have no previous history of tumor disease, be free of tumors other than MT at the time of diagnosis, and have an owner who consented to surgery with curative intents but declined postoperative adjuvant treatments. Owners also consented to postoperative evaluation of their dog for a period of 2 years.

During surgical treatment, MTs were removed and 1 entire tumor from each dog was selected for purposes of this investigation. Regional lymph nodes were collected from 72 dogs with malignant tumors. In nearly half of those cases, intramammary lymph nodes (1 to 7 nodes/dog) were identified and examined.

In addition, samples of mammary gland tissues, uterus, and bone were obtained from 8 dogs without MTs that had been euthanized. Euthanasia was performed via slow IV injection of pentobarbital sodium solution at a municipal kennel as part of the national stray dog control program.

The study protocol was performed in compliance with institutional guidelines for research on animals and approved by the Animal Care and Ethics Committee of the University of Porto. All diagnostic or therapeutic procedures were in the best interest of the patients and approved by the owners. No procedures were conducted for purposes of the study alone.

Characterization of MTs—All tumors and clinically normal mammary gland tissues were fixed in neutral-buffered 10% formalin for 48 hours. The largest cross-sectional diameter of each tumor was recorded and categorized as either < 3 or ≥ 3 cm. Tumors < 1 cm in diameter were paraffin embedded in 1 block, and larger tumors were cut sequentially at 5-mm intervals to provide a series of tissue blocks representative of the entire lesion. The samples of clinically normal mammary glands were also cut sequentially at 5-mm intervals and paraffin embedded.

After dehydration and embedding in paraffin wax, 3-μm sections were cut from each block. For diagnostic purposes, 1 section/block was stained with H&E stain. One adjacent section to the H&E-stained section from each block was selected for immunohistochemical analysis; for tumors > 1 cm, a section from each of 2 representative blocks was collected. When available, local and regional lymph nodes were processed and examined as previously described.

The H&E-stained sections were evaluated independently by 2 observers (MFG and IFA) according to the criteria of the World Health Organization for the histologic classification of mammary gland tumors of domestic animals. The histologic classification of the MTs was performed as part of this study. Histologic grading of each tumor was performed according to the Nottingham grading method for human breast tumors, and tumors were classified as grade I (well differentiated), grade II (moderately differentiated), or grade III (poorly differentiated). The mode of growth of each tumor was assessed and classified as expansive (cohesive and well-delineated growth of the tumor mass pushing normal surrounding tissue) or invasive (infiltrative growth or lymphatic or blood vessel invasion). In dogs with > 1 MT, the tumor with more aggressive clinicopathologic features was selected for analysis.

Immunohistochemical analysis of MMP-9 expression—Tumors and clinically normal mammary glands sections adjacent to those that underwent H&E staining were analyzed immunohistochemically by use of the modified avidin–biotin–peroxidase complex method. After sections were dewaxed and rehydrated, endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide in methanol for 10 minutes. Slides were then incubated with rabbit serum for 20 minutes at room temperature (20°C) and then with the anti–MMP-9 (C-20) goat polyclonal antibody (diluted 1:200 in Tris-buffered saline solution with 5% bovine serum albumin), overnight at 4°C in a humid chamber. Sections were then incubated with biotinylated rabbit anti-goat antibody (diluted 1:100) for 30 minutes, followed by incubation with avidin–biotin–peroxidase complex for a further 30 minutes. Color was developed with a solution of 3,3′-diaminobenzidine, and sections were then counterstained with hematoxylin, dehydrated, and mounted.

In negative controls, the primary antibody was replaced with nonimmune goat immunoglobulin to confirm the specificity of the immunohistochemical staining. Positive controls consisted of sections of human breast cancer.
tissue (samples from an invasive ductal carcinoma\(^a\)) known to express MMP-9 and clinically normal canine tissues (uterus and bone) collected from the euthanized stray dogs.\(^b\)

For each tumor, MMP-9 expression was evaluated in a semiquantitative manner. Each section was examined microscopically by 2 independent observers (AAS and AJFD), who each estimated the percentage of tumor-adjacent stromal cells (fibroblasts) with cytoplasmic staining and the percentage of neoplastic cells with cytoplasmic staining. Tumors were classified as having low or high MMP-9 expression in stromal cells (ie, \(< 50\%\) or \(\geq 50\%\) of stromal cells with cytoplasmic staining, respectively) and low or high MMP-9 expression in neoplastic cells (ie, \(< 25\%\) or \(\geq 25\%\) of neoplastic cells with cytoplasmic staining, respectively). In tumors \(> 1\) cm, both sections were examined and the consensus estimation, in a global perspective, was considered instead of the 2 different individual estimations. When the estimations of the 2 observers were dissimilar (\(\leq 5\%\) of the cases), a consensus was obtained from the observers by use of a multiheaded microscope.

**Immunohistochemical analysis of MIB-1 and uPA expressions**—Sequential tumor sections of the MMTs were also immunostained and evaluated for MIB-1 labeling index (Ki-67 expression in neoplastic cells) and for uPA expression (in stromal cells, according to the previously described methods.\(^21,25\) The MIB-1 labeling index was determined by counting the number of immunopositive cells in 1,000 neoplastic cells (at 400X magnification) in the area of highest labeling, and the index was expressed as a percentage. Tumors were classified as having low or high MIB-1 expression in neoplastic cells (ie, \(< 43\%\) or \(\geq 43\%\) of neoplastic cells with MIB-1–specific cytoplasmic staining). The expression of uPA was assessed in a semiquantitative manner. Sections from MMT were examined microscopically, and the percentage of stromal cells (fibroblasts) with cytoplasmic staining for uPA was estimated. Tumors were classified as having low or high uPA expression in stromal cells (ie, \(< 10\%\) or \(\geq 10\%\) of stromal cells with uPA–specific cytoplasmic staining). In tumors \(> 1\) cm, both sections were examined and the consensus estimation, in a global perspective, was considered instead of the 2 individual estimations.

**SDS-PAGE and western blot analysis of MMP-9**—To confirm the specificity of the antibody for canine MMP-9 protein, canine tissue samples (mammary gland carcinoma and clinically normal canine tissues collected from the euthanized stray dogs) were evaluated by western blot analysis. Cytosolic fractions of samples were obtained by homogenization and incubation in a lysis buffer containing protease inhibitors at room temperature over a period of 10 minutes. Lysates were centrifuged at 10,000 \(\times\) g at 4\(^\circ\)C for 5 minutes. Supernatants were then collected and stored at –80\(\circ\)C.

For western blot analysis, cytosolic fractions (55 mg of total protein/well) contained on a 10% SDS gel were blotted for 3.5 hours (40 V and 300 mA) into a polyvinylidene difluoride membrane.\(^6\) The membrane was incubated overnight in a buffer of PBS-Tween 20 solution containing 3% bovine serum albumin to block nonspecific binding and further incubated for 3 hours at room temperature with the anti–MMP-9 (C-20) goat polyclonal IgG\(^b\) diluted 1:75 in PBS-Tween 20 solution. After the membrane was washed in PBS-Tween 20 solution (three 5-minute washes and two 10-minute washes), it was incubated for 45 minutes with horseradish peroxidase–coupled polyclonal donkey anti-goat IgG\(^b\) diluted 1:5,000 in PBS-Tween 20 solution. Bound antibodies were detected by use of a 3,3′,5,5′ tetramethylbenzidine\(^b\) liquid substrate system for membranes.

**Follow-up assessment of dogs with MMTs**—Dogs were routinely examined prior to surgery, 3 weeks after surgery, and every 3 months thereafter for a 2-year period if no clinical signs were detected. Owners were instructed to report and discuss with the researchers any detected abnormalities, even if those abnormalities were not obviously related to the MTS, at any time. Each examination consisted of a complete physical examination, thoracic radiography (3 views), and complete abdominal ultrasonographic examination. In case of the development of new skin or mammary gland nodules, lymph nodes alterations, or any other organ abnormalities, appropriate additional examinations (eg, cytologic examination of fine-needle aspiration specimens, histologic examination of excisional biopsy specimens, or skeletal radiography) were performed with the owner's consent to rule out other neoplasms or confirm metastatic disease. Complete necropsies were performed in all dogs that died or were euthanized (via IV administration of sodium pentobarbital) during the 2-year follow-up period. Metastases were histologically confirmed.

**Statistical analysis**—The Fisher exact test was used to analyze differences in stromal and neoplastic cell expressions of MMP-9 between benign and malignant MTs and to evaluate the relationship between MMP-9 expression in MMTs and tumor size, mode of growth, MIB-1 labeling index, regional lymph node status, and distant metastasis.\(^26\) The relationship between MMP-9 expression and histologic grade of the tumors was analyzed by use of the Pearson \(\chi^2\) test.\(^26\)

For dogs with MMTs, overall survival time was calculated from the date of tumor removal to the date of the dog's death or euthanasia due to tumor metastasis. Disease-free interval among dogs with MMTs 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 overall survival time and disease-free interval and to construct the survival curves.\(^23\) A log-rank test was used to analyze the significance of differences between groups.\(^23\) In the overall survival time assessment, dogs were censored when they died of causes unrelated to MTs, were lost to follow-up, or were alive 2 years after surgery. In the disease-free interval assessment, dogs were censored when they were lost during follow-up, died for causes unrelated to MTs before developing signs of metastatic disease, or were free of distant metastases 2 years after surgery. Values of \(P < 0.05\) were considered significant. Statistical analysis was performed by use of statistical software.\(^1\)
Results

Dogs and MTs—Among the 118 dogs with MTs, 24 dogs had only benign tumors. Of those 24 dogs, 8 had 1 tumor, 5 had 2 tumors, 7 had 3 tumors; 1 dog each had 4, 5, 8, or 9 tumors. Ninety-four dogs had malignant tumors. Of those 94 dogs, 62 had 1 tumor, 23 had 2 tumors, 6 had 3 tumors, 2 had 4 tumors, and 1 had 5 tumors. One tumor from each dog was selected and analyzed in the study. The selected BMTs included 6 simple adenomas, 11 complex adenomas, and 7 benign mixed tumors. The selected MMTs included 31 solid carcinomas, 21 complex carcinomas, 20 tubulopapillary carcinomas, 2 microcystic carcinomas, 2 mucinous carcinomas, 2 anaplastic carcinomas, 1 spindle cell carcinoma, 13 carcinosarcomas, and 2 carcinomas in benign tumors (a malignant MT type with foci or distinct nodules of malignant cells in complex adenomas or benign mixed tumors). Because of the low number of some histologic types, MMTs were grouped as follows: complex carcinomas (n = 21), simple carcinomas (solid, tubulopapillary, anaplastic, and micropapillary carcinomas [55]), and others (carcinosarcomas, carcinomas in benign tumor, spindle cell carcinomas, and mucinous carcinomas [18]). Among the BMTs, 19 were < 3 cm in diameter and 5 were ≥ 3 cm in diameter. Among the MMTs, 48 were < 3 cm in diameter and 45 were ≥ 3 cm in diameter.

Sections of each MMT were examined histologically to determine mode of growth and histologic grade. Among the 94 MMTs, 24 were considered expansive and 70 were considered invasive. Histologic grades I, II, and III were assigned to 21, 43, and 30 MMTs, respectively. Presence or absence of regional lymph node metastases at the time of surgery was also determined histologically for 72 dogs with MMTs; lymph nodes were not obtained from 22 dogs with MMTs.

Clinically normal mammary gland tissue samples—Following euthanasia of 8 dogs without any palpable mammary gland masses, 8 apparently normal mammary glands were collected. Histologic examination of H&E-staining sections of those tissues confirmed the absence of tissue abnormalities.

Figure 1—Representative results of western blot analysis of MMP-9 expression in neoplastic and clinically normal canine tissues. The 4 lanes included a molecular weight marker (lane 1), a sample of uterus from a clinically normal dog (lane 2), a sample of liver from a clinically normal dog (lane 3), and a sample of mammary gland carcinoma from a dog (lane 4). Notice the presence of the active form of MMP-9 (82 kDa) in all 3 tissues (although weakly in the liver). The band presenting the proenzyme (proMMP-9 [92 kDa]) was least marked in the mammary gland carcinoma tissue and most marked in the hepatic tissue.

Figure 2—Representative photomicrographs of sections of a mammary gland tubulopapillary carcinoma (A, B, and C) and a mammary gland solid carcinoma (D, E, and F) obtained from 2 dogs following immunohistochemical staining for MMP-9 (A and D), uPA (B and E), and MIB-1 (C and F). A—Fibroblasts adjacent to the tubulopapillary carcinoma (arrowheads) have high MMP-9 expression. B—Expression of uPA in stromal fibroblasts (arrowheads) is high in this section. C—Nuclear labeling for MIB-1 is evident in the tubulopapillary carcinoma cells (arrow). D—Fibroblasts adjacent to the solid carcinoma (arrowheads) have low MMP-9 expression, and a few neoplastic cells are weakly immunoreactive for MMP-9 (arrows). E—Expression of uPA is scattered among a few stromal cells adjacent to the solid carcinoma (arrowheads). F—Nuclear labeling for MIB-1 is evident in a few solid carcinoma cells (arrows). Immunohistochemical stains; in all panels, bar = 50 µm.
Immunohistochemical and western blot analysis of MMP-9 expression—Sections of canine bone and uterus (positive controls) had intense MMP-9 immunoreactivity in bone osteoclasts and in the myomètreum, as expected. Sections of tumors that were treated with nonimmune goat immunoglobulin instead of the anti-MMP-9 (C-20) goat polyclonal antibody (negative controls) did not have any MMP-9–specific staining. Western blot analysis revealed the presence of an 82-kDa band mostly in mammary gland carcinomas and uterus, whereas hepatic tissue had weak bands at 82 and 92 kDa (Figure 1). These bands corresponded to the active (82 kDa) and inactive (proenzyme) forms of canine MMP-9.

In clinically normal mammary gland samples, only keratinocytes from epidermis and the smooth muscle layer of vascular structures were immunopositive for MMP-9; the epithelial and myoepithelial cells and the stromal fibroblasts were negative for MMP-9 expression. The staining of both keratinocytes and vascular smooth muscle were used as internal positive controls.

The expression patterns of MMP-9 in tumor-adjacent stromal cells (fibroblasts) and in neoplastic cells were cytoplasmic (Figure 2). Malignant MTs had significantly higher MMP-9 expression than did BMTs (Table 1); in each BMT, < 50% of the stromal cells were MMP-9 positive, which was the rationale for use of 50% as a cutoff in the grouping of MMTs on the basis of stromal expression. Neoplastic cell reactivity was not as remarkable (both in percentage and staining intensity) as was stromal cell expression in both BMT and MMTs. The cutoff of 25% for neoplastic cell reactivity was considered to be representative of the detected differences between BMTs and MMTs.

Matrix metalloproteinase-9 expression by stromal cells was not significantly associated with histologic type (Table 2), although complex carcinomas had lower ($P = 0.033$) stromal cell MMP-9 expression than did the other MMT groups considered together. In contrast, there was a significant association between histologic type and neoplastic cell expression of MMP-9. Complex carcinomas had a lower ($P = 0.004$) neoplastic cell expression of MMP-9, compared with findings for the other MMT groups considered together.

Stromal MMP-9 expression was significantly higher in MMTs that were ≥ 3 cm in diameter ($P = 0.001$); in histologic grade III tumors, compared with grade II or I tumors ($P = 0.005$); in tumors with invasive growth, compared with tumors with expansive growth ($P = 0.034$); in tumors with a high MIB-1 labeling index, compared with tumors with a low MIB-1 labeling index ($P = 0.010$); and in tumors with a high expression of uPA in stromal cells, compared with tumors with a low expression of uPA in stromal cells ($P < 0.001$; Table 2). There were also significant associations between high stromal expression of MMP-9 and regional lymph node and distant metastases ($P = 0.032$ and 0.014, respectively). The expression of MMP-9 in neoplastic cells was not related to the aforementioned characteristics, with the exception of regional lymph node metastases ($P = 0.006$).

Overall survival times and disease-free intervals—The assessment of overall survival times involved 70 dogs with MMTs (for 12 dogs, the follow-up period was not yet completed; another 7 dogs were lost to follow-up, and another 5 dogs died because of unknown reasons during the immediate postoperative period), and the assessment of disease-free intervals involved 74 dogs with MMTs (for 8 dogs, the follow-up period was not yet completed although they remained free of detectable metastases at the last examination performed; another 7 dogs were lost to follow-up, and another 5 dogs died for unknown reasons during the immediate postoperative period). The mean ± SE overall survival time for dogs with tumors with high MMP-9 expression in stromal cells (15.13 ± 1.7 months) was significantly ($P < 0.001$) lower than the mean overall survival time (22.22 ± 0.83 months) for dogs with tumors with low stromal cell MMP-9 expression (Figure 3). Furthermore, there was a significant ($P < 0.001$) relationship between high expression of MMP-9 in stromal cells and shorter disease-free interval (mean ± SE, 12.94 ± 1.94 months), compared with tumors with low stromal cell MMP-9 expression (20.55 ± 1.13 months; Figure 4). Matrix metalloproteinase-9 expression in neoplastic cells was not significantly associated with either overall survival time or disease-free interval. Dogs that had MMTs with a low MIB-1 labeling index (< 43% MIB-1–positive cells)

Table 1—Expression* of MMP-9 (determined semiquantitatively via immunohistochemical staining) in tumor-adjacent stromal and neoplastic cells in BMTs and MMTs removed surgically from 118 dogs.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Total No. of tumors</th>
<th>Low expression</th>
<th>High expression</th>
<th>P value</th>
<th>Low expression</th>
<th>High expression</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
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<tr>
<td>BMT</td>
<td>24</td>
<td>24 (100.0)</td>
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<td>&lt; 0.001</td>
<td>20 (83.3)</td>
<td>4 (16.7)</td>
<td>0.023</td>
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<tr>
<td>MMT</td>
<td>94</td>
<td>56 (61.7)</td>
<td>36 (38.3)</td>
<td>0.033</td>
<td>56 (59.6)</td>
<td>38 (40.4)</td>
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</table>

*Sections of tumors were stained immunohistochemically for MMP-9 and examined microscopically (1 section was examined in tumors < 1 cm and 2 sections were examined in tumors > 1 cm; in tumors > 1 cm, both slides were examined, and the global estimation was considered instead of the 2 individual estimations). Tumors were classified as having low or high MMP-9 expression in stromal cells (ie, < 50% or ≥ 50% of stromal cells with cytoplasmic staining, respectively) and as having low or high MMP-9 expression in neoplastic cells (ie, < 25% or ≥ 25% of neoplastic cells with cytoplasmic staining, respectively).
had a significantly (P < 0.001) longer overall survival time (22.13 ± 0.86 months) and disease-free interval (20.32 ± 1.20 months) than did dogs that had MMTs with a high MIB-1 labeling index (mean overall survival time, 15.95 ± 1.64 months; mean disease-free interval, 13.62 ± 1.87 months).

Discussion

Currently, cancer researchers have widened their focus from neoplastic cells alone to neoplastic cells and the surrounding tumor stroma. Tumor-adjacent stromal cells are considered to have a role in the process of tumor invasion. In recent years, investigation of MMPs has amplified the interest in stromal cell involvement in tumor progression and metastasis.10

The present study evaluated the expression of MMP-9 in stromal and neoplastic cells of MTs in dogs. The MMP-9 expression in tumor-adjacent fibroblasts in MMTs was significantly higher than that in BMTs or clinically normal mammary gland tissues, as previously confirmed significantly higher MMP-9 activity and expression.1

<table>
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<th>Characteristic</th>
<th>Total No. of tumors</th>
<th>Low expression</th>
<th>High expression</th>
<th>P value</th>
<th>No. (%) of tumors with MMP-9–positive stromal cells</th>
<th>No. (%) of tumors with MMP-9–positive neoplastic cells</th>
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<tr>
<td>Tumor histologic type†</td>
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<td>31 (56.4)</td>
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<td>8 (44.4)</td>
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<td>Tumor diameter‡</td>
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<td>&lt; 3 cm</td>
<td>48</td>
<td>37 (77.1)</td>
<td>11 (22.9)</td>
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<td>≥ 3 cm</td>
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<td>20 (44.4)</td>
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<td>11 (47.8)</td>
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<td>Distant metastases¶</td>
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<td>High</td>
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<td>18 (46.2)</td>
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<td>uPA expression in stromal cells*</td>
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†Because of the low number of some histologic types, MMTs were grouped as follows: complex carcinomas, simple carcinomas (solid, tubulopapillary, anaplastic, and micropapillary carcinomas), and others (carcinosarcomas, carcinomas in benign tumors, spindle cell carcinomas, and mucinous carcinomas). In 1 case, tumor size was impossible to measure because it was multifocal throughout the mammary gland chain. §Lymph nodes from 22 dogs were not submitted for examination (1 regional lymph node/dog). ¶The presence of distant metastases was assessed via physical examination, 3-view thoracic radiography, and abdominal ultrasonography, and confirmed via cytologic or histologic analysis. At the end of the study, 12 dogs were alive and still undergoing follow-up; 7 dogs were lost to follow-up. ¶¶Tumor sections were immunostained for uPA and MIB-1. $Tumor sections were immunostained for uPA (at 200X magnification) in the area of highest labeling, and the index was expressed as a percentage. $Tumor sections were immunostained for MIB-1 labeling (assessment of Ki-67 expression) in neoplastic cells and evaluated microscopically. The MIB-1 labeling index was determined by counting the number of immunopositive cells among 1,000 neoplastic cells (at 400X magnification) in the area of highest labeling, and the index was expressed as a percentage. "Present" means that there is a staining in the neoplastic cells (at 400X magnification) in the area of highest labeling. "Absent" means that there is no staining at all in the neoplastic cells. NS = Not significant (P ≥ 0.05). See Table 1 for remainder of key.
expression of MMP-9 by neoplastic cells was significantly related to higher MIB-1 indexes, suggesting that neoplastic cell proliferation is stimulated by stromal MMP-9. Stromal-derived MMPs may facilitate tumor growth through the activation of latent growth factors or inactivation of growth inhibitory molecules and by providing space for the expanding tumor mass.\[2,31\] One important signaling pathway for tumor growth involves transforming growth factor-\(\beta\), which can be activated by MMP-9.\[22,25\] Matrix metalloproteinase-9 is also an important promoter of tumor growth by increasing the bioavailability of angiogenic factors.\[8,12,23\]

The expression of MMP-9 by neoplastic cells was not significantly associated with MIB-1 index in the...
present study, in contrast to the results of Nowak et al. Differences in field of view selection and counting procedures used to determine MIB-1 may explain the discordant results, but this is uncertain because there are no details of the counting protocol in that other report.

In human breast cancers, MMP-9 expression by tumor-associated fibroblasts has been related with histologic grade of the tumor. In the present study, grade III tumors had a significantly higher expression of MMP-9 in stromal cells, compared with findings for grade II or I tumors. By use of western blot analysis, Vinothini et al. detected a positive association between high concentrations of MMP-9 and less differentiated MTs in dogs. In addition to the upregulation of mitogenic signaling via release and activation of cytokines and growth factors by MMP-9, this could also be explained by the capacity of MMP-9 to modify integrin-mediated anchorage of epithelial cells (through proteolysis of ECM and cleavage of adhesion molecules such as cadherins), which contributes to the disruption of tissue architecture.

Recently, we determined that uPA expression by fibroblasts in the tumor vicinity is associated with poor prognostic factors and poor outcome in dogs with MMTs. Urokinase-type plasminogen activator, a serine protease, participates in the activation cascade of MMP-9. To our knowledge, the present study is the first to investigate the possible relationship between uPA expression and MMP-9 expression in MMTs in dogs. We found a significant association between high expressions of both proteins, suggesting that one of the mechanisms by which uPA promotes malignancy of MTs in dogs may be through an increase in MMP-9 activity in tumor-associated fibroblasts.

Although there is a wide range of biological functions of MMPs in neoplastic processes, the proteolytic degradation of the endothelial basement membrane and other matrix components by type IV collagenases is crucial for the tissue infiltration and intravasation of neoplastic cells into circulation. Matrix metalloproteinase-9 is also an important molecule for metastatic niche formation, thereby establishing a metastasis-supportive microenvironment. In the present study, stromal expression of MMP-9 in MMTs with invasive growth and regional lymph node and distant metastases was significantly higher than the stromal expression in MMTs with expansive growth and without regional lymph node and distant metastasis, which reinforces the hypothesis that stromal MMP-9 facilitates cancer cell migration and contributes to the development of a metastatic phenotype. Similar results have been reported for human breast cancers. Loukopoulos et al. found that in tumors of dogs, metastatic malignancies produced higher concentrations of MMP-9 than did nonmetastatic tumors, although this association did not reach significance.

In the present study, high MMP-9 expression by tumor cells was significantly related to regional lymph node metastasis but not to tumor size, mode of growth, histologic grade, stromal cell expression of uPA, or distant metastasis. With regard to human breast cancers, Kim et al. and Jobim et al. reported the absence of significant associations between tumor cell MMP-9 expression and poor prognostic factors, such as tumor size, histologic grade, or metastasis. After a thorough search in the literature, we were unable to locate any reports of immunohistochemical studies investigating the association of MMP-9 expression and any of the aforementioned factors in MTs in dogs.

As reported previously by our group and in accordance with findings of other researchers, high MIB-1 indexes were negatively associated with overall survival time and disease-free interval in dogs with MMTs. Furthermore, dogs with MMTs that had high stromal cell expression of MMP-9 had significantly shorter disease-free intervals and overall survival times, compared with those of dogs with MMTs with low stromal cell expression of MMP-9, similar to findings in human breast cancer studies. It is possible that higher MMP-9 activity in stromal cells translates into a more invasive and metastatic tumor. In contrast, the MMP-9 expression in neoplastic cells of MMTs was not significantly associated with survival time in dogs of the present study. To our knowledge, this is the first study of MMP-9 expression in MMTs and survival time in dogs.

In dogs with MTs, MMP-9 expression by tumor-associated fibroblasts appears to be significantly related to poor prognostic factors and shorter disease-free intervals and overall survival time. The results of the present study have suggested that tumor-adjacent stromal cells contribute to progression of MTs in dogs and that the stromal cell MMP-9 expression may be of prognostic value.

References


8. Gonzalez LO, Corte MD, Junqueira S, et al. Expression and prog-


