Tumor invasion, metastasis, and angiogenesis require controlled degradation of the ECM. There are several families of ECM-degrading enzymes, the most extensive of which are the MMPs. They belong to a multigene family of zinc-containing, calcium-dependent endopeptidases, which are involved in the degradation of ECM components in both physiologic and pathologic processes. Metalloproteinases are classified into 5 subgroups (gelatinases, collagenases, stromelysins, MT1-MMPs, and other MMPs) on the basis of their structure, substrate specificity, and cellular localization. Their combined action is capable of degrading almost all of the macromolecular ECM components. Matrix metalloproteinase-2 (gelatinase A) and MMP-9 (gelatinase B) are the main gelatinases and are type IV collagenases. Membrane-type 1 MMP is involved in the activation of MMP-2. Because these enzymes are able to degrade critical basement membrane and ECM components (different types of collagen, elastin, and fibronectin), they play an important role in tumor invasion and

Expression of matrix metalloproteinase-2 and -9 and membrane-type 1 matrix metalloproteinase in melanocytic tumors of dogs and canine melanoma cell lines

María-José Docampo, DVM, PhD; Jennifer Cabrera, MSc; Rosa M. Rabanal, PhD; Anna Bassols, PhD

**Objective**—To evaluate expression of matrix metalloproteinase (MMP)-2 and -9 and membrane-type 1 MMP (MT1-MMP) in melanocytomas and malignant melanomas of dogs, analyze in vitro production of MMPs by canine melanoma cell lines and primary dermal fibroblasts, and investigate mutual communication between tumor cells and fibroblasts and the influence of collagen on MMP regulation.

**Sample**—35 biopsy specimens from melanocytic tumors and primary dermal fibroblasts of dogs and 3 canine melanoma cell lines (CML-1, CML-10c2, and CML-6M).

**Procedures**—MMP-2, MMP-9, and MT1-MMP were detected in tumor samples by use of immunohistochemical analysis. In vitro production was analyzed via reverse transcriptase–PCR assay, immunocytochemical analysis, zymography, and immunoblotting.

**Results**—MMP-9 was overexpressed in malignant melanomas, compared with expression in melanocytomas, whereas no significant differences in MMP-2 and MT1-MMP immunostaining were detected. Stromal cells also often had positive staining results. In vitro, all 3 melanoma cell lines and dermal fibroblasts had evidence of MMP-2 and MT1-MMP but only melanoma cells had evidence of MMP-9. Coculture of CML-1 or CML-10c2 cells and dermal fibroblasts induced an increase in expression of the active form of MMP-2. Culture of melanoma cells on type I collagen increased the activation state of MT1-MMP.

**Conclusions and Clinical Relevance**—MMP-9 expression was increased in malignant melanomas of dogs. Stromal cells were a source for MMPs. Stromal cells, in combination with matrix components such as type I collagen, can interact with tumor cells to regulate MMP production. Information about MMP production and regulation could help in the development of new treatments. (Am J Vet Res 2011;72:1087–1096)

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco modified Eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>Membrane-type 1 matrix metalloproteinase</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with Tween-20</td>
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metastasis. The activity of these enzymes is controlled at several biological levels, including transcriptional regulation, release of inactive zymogens or proenzymes that must be activated, and production of endogenous tissue inhibitor of metalloproteinases. Furthermore, coexpression of activated MMPs with certain membrane receptors or adhesion molecules, such as CD44, may be essential in positioning the active proteases at the invasive edge of a tumor.

Increased concentrations of gelatinases are associated with invasion, metastasis, and poor prognosis in numerous malignancies of humans. This degradative ability can derive from tumor cells or other cellular components of the stroma, such as fibroblasts. It has been suggested that both tumor and stromal cell components may cooperate, which enables tumor cells to reach their target organ and survive the metastatic process. Thus, MMP-2 and -9 in tumor stromal elements has been reported in several types of carcinomas (including ovarian, thyroid, hepatocellular, and gastrointestinal) in humans. In tumors of dogs, gelatinase activity was first detected by use of gelatin zymography as a 92-kDa band secreted from cultured mastocyteoma cells. Subsequently, production of gelatinases has been described in several tumor types (eg, osteosarcomas, mast cell tumors, sarcomas and carcinomas, oronal tu-
mors, mammary gland tumors, and meningio-
mas) of dogs.

Melanoma is a common neoplastic disease of dogs, with variable clinical manifestations and biological behavior. Melanocytic tumors comprise 4% to 7% of all neoplasms in dogs. Melanomas account for 9% to 20% of skin tumors in dogs, and they are the most common malignant tumors of the oral cavity and digits of dogs. Oral and mucocutaneous melanomas are aggressive tu-
mors that commonly metastasize to the regional lymph nodes and lungs, although the existence of benign oral melanocytic lesions has been reported. Cutaneous melanocytic lesions are benign in most dogs. Data are available regarding evidence of MMPs in mel-
анocytes in humans, although the functional role of specific enzymes is still controversial. Little is known about the importance and distribution of MMPs in melanocytic tumors of dogs.

The purpose of the study reported here was to determine MMP-2, MMP-9, and MT1-MMP activity in melanocytic lesions of dogs. Furthermore, an in vitro analysis was conducted of the production of these enzymes by canine melanoma cell lines and primary dermal fibroblasts as well as the mutual communication between tumor cells and fibroblasts in relation to MMP production.

Materials and Methods

Sample—Biopsy specimens from 35 melanocytic tumors (14 melanocytomas and 21 malignant melano-

mers) of dogs were used in the study. All melanocytomas were of dermal origin. Fourteen of 21 malignant melanomas were within the oral cavity, and the other 7 were cutaneous melanomas. Tumors were on slides and in paraffin blocks of the archives of the Pathol-

ogy Department at the Facultat de Veterinària de the Universitat Autònoma de Barcelona. Serial sections (4 µm thick) were cut from paraffin blocks, mounted on slides, and stained with H&E. Tumors were classified as malignant or benign on the basis of cytologic features, mitotic activity, ulcers and necrosis, melanin production, junctional activity, and infiltration into deeper tissues in accordance with the World Health Or-

ganization classification. Clinical follow-up was not available for the 35 dogs.

Antibodies—Antibodies used in the study were monoclonal murine antibodies against MMP-2 (clone 42-5D11, Ab-3), MMP-9 (clone 36-2A4, Ab-3), and MT1-MMP (clone 113-5B7, Ab-4). Other antibodies used in the study were a polyclonal goat antibody against MMP-2 (K-20) and a monoclonal murine antibody against β-actin.

Immunohistochemical analysis—For MMP-2 and MMP-9 immunostaining, an adapted protocol reported elsewhere was used. Briefly, paraffin was removed from the slides, and the sections were rehydrated by use of a series of xylol and graded alcohol solutions. Activity of endogenous peroxidase was blocked by immersing slides in 0.3% H2O2 in methanol for 30 minutes. For antigen retrieval, a 20-minute heating step was performed in citrate buffer (pH, 6.0). After nonspecific sites were blocked by incubation with 20% normal goat serum in TBS solution (0.02M Tris-HCl and 150mM NaCl [pH, 7.4]) for 1 hour at 25°C, sections were incubated overnight at 4°C with anti–MMP-2 or anti–MMP-9 antibodies (which recognized both proenzyme and active forms of the enzyme) diluted 1 in 50 in blocking solution. Slides were then incubated with biotinylated goat anti-mouse immunoglobulin at a dilution of 1 in 200 in blocking solution for 1 hour at 25°C. Sections were incubated with the secondary antibody at a dilution of 1 in 50 in TBS solution (0.02M Tris-HCl and 150mM NaCl [pH, 7.4]) for 1 hour at 25°C, which was followed by incubation with the avidin-biotin-peroxidase complex. The reaction was developed by use of a commercial staining kit, and sections were counterstained with Mayer’s hematoxylin stain. Sections incubated with an irrelevant isotype-matched monoclonal antibody were used as negative control samples.

For MT1-MMP analysis, an adaptation of a protocol described elsewhere was used. Paraffin was removed, sections were rehydrated, endogenous peroxidase activity was inhibited, and nonspecific sites were blocked by incubation with 20% goat serum in TBS solution for 1 hour at 25°C. Sections were incubated with the anti–MT1-MMP antibody diluted 1 in 50 in blocking solution at 4°C overnight. Sections were washed and then incubated with the secondary antibody at a dilution of 1 in 200 in blocking solution for 1 hour at 25°C, which was followed by incubation with the avidin-biotin-peroxidase complex. The reaction was developed by use of 0.04% 3-amino-9-ethylcarbazole with 0.015% H2O2 in 50mM acetate buffer (pH, 5.0) containing 0.05% dimethylformamide. Slides were counterstained with Mayer’s hematoxylin stain. Sections incubated with an irrelevant isotype-matched monoclonal antibody were used as negative control samples.

Sections were examined separately by 2 investigators (MJD and RMR). Sections were considered to have negative or positive results on the basis of the absence or presence of cytoplasmic staining. Slides were graded 1088 AJVR, Vol 72, No. 8, August 2011 Unauthenticated | Downloaded 08/07/24 08:50 AM UTC
on the basis of the intensity and pattern of immunoreaction by use of a 4-tier grading system (0 = no staining, 1 = weak staining, 2 = multifocal expression with strong staining intensity, and 3 = diffuse expression with strong staining intensity). Slides were examined via a microscope and photographed with an integrated digital camera system.10

**Statistical analysis**—Statistical analysis was performed by use of commercial software. The χ² test was used to examine the association between MMP expression and tumor type. Significance was set at values of P < 0.05.

**Comparison of expression of CD44, hyaluronan, and MMPs**—Expression of CD44 and hyaluronan was investigated in a previous study conducted with serial sections of the same set of melanocytic tumors. Therefore, expression of MMPs for the study reported here was compared with expression for the ECM-related molecules reported in the other study by use of a χ² test.

**Cell lines and cell culture**—Canine melanoma cell lines CML-1, CML-10c2, and CML-6M were originally derived from melanoma tumors of dogs.32,33 Canine primary fibroblasts were obtained from skin biopsy specimens.4 Cells were grown in a humidified atmosphere at 37°C with 5% CO₂ in DMEM supplemented with 10% FCS, 100 U of penicillin/mL, and 100 µg of streptomycin/mL.

For gel zymography, subconfluent cell cultures were washed twice with PBS solution and once with DMEM without FCS for 4 hours and subsequently cultured in FCS-free medium for 24 hours. Conditioned media were collected, centrifuged to clarify debris, and subjected to gelatin zymography.

**Coculture experiments**—Cells were trypsinized and diluted to 1 X 10⁶ cells/mL for fibroblasts and 2 X 10⁵ cells/mL for melanoma cells (CML-1, CML-10c2, or CML-6M) in DMEM with 10% FCS. For cocultures, 1:1 mixtures of fibroblasts (1 X 10⁶ cells/mL) and each melanoma cell line (2 X 10⁵ cells/mL) in DMEM with 10% FCS were prepared. Two milliliters of each single-cell or mixed-cell suspension was seeded on 6-well coated dishes. Cells were allowed to attach overnight. Adherent cells were rinsed and diluted to 1 X 10⁵ cells/mL for melanoma cells (CML-1, CML-10c2, and CML-6M) in DMEM with 10% FCS, 100 U of penicillin/mL, and 100 µg of streptomycin/mL.

Collagen-coating experiments—Culture dishes were coated with a solution of type I or type IV collagen (300 µg/mL) to achieve a coating concentration of 50 µg/cm². Coated dishes were incubated for 3 hours at 37°C and then left to dry overnight in a sterile tissue culture hood, rinsed with sterile distilled water to remove salt precipitates, and rehydrated with PBS solution. This protocol allows a film of fibrillar collagen I to form.33 Cells were seeded on collagen-coated or uncoated dishes in DMEM with 10% FCS and allowed to attach overnight. Adherent cells were rinsed and then incubated for 48 hours with FCS-free DMEM.

Gelatin zymography—Gelatin zymography was performed as described elsewhere.36 Briefly, samples were mixed with SDS sample buffer without a reducing agent (50% glycerol, 10% SDS, and 1% bromophenol blue in 1M Tris HCl [pH, 6.8]) and separated on 7.5% SDS-polyacrylamide gels containing 1 mg of gelatin/mL. After electrophoresis, gels were thoroughly rinsed with 2.5% Triton X-100 in 50mM Tris HCl (pH, 7.5) followed by distilled water to completely eliminate SDS. Gels were incubated for 20 hours at 37°C with developing buffer (150mM NaCl, 10mM CaCl₂, and 50mM Tris HCl [pH, 7.5]). To develop the gelatin-degrading proteinase activity, gels were stained with 0.1% Coomassie brilliant blue R250 in 10% propanol–10% acetic acid and then destained in 10% propanol–10% acetic acid. Gelatinolytic activities were detected as clear bands against a blue background. Images were recorded and analyzed.36 Conditioned media from MXT-c1.1 cells (a metastatic mouse mammary gland carcinoma cell line) were included as control samples for secreted gelatinases.

Western immunoblotting—For MT1-MMP detection, cell extracts from subconfluent cultures were prepared by lysing cells via incubation in buffer containing protease inhibitors (50mM Tris-HCl [pH, 7.5], 0.27mM sucrose, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 1mM NaVO₄, 10mM sodium glycerophosphate, 50mM NaF, 5mM sodium pyrophosphate, 0.5mM benzamidine, 0.1% β-mercaptoethanol, and 0.2mM phenylmethyl sulfonyl fluoride) for 30 minutes at 4°C under agitation. Cell lysates were centrifuged at 14,000 g for 15 minutes at 4°C to remove insoluble debris, and supernatants were collected. The amount of protein applied to the gel was adjusted on the basis of protein content after quantification via the Bradford assay. Samples (50 µg of protein) were mixed with 6X reducing sample buffer (1.5% SDS, 32.5mM Tris-HCL, 3.75% β-mercaptoethanol, 0.1

| Table 1—Immunohistochemical expression of MMP-9, MMP-2, and MT1-MMP in melanocytic tumors of dogs. |

<table>
<thead>
<tr>
<th>Tumors</th>
<th>MMP-9</th>
<th>MMP-2</th>
<th>MT1-MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Melanocytoma (n = 14)</td>
<td>0</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Malignant melanoma (n = 21)</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Oral (n = 14)</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cutaneous (n = 7)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Slides were graded on the basis of the intensity and pattern of immunoreaction by use of a 4-tier grading system (0 = no staining, 1 = weak staining, 2 = multifocal expression with strong staining intensity, and 3 = diffuse expression with strong staining intensity).
g of saccharose/mL, and 0.0025% bromophenol blue), denatured at 95°C for 10 minutes, and resolved in a 12% polyacrylamide gel under reducing conditions. After SDS-PAGE, proteins were transferred onto polyvinylidene fluoride membranes and nonspecific binding sites on the blot were blocked by incubation with 5% (vol/vol) dried skim milk in TBS-T solution (0.02M Tris and 0.15M NaCl [pH, 7.4] containing 0.1% Tween 20) for 1 hour at 25°C. Membranes then were incubated with antibody against MT1-MMP (1 in 20 dilution) in blocking solution overnight at 4°C, washed with TBS-T solution, and incubated with horseradish peroxidase–conjugated anti-mouse IgG (1 in 5,000 dilution) in 2.5% dried skim milk in TBS-T solution for 1 hour at 25°C. Antibody binding was detected via enhanced chemiluminiscence. Blots were then stripped of antibodies by incubation in 2% SDS in 62.5mM Tris-HCl (pH, 6.7) containing 100mM β-mercaptoethanol for 30 minutes at 55°C, washed with TBS-T solution, blocked again, and then probed with anti-β-actin at a dilution of 1 in 20,000 to verify uniform total protein loading of each well on the gels.

Fluorescence immunocytochemical analysis—Cells were seeded and grown on coverslips, rinsed with PBS solution, and fixed with 3% paraformaldehyde–2% saccharose for 20 minutes at 37°C. After cells were rinsed with PBS solution, they were permeabilized by incubation with 0.1% Triton X-100 in PBS solution for 10 minutes, and nonspecific binding sites were blocked by incubation with 1% bovine serum albumin and 10% normal rabbit serum in PBS solution for 1 hour. Cells then were incubated overnight at 4°C with the polyclonal anti–MMP-2 antibody (diluted 1 in 10) in PBS solution. Subsequently, cells were washed with PBS solution and incubated with anti-goat secondary antibody labeled with fluorescein isothiocyanate (diluted 1 in 400) in PBS solution. Nuclei were revealed by incubating the coverslips with 0.1 mg of Hoechst 33342/mL in PBS solution for 3 minutes. Cultures were evaluated with an epifluorescence microscope and photographed with an integrated digital camera system.

RNA isolation and RT-PCR assay—Total RNA was extracted from subconfluent cells by use of an isolation kit. For RT-PCR assays, 2 µg of total RNA was reverse transcribed in a 40-µL reaction volume with the transcriptase, as described in a protocol provided by the manufacturer. An aliquot (2.5 µL) of the reaction mixture was subsequently used for PCR assay with specific primers for canine MMP-2, MMP-9, and MT1-MMP (Appendix). The assay included use of an initial denaturing step at 94°C for 2 minutes; followed

Figure 1.—Photomicrographs of sections of melanocytic tumors of dogs in which MMP-9 (A–D), MMP-2 (E and F), and MT1-MMP (G and H) were analyzed immunohistochemically. Melanocytomas (A and B) had moderate diffuse MMP-9 expression, whereas cutaneous (C) and oral (D) malignant melanomas had intense and diffuse, respectively, cytoplasmic expression of MMP-9 in tumor cells arranged in nests (C and D) and strands (C). Notice the positively stained inflammatory cell (thin arrow) in the cutaneous malignant melanoma (C). Cutaneous (E) and oral (F) malignant melanomas had moderate cytoplasmic expression of MMP-2 in tumor cells. Notice the strongly positive stromal cells primarily located in the septa between tumor cell nests (white arrowheads) in the cutaneous malignant melanoma (E). Intensity of the signal may be heterogeneous inside the lesion of the oral malignant melanoma (F). A cutaneous malignant melanoma with moderate diffuse cytoplasmic expression of MT1-MMP in tumor cells (G) and an oral malignant melanoma with weak cytoplasmic expression of MT1-MMP in tumor cells (H). Notice the strongly positive peritumoral stromal cells in the cutaneous malignant melanoma (thick arrow [G]) and the positively stained endothelial cells in intratumoral blood vessels (black arrowheads [H]). Avidin-biotin-peroxidase staining with Mayer’s hematoxylin counterstain. Bars = 100 µm.
by 30 cycles of reaction, with each cycle including 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C; and a final extension step at 72°C for 5 minutes. Amplification of a fragment of GAPDH was used as an internal control sample. The PCR amplification products were resolved on 2% agarose gels, stained with ethidium bromide, developed and photographed under UV light, and recorded for analysis.

Results

Immunohistochemical detection of MMP-2, MMP-9, and MT1-MMP in melanocytic tumors of dogs—Immunoreactivity of MMP-2, MMP-9, and MT1-MMP was assayed in 35 tissue samples obtained from melanocytomas and oral and cutaneous melanomas of dogs. Intensity of labeling was assessed (Table 1).

Matrix metalloproteinase-9 was widely expressed in tumor samples. There was a significant (P = 0.018) increase in the amount of expression of MMP-9 in melanomas, compared with the amount of expression in melanocytomas. This difference was found when comparing expression for the total number of benign tumors with that of malignant tumors as well as when comparing expression for melanocytomas with expression of melanomas of oral or cutaneous origin separately. No significant difference in expression was found between oral and cutaneous melanomas. There was a cytoplasmic and diffuse pattern of labeling for MMP-9 in tumor cells. Distribution of MMP-9 inside tumors was quite homogeneous, and cell types (including neutrophils, mast cells, and some stromal fibroblasts) other than tumor cells also had positive results for MMP-9 (Figure 1).

Expression of MMP-2 was heterogeneous among tumors and also inside a specific tumor. There was often a perinuclear pattern of reactivity in the neoplastic cells (Figure 1). Individual tumors had variations in the number of tumor cells with positive results for MMP-2 and in the labeling intensity, which ranged from tumors with no staining to tumors with intense labeling for benign and malignant tumors. In concordance with this,
no significant ($P = 0.220$) differences were detected in expression of MMP-2 between melanocytomas and oral or cutaneous melanomas. It was interesting that stromal cells inside the tumors often had strong positive results for MMP-2.

The MT1-MMP labeling was weak, or there was no staining, in 28 of 35 samples. Interestingly, peritumoral stromal cells and tumor blood vessels were often labeled (Figure 1). No significant ($P = 0.839$) difference was detected for MT1-MMP labeling between benign and malignant tumors.

**Comparison of expression of CD44, hyaluronan, and MMPs**—Examination of the labeling intensity revealed that MMP-9 expression was significantly ($P = 0.019$) associated with expression of hyaluronan but not with expression of CD44 ($P = 0.746$) or versican ($P = 0.146$). Expression of MT1-MMP was significantly ($P = 0.029$) associated with expression of CD44 but not with expression of hyaluronan ($P = 0.937$) or versican ($P = 0.231$). Expression of MMP-2 was not significantly associated with expression of CD44 ($P = 0.070$), hyaluronan ($P = 0.683$), or versican ($P = 0.483$).

**MMPs of canine melanoma cell lines and canine dermal fibroblast cultures**—To obtain further insight into the cell origin of MMPs, RT-PCR assays were performed with RNA extracted from canine dermal fibroblasts and melanoma cell lines by use of specific primers for canine sequences. Matrix metalloproteinase-2 and MT1-MMP were expressed in fibroblasts as well as in all 3 melanoma cell lines (Figure 2). In contrast, only melanoma cells expressed MMP-9 in variable amounts. To validate results of the RT-PCR analysis, MMP-2 was detected via immunocytochemical analysis in all melanoma cell lines as well as in dermal fibroblasts. Because immunolocalization does not distinguish between active and proactive forms of MMP, in vitro evaluations were conducted to analyze whether tumor cells and stromal cells produce the active forms of gelatinases MMP-2 and MMP-9. Gelatin zymograms of conditioned media from canine melanoma cell lines and canine dermal fibroblasts had gelatinolytic bands with different molecular masses. The main MMP in the media was MMP-2 in its proactive form. Active and intermediate forms of MMP-2 were also visible in the zymography gel, with variations in the amounts depending on the cell line. The proactive and active forms of MMP-9 were evident as minor bands in melanoma cells but were not evident in dermal fibroblasts. In addition, MT1-MMP was detected in all cell types by use of western immunoblotting with an antibody against the human protein; MT1-MMP was more abundant in melanoma cells than in fibroblasts. This technique allowed us to identify 2 bands: the first band at approximately 57 kDa that corresponded to the active form of MT1-MMP and the second approximately 60 kDa that corresponded to the proactive form of MT1-MMP.

**MMPs of tumor melanoma cells cocultured with dermal fibroblasts and on collagen-coated wells**—To evaluate whether melanoma and dermal fibroblast
cells can mutually interact, leading to modulation of proteolytic activity, a coculture of both cell types was established and maintained for 48 hours in FCS-free medium. Following this, the gelatinolytic activity of the cultures was determined by use of gelatin zymography. Cocultures of CML-1 and CML-10c2 cells and fibroblasts had an increase in the intensity of the band corresponding to active MMP-2 (Figure 3). No difference was detected when CML-6M cells and fibroblasts were cocultured, probably because of the already high expression of the active form of MMP-2.

It has been reported that some ECM components (eg, type I collagen) can modulate MMP activity in tumor cells. Thus, canine melanoma cells were cultured on type I and type IV collagen-coated wells for 48 hours, and the presence and activation state of MT1-MMP were analyzed by use of western immunoblotting. An important change in the ratio between the active and proactive forms resulted when cells were cultured with type I collagen (Figure 4). This activation was especially noticeable in CML-1 cells, which were able to strongly attach to collagen (data not shown). In contrast, culture of any of the melanoma cell lines with type IV collagen did not yield substantial changes in MT1-MMP amounts.

Discussion

In the study reported here, expression of gelatinases MMP-2, MMP-9, and MT1-MMP in canine melanoma and melanocytomas was evaluated. Diagnosis of the tumor type was made on the basis of cytologic criteria, and no follow-up monitoring of the dogs was available. Analysis of the results revealed that there was an increase in MMP-9 expression in melanomas, compared with the expression of MMP-9 in benign melanocytic tumors. Matrix metalloproteinase-9 was abundant in melanomas and had a homogeneous distribution in both tumor and stromal cells. Other cell types, particularly tumor-infiltrating neutrophils and mast cells, also produced MMP-9, which suggested that MMP-9 can be generated as a result of the reciprocal interaction between tumor and nontumor cells. The production of MMP-9 by inflammatory cells has been reported by other investigators.

In contrast to results for MMP-9, we did not detect significant differences in expression of MMP-2 and MT1-MMP between melanomas and melanocytomas. Contradictory results have been reported for melanocytic tumors in humans, even though these tumors had an established pattern of progression from benign nevi to dysplastic nevi to primary melanoma to metastatic melanoma. Expression of MMP-2 is detected in the early stage of tumor progression because > 50% of common nevi have positive results for MMP-2. In another study, MMP-2 expression was not detected in benign lesions, but most of the primary and metastatic melanomas had positive results for MMP-2. Investigators in other studies proposed that MMP-2 is a separate prognostic factor because overexpression is associated with an increased risk of death as a result of

![Figure 4](image-url)

**Figure 4**—Expression of MT1-MMP in canine melanoma cell lines cultured on collagen-coated wells as determined by use of western immunoblotting. Cells were cultured on plastic (P), type I collagen (Col I; A), or type IV collagen (Col IV; B) for 48 hours. Notice the change between the proactive (Pro-) and active form of MT1-MMP in cells (particularly CML-1 cells) cultured on type I collagen. β-Actin was used as a loading control sample.
melanoma. In the present study, we detected MMP-2 immunoreactivity in a high number of melanocytomas and melanomas; unfortunately, follow-up monitoring of the dogs was not possible. In other tumor types in dogs, increased expression of MMP-2 and MT1-MMP is found in mammary gland carcinomas, when compared with expression in normal tissue, but the labeling pattern for MMP-2 and MT1-MMP was not significantly associated with the histologic subtype or tumor stage. Investigators in 1 study detected increased MMP-2 activity in malignant mammary gland tumors of dogs, whereas in another study, investigators found several tumor types (eg, carcinomas, sarcomas of various origins, or mast cell tumors) of dogs in which there was no correlation between gelatinase activity of tumor extracts (as quantified by use of zymography and the biological behavior of the tumors) for benign or malignant tumors. The authors of that study claimed that some benign and malignant tumors may have higher and lower gelatinolytic activity, respectively, and argued that the inflammatory component (evident in a high percentage of benign tumors) may affect total gelatinase activity of the tumor. Lack of correlation between MMP activity and protein immunoreactivity has been reported in melanomas in humans, and this may explain contradictory data. Thus, the role of MMP-2 in tumor progression is controversial. It is interesting that peritumoral stromal cells as well as blood vessels were often labeled with MT1-MMP, which reinforced the proposed role of stromal cells in invasion and angiogenesis.

In addition to their role as ECM-degrading enzymes, MMPs have important roles in the interaction with nonmatrix proteins, including cell adhesion molecules (such as members of the integrin family and CD44) and the hyaluronan receptor. Both MMP-9 and hyaluronan are related to CD44, the membrane receptor for hyaluronan, and an anchorage molecule for the proteolytically active form of MMP-9 on the cell surface of tumor cells. Examination of the labeling intensity and distribution of these molecules revealed a significant (P = 0.029) relationship between MT1-MMP and CD44 expression and similar intratumor distribution of MT1-MMP and CD44 (data not shown). Results for the study reported here are in agreement with those reported elsewhere, in which MT1-MMP and CD44 were colocalized at the adherent edge of migrating cells and may be functionally related.

Canine melanoma cell lines and dermal fibroblasts were able to produce MT1-MMP and active MMP-2. Nevertheless, only canine melanoma cell lines, but not dermal fibroblasts, expressed MMP-9, as detected by use of RT-PCR assay and zymography. Interestingly, the CML-6M cell line, which represents metastatic melanoma cells, had a higher expression of MMP-9. In mice, MMP-9 is especially important in the early steps of metastasis of melanoma cells to the lungs, and it has been suggested that the in vivo selection process for metastatic subclones favors those that express MMP-9. It has become increasingly evident that stromal cells and the microenvironment play a key role in tumor progression. Analysis of results of the present study indicated that cells of canine melanoma cell lines and normal fibroblasts reciprocally communicate, leading to higher synergic MMP-2 activity in cocultured cells. This synergic effect was more important in CML-1 and CML-10c2 cells than in the CML-6M cells, possibly because of the high basal MMP-2 activity in the CML-6M cell line. Similar results have been described for oral epithelial cancer cells and human melanoma cells cocultured with dermal fibroblasts. An explanation for the reason that the tumor cells and adjacent stromal cells express MT1-MMP, MMP-2, and MMP-9 may be that both cellular components contribute to different parts of the metastatic cascade. The MMPs in tumor cells may contribute to invasive growth of the tumors, whereas stromal elements may contribute to the remodeling process and the desmoplastic reaction in tissues adjacent to tumors.

Finally, cell culture on type I collagen films was able to modulate MMP expression in cells of canine melanoma cell lines. A change in the ratio between the proactive and active forms of MT1-MMP protein detected by the use of western immunoblotting was evident in CML-1 cells. This effect appeared to be cell-type specific because it was more noticeable in CML-1 cells than in CML-10c2 or CML-6M cells. Regulation of MMP activity by collagen has been described for several tumor types, but the effects differ depending on the MMP (MMP-2 and MT1-MMP), collagen type, or collagen-coating procedures. Analysis of these results suggested that the cell component of the tumor stroma may have a role in melanoma tumor progression and that some specific ECM components may modulate the matrix-degradative abilities of melanoma cells.

Analysis of results of the study reported here suggested a collaboration between tumor and stromal cells in the production of MMPs as well as a potential relationship between MMPs and other ECM-related molecules (ie, hyaluronan and CD44) that would contribute to tumor progression in melanomas of dogs. Elucidation of critical steps in the regulation of MMPs may allow the identification of new targets for the development of specific antitumor drugs.
References


### Appendix

Primer sets used for RT-PCR assay of MMPs in canine melanoma cell lines.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>GenBank accession No.</th>
<th>Primer sequence (5′–3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>XM_535300*</td>
<td>Forward: GAGACGGCCATGTCCACTAT Reverse: TTGTCCGTGTTGTCACGTG</td>
<td>506</td>
</tr>
<tr>
<td>MMP-9</td>
<td>NM_001002319</td>
<td>Forward: GCAGCTGGCGAGAGGATATC Reverse: TGGCCAGAGTCCATTACCC</td>
<td>515</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>XM_843964 (transcript variant 1)*</td>
<td>Forward: CTTACTTCTTCGCGGAAAC</td>
<td>521</td>
</tr>
<tr>
<td></td>
<td>XM_851854 (transcript variant 2)</td>
<td>Reverse: TTCAGAGCAGAATCTCGCTT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>AB039240</td>
<td>Forward: TGAATTGCAGATCTCAAGG Reverse: CCAGAAATGAGCTTGACAA</td>
<td>929</td>
</tr>
</tbody>
</table>

*Predicted canine sequence. †Primer amplifies a fragment common to both variants.