MMP-2, MMP-9 AND MT1-MMP EXPRESSION IN MELANOCYTIC TUMORS OF DOGS AND CANINE MELANOMA CELL LINES

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Abstract

Objective: To study the expression of MMP-2, MMP-9 and MT1-MMP in melanocytomas and malignant melanomas of dogs. Secondly, to analyze *in vitro* the production of MMPs by canine melanoma cell lines and primary dermal fibroblasts. Third, to investigate the crosstalk between both cell types and the influence of collagen on MMP regulation.

Sample Population: 35 biopsies corresponding to canine melanocytic tumors, canine primary dermal fibroblasts, and 3 canine melanoma cell lines.

Procedures: MMP-9, MMP-2 and MT1-MMP were detected in tumor samples by immunohistochemistry. *In vitro* detection was analyzed by RT-PCR, immunocytochemistry, zymography and western blot.

Results: MMP-9 was found to be overexpressed in malignant melanoma compared to melanocytoma, whereas no significant differences in MMP-2 and MT1-MMP immunostaining were observed. Stromal cells were also often positive. *In vitro,* all three melanoma cell lines and dermal fibroblasts produced MMP-2 and MT1-MMP, but only melanoma cells produced MMP-9. Co-culture of CML-1 and CML-10c2 melanoma cells and dermal fibroblasts induced an increase in 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 is increased in malignant melanomas of dogs. Stromal cells are a source for MMPs and, together with matrix components as type I collagen, can interact with tumor cells to regulate MMP production. Because MMPs are responsible for the degradation of the extracellular matrix and are involved in tumor invasion and metastasis, the knowledge of MMP production and regulation could help in the development of new potential therapies.

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Tumor invasion, metastasis and angiogenesis require controlled degradation of the extracellular matrix (ECM). There are several families of ECM-degrading enzymes, the most extensive of which are the matrix metalloproteinases (MMPs). They belong to a multigene family of zinc-containing, calcium-dependent endopeptidases that are involved in the degradation of ECM components in both physiological and pathological processes. The MMPs are classified into five different subgroups according to their structure, substrate specificity and cellular localization: gelatinases, collagenases, stromelysins, membrane-type MMPs (MT-MMP) and other MMPs. Their combined action is capable of degrading almost all of the macromolecular ECM components.[1, 2] Amongst the MMPs, the gelatinases or type-IV collagenases group includes MMP-2 (gelatinase A) and MMP-9 (gelatinase B). MT1-MMP is involved in the activation of MMP-2. Since these enzymes are able to degrade critical basement membrane and ECM components (different collagen types, elastin, fibronectin), they play an important role in tumor invasion and metastasis.[3] The activity of these enzymes is controlled at different biological levels, including transcriptional regulation, the release of inactive zymogens or proenzymes that must be activated and the production of endogenous tissue inhibitors (TIMPs).[4, 5] 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 front of the tumor.[6]

Increased concentrations of gelatinases are associated with invasion, metastasis and poor prognosis in numerous human malignancies. This degradative ability can be through either enzymatic capacities of the tumor cell or other cellular components of the stroma, such as fibroblasts. Likely there is a cooperation between the two components enabling the tumor cell to reach its target organ and survive in the metastatic process.[3, 7-9] Thus, the presence of MMP-2 and MMP-9 in tumor stromal elements has been

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reported in several types of human carcinomas including ovarian, thyroid, hepatocellular, and gastrointestinal.[1]

In canine tumors, gelatinase activity was first detected by gelatin zymography as a 92-kDa band secreted from cultured mastocytoma cells.[10] Subsequently, production of gelatinases has been described in several canine tumor types, such as osteosarcomas,[11] mast cell tumors,[12] sarcomas and carcinomas,[13, 14] oronasal tumors,[15] mammary tumors,[16-19] and meningiomas.[20]

Melanoma is a common neoplastic disease of dogs with a variable presentation and biological behavior. Melanocytic tumors comprise 4-7% of all canine neoplasms. Melanomas account for 9-20% of skin tumors in dogs, and they are the most common malignant tumors of the oral cavity and the digits of the dog. Oral and mucocutaneous melanomas are aggressive tumors that commonly metastasize to the regional lymph nodes and to lungs,[21] although the existence of benign oral melanocytic lesions have been reported.[22, 23] Cutaneous melanocytic lesions are benign in most dogs.[21, 22] Some data is available about the presence of MMPs in human melanocytic lesions,[24, 25] although the functional role of the specific enzymes is still controversial. [26-29] Nevertheless, little is known about the importance and distribution of these molecules in canine melanocytic tumors.

The purpose of the present study was to determine MMP-2, MMP-9 and MT1-MMP in canine melanocytic lesions. Furthermore, a *in vitro* analysis of the production of these enzymes by canine melanoma cell lines and primary dermal fibroblasts, as well as the crosstalk between both cell types in relation to MMP production, was also undertaken.

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Materials and Methods

Case material

Thirty-five melanocytic tumors (14 melanocytomas and 21 malignant melanomas) 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. Tumors were on slides and in paraffin blocks of the archives of the Pathology Department at the Facultat de Veterinària of the Universitat Autònoma de Barcelona. Serial sections (4 μm) 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 Organization classification.[30, 31]

Follow-up of most animals was not available in this retrospective study.

Antibodies

The antibodies used in this study were monoclonal murine antibodies against MMP-2 (clone 42-5D11, Ab-3), MMP-9 (clone 56-2A4, Ab-3) and MT1-MMP (clone 113-5B7, Ab-4),[a] a polyclonal goat antibody against MMP-2 (K-20)[b] and a monoclonal murine antibody against β-actin.[b]

Immunohistochemistry

For MMP-2 and MMP-9 immunostaining, an adapted protocol from Hirayama et al. was used.[17]

Paraffin was removed from the slides and the sections were rehydrated by use

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of a series of xylol and graded alcohol solutions. Activity of endogenous peroxidase was blocked by immersing slides in 0.3% H_2O_2 in methanol for 30 minutes. For antigen retrieval, a 20 minute heating step was performed in citrate buffer (pH 6). After non-specific sites were blocked by incubation with 20% normal goat serum in 0.02M Tris-HCl, 150 mM Na Cl, pH 7.4 (TBS) solution for 1 hour at room temperature, sections were incubated overnight at 4ºC with anti-MMP2 or anti-MMP9 antibodies,[a] which recognize both latent and active forms of the enzyme, diluted 1 in 50 in blocking solution. Slides were then incubated with biotinylated goat anti-mouse immunoglobulin[c] 1 in 100 in TBS solution at room temperature for 1 hour, which was followed by incubation with the avidin-biotin-peroxidase complex.[d] The reaction was developed by use of a commercial staining kit[e] and sections were counterstained with Mayer's hematoxyilin. Sections incubated with an irrelevant isotype-matched monoclonal antibody were used as negative control samples.

For MT1-MMP, we used an adaptation of the protocol described by Paparella et al.[18] After removal of paraffin, rehydration, inhibition of endogenous peroxidase activity and blocking the nonspecific sites with goat serum 20% in TBS for 1h at room temperature, the sections were incubated with the anti MT1-MMP antibody diluted 1 in 50 in blocking solution overnight at 4ºC. After washing, they were incubated with the secondary antibody at 1 in 200 dilution in blocking solution for 1 hour at room temperature, followed by the avidin-biotin-peroxidase complex. The reaction was developed by use of 0.04% 3-amino-9-ethylcarbazole (AEC)[f] with 0.015% H_2O_2 in 50mM acetate buffer pH 5.0 containing 0.05% dimethylformamide and the slides were counterstained with Mayer's haematoxylin. Sections incubated with an irrelevant isotype-matched monoclonal antibody were used as negative control samples

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Immunohistochemical evaluation and statistical analysis

The sections were examined independently by two observers (MJD, RMR). Sections were considered negative or positive according to the absence or presence of cytoplasmic staining. Slides were graded based on the intensity and pattern of immunoreaction using a four tier grading system of 0 (negative), 1 (weak staining), 2 (multifocal expression with strong intensity) and 3 (diffuse expression with strong positivity). Slides were examined with a microscope[g] and photographed with an integrated digital camera system.[h] Statistical analysis was performed by use of commercial software.[i] The χ^2 test was used to examine the correlation between MMP expression and tumor type. Significance was set at values of $P < 0.05$.

Cell Lines and Cell Culture

Canine melanoma cell lines CML-1, CML-10c2 and CML-6M[j] were originally derived from melanoma tumors of dogs.[32, 33] Canine primary fibroblasts were obtained from skin biopsy specimens.[k] Cells were grown in a humidified atmosphere at 37° C with 5% CO_2 in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.[l].

For gel zymography, subconfluent cell cultures were washed twice with PBS and once with DMEM without FCS for 4 hours and subsequently cultured in FCS free-medium for 24 hours. The conditioned media were collected, centrifuged to clarify debris and frozen at -20ºC until use. Volumes for each cell line were normalized to the protein content.

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177 **Co-culture experiments**

178 Cells were trypsinized and diluted to 1 x 10^5 cells/ml for fibroblasts and 2 x 10^5
179 cells/ml for melanoma cells (CML-1, CML-10c2 or CML-6M) in DMEM with 10%
180 FCS. For co-cultures, 1:1 mixtures of fibroblasts (1 x 10^5 cells/ml) and each melanoma
181 cell line (2 x 10^5 cells/ml) in DMEM with 10% FCS were prepared. Two ml of each
182 single or mixed cell suspension were seeded on 6 well dishes.[m] The cells were allowed
183 to attach overnight, washed with PBS and once with FCS free-DMEM, and further
184 incubated in FCS free-medium for 48h. Conditioned media was collected, centrifuged to
185 clarify debris and subjected to gelatin zymography.

186

187 **Collagen coating experiments**

188 Culture dishes where coated with a solution of 300 µg/ml of type I or type IV
189 collagen[n] to get a coating concentration of 50 µg/cm^2. Coated dishes were incubated for
190 3 h at 37ºC and then left to dry overnight in the sterile tissue culture hood, rinsed with
191 sterile distilled water to remove salt precipitates and rehydrated with PBS. This protocol
192 allows a film of fibrillar collagen I to form.[34] Cells were seeded on collagen-coated or -
193 uncoated dishes in DMEM with 10% FCS and allowed to attach overnight. Adherent
194 cells were rinsed and then incubated for 48 h with FCS-free DMEM.

195

196 **Gelatin zimography**

197 Gelatin zimography was performed as described by Llorens et al..[35] In brief,
198 samples were mixed with SDS sample buffer without reducing agent (50% glycerol,
199 10% SDS and 1% bromophenol blue in 1M Tris HCl pH 6.8) and separated on 7.5%
200 SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, gels were

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thoroughly rinsed with 2.5% Triton X-100 in 50 mM Tris HCl pH 7.5 followed by distilled water to completely eliminate SDS. The gels were incubated for 20 h at 37ºC with developing buffer (150 mM NaCl, 10 mM CaCl2, 50 mM Tris HCl pH 7.5). To visualize 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 captured[o] and analyzed [p]. Conditioned media from MXT-c1.1 cells (a metastatic mouse mammary carcinoma cell line) were included as control for secreted gelatinases.[36]

Immunoblotting

For MT1-MMP detection, cell extracts from subconfluent cultures were prepared by lysing cells in buffer containing protease inhibitors (50 mM Tris-HCl pH 7.5, 0.27 mM sucrose, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO4, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM benzamidine, 0.1% β-mercaptoethanol, 0.2 mM PMSF) for 30 min at 4ºC under agitation. Cell lysates were centrifuged at 14000g for 15 min at 4ºC, in order to remove insoluble debris, and the supernatants were collected. The amount of protein applied to the gel was normalized after Bradford quantification.[q] Samples (50 µg of protein) were mixed with 6X reducing sample buffer (1.5% SDS, 32.5 mM Tris-HCL, 3.75% β-mercaptoethanol, 0.1 g/ml saccharose and 0.0025% bromophenol blue), denatured at 95ºC for 10 minutes and resolved in a 12% polyacrilamide gel under reducing conditions (SDS-PAGE). After electroforesis, proteins were transferred onto Immobilon-P membranes[r] and non-specific binding sites on the blot were blocked with 5% (v/v) skimmed dried milk in 0.02 M Tris Buffer, 0.15 M NaCl pH 7.4 containing

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0.1% Tween-20 (TBS-T) for 1 h at room temperature. The membrane was then incubated with the antibody against MT1-MMP (1 in 20) in blocking solution overnight at 4ºC, washed with TBS-T and further incubated with horseradish peroxidase-congugated anti-mouse IgG[s] diluted 1 in 5000 in 2.5% skimmed dried milk in TBS-T for 1 h at room temperature. Antibody binding was detected by enhanced chemiluminiscence.[t,o,p] The blots were then stripped of antibodies by incubation in 2% SDS in 62.5 mM Tris-HCl pH 6.7 containing 100 mM β-mercaptoethanol for 30 min at 55ºC, washed with TBS-T and then reblocked and reprobed with anti-β-actin at a 1 in 20000 dilution, to verify uniform total protein loading of each well on gels.

Fluorescence immunocytochemistry

Cells were seeded and grown in coverslips, rinsed with PBS and fixed with 3% paraformaldehyde-2% sacarose for 20 min at room temperature. After rinsing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and non-specific binding sites were blocked for 1 hour with 1% BSA and 10% normal rabbit serum in PBS. Cells were then incubated overnight at 4ºC with the polyclonal anti-MMP2 antibody 1 in 10 dilution in PBS. Subsequently, cells were washed with PBS and incubated with anti-goat secondary antibody labelled with FTIC[u] diluted 1 in 400 in PBS. Nuclei were visualized by incubating the cover slips with 0.1 mg/ml Hoetsch 33342[v] in PBS for 3 min. Cultures were studied with a epifluorescence microscope[x] and photographed with an integrated digital camera system[y].

RNA isolation and RT-PCR

Total RNA was extracted from subconfluent cells by use of an isolation kit[z]. For RT-PCR, 2 µg of total RNA was reverse transcribed in a 40 µl reaction volume with the

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Expand Reverse Transcriptase,[α] as described in the protocol provided by the manufacturer. An aliquot (2.5 µl) of the reaction mix was subsequently used for PCR assay[β] with specific primers for canine MMP-2, MMP-9 and MT1-MMP. The PCR primer sets and product sizes are shown in Table 1. The assay used an initial denaturing step at 94ºC for 2 min, which was followed by 30 cycles of reaction, each cycle including 1 min at 94ºC, 1 min at 55ºC and 1 min at 72ºC, and a final extension step at 72ºC for 5 minutes. Amplification of a fragment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. PCR amplification products were resolved on 2% agarose gels, stained with ethidium bromide and developed and photographed under ultraviolet light.[χ]

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Results

Immunohistochemical detection of MMP-2, MMP-9 and MT1-MMP in melanocytic tumors of dogs.

MMP-2, MMP-9 and MT1-MMP immunoreactivity was assayed in 35 tissue samples corresponding to melanocytomas, and oral and cutaneous melanomas. Intensity of labeling was assessed and the results are shown in Table 2.

MMP-9 was widely expressed in tumor samples. A significant increase in the level of expression of MMP-9 in melanomas was found when compared to melanocytomas ($P < 0,05$). This difference was found when comparing the total number of benign and malignant tumors, as well as comparing melanocytomas with melanomas of oral or cutaneous origin separately. No significant difference was found between oral and cutaneous melanomas. MMP-9 showed a cytoplasmic and diffuse pattern of labeling in tumor cells. The distribution of MMP-9 inside the tumor was quite homogeneous, and other cell types besides tumor cells were also positive for MMP-9, including neutrophils, mast cells and some stromal fibroblasts (Figure 1).

The expression of MMP-2 was heterogeneous between tumors and also inside the tumors. The pattern of reactivity was often perinuclear in the neoplastic cells (Figure 1). Individual tumors showed variation in the number of positive tumor cells and in the labeling intensity, ranging from negative tumors to markedly labeled tumors in benign as well as malignant cases. In concordance with this appreciation, no significant differences were observed between melanocytomas and oral/cutaneous melanomas ($P = 0,220$). It is interesting to note that stromal cells inside the tumors were often strongly positive for MMP-2 (Figure 1).

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MT1-MMP labeling was weak or absent in 28 out of the 35 samples. Interestingly, peritumoral stromal cells were often labeled as well as tumor blood vessels (Figure 1). No significant difference was observed between benign and malignant tumors (P = 0,839).

MMPs of canine melanoma cell lines and canine dermal fibroblast cultures

To get further insight into the cell origin of these enzymes, RT-PCR were performed from RNA extracted from canine dermal fibroblasts and melanoma cell lines using specific primers for the canine sequences. As observed in Figure 2A, MMP-2 and MT1-MMP were expressed in fibroblasts as well as in all three melanoma cell lines. In contrast, only melanoma cells expressed MMP-9 in variable amounts. To validate the results of the RT-PCR analysis, MMP-2 was detected by immunocytochemistry in all melanoma cell lines, as well as in dermal fibroblasts (Figure 2B). Since immunolocalization does not distinguish the active and proactive MMP forms, *in vitro* studies were undertaken to analyze whether both 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 showed gelatinolytic bands of different molecular mass. The main MMP present in media was MMP-2 in its proactive form. Active and intermediate forms of MMP-2 were also visible in the zymography gel in variable amounts depending on the cell line. MMP-9 in its proactive and active forms were minor bands in melanoma cells and they were absent in dermal fibroblasts (Figure 2C). MT1-MMP was detected in all cell types by western blot using an antibody against the human protein. The enzyme was more abundant in melanoma cells than in fibroblasts. This technique allowed us to identify two bands, one

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of ~ 57 kDa corresponding to the active form, and another band of ~ 60 kDa corresponding to the proenzyme form (Figure 2D).

MMPs of tumor melanoma cells cultured in the presence of dermal fibroblasts and on collagen-coated wells

To study whether melanoma and dermal fibroblast cells may crosstalk leading to a modulation of the proteolytic activity, a co-culture of both cell types was established and kept for 48 hours in a serum-free medium. After this time, the gelatinolytic activity of the cultures was visualized through gelatin zymography. As observed in Figure 3, co-cultures of CML-1 and CML-10c2 and fibroblasts showed an increase in the intensity of the band corresponding to active MMP-2. No difference was observed when co-culturing CML-6M melanoma cells and fibroblasts, probably due to the already high expression of the active form of MMP-2.

Finally, it has been described that some ECM components, such as type I collagen, may modulate MMP activity in tumor cells.[34] Thus, canine melanoma cells were cultured on type I and type IV collagen-coated wells for 48h and the presence and activation state of MT1-MMP was analyzed by western blot. As shown in Figure 4, an important change in the ratio between the enzyme and proenzymes forms is produced when culturing the cells in the presence of type I collagen. This activation is especially noticeably in CML-1 cells, which are able to strongly attach to collagen (not shown). In contrast, culture on type IV collagen did not produce significant changes in MT1-MMP levels in any of the melanoma cell lines.

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Discussion

In the present work, we have studied the expression of gelatinases MMP-2, MMP-9, and MT1-MMP in canine melanoma and melanocytomas. Our results show that there is an increase in MMP-9 expression in melanomas compared to benign melanocytic tumors. MMP-9 is abundant in this kind of tumors, and has a homogeneous distribution, being present in tumor and stromal cells. Other cell types also produce MMP-9, specially tumor-infiltrating neutrophils and mast cells, suggesting that MMP-9 can be generated as a result of the reciprocal interaction between tumor and non-tumor cells.[29, 37] The production of MMP-9 by inflammatory cells has been previously reported.[25, 37]

In contrast, we did not find significant differences in MMP-2 and MT1-MMP expression when comparing melanomas and melanocytomas. Some contradictory results have been reported in human melanocytic tumors, which follow a well established progression pattern from benign nevi, dysplastic nevi, primary melanoma and metastatic melanoma. MMP-2 expression has been found to occur in the early stage of tumor progression, being more than 50% of common nevi positive for MMP-2 [38], whereas in another study MMP-2 expression was not detected in benign lesions, but the majority of primary and metastatic melanomas were positive.[27] Other studies proposed MMP-2 as an independent prognostic factor since overexpression was associated to an increased risk of dying from melanoma. [26, 39] In the present study, we have detected MMP-2 immunoreactivity in a high number of melanocytomas and melanomas, but unfortunately follow-up of the cases was not possible. In other canine tumor types, an increased expression of MMP-2 and MT1-MMP was found in mammary carcinomas when compared to normal tissue but the labeling pattern showed no significant

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association with either the histological subtype or tumor stage.[18] Kawai et al showed an increased MMP-2 activity in canine malignant mammary tumors,[19] although another study in several canine tumor types (carcinomas, sarcomas of different origins, mast cell tumors and others) did not find a correlation between gelatinase activity of tumor extracts as quantified by zymography, and the biologic behavior of the tumors, either benign or malignant.[14] The authors claimed that some benign and malignant tumors may have higher and lower gelatinolytic activity, respectively, and argued that the inflammatory component (present in a high percentage of benign tumors) may affect total gelatinase activity of the tumor.[14] Lack of correlation between MMP activity and protein immunoreactivity has been reported in human melanoma and it may explain contradictory data.[40] Thus, the role of MMP-2 in tumor progression is still controversial. Regarding MT1-MMP, it is interesting to note that peritumoral stromal cells as well as blood vessels were often labeled with MT1-MMP which reinforces the proposed role of stromal cells in invasion and angiogenesis.[3].

Besides their role as ECM-degrading enzymes, MMPs have important roles in the interaction with non-matrix proteins including cell adhesion molecules, such as members of the integrin family and CD44, the hyaluronan receptor.[6, 24] Since the expression of CD44 and hyaluronan was previously investigated in serial sections of the same set of canine melanocytic tumors,[41] we compared the level of expression of both type of molecules. The statistical analysis of the complete results showed a significant correlation between intensity of MMP-9 expression and hyaluronan (P<0,05). Both molecules 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.[6, 42, 43] Careful examination of the labeling intensity and distribution of these molecules also showed a relationship between MT1-MMP and CD44 expression (P =

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0,029) and similar intratumor distribution of both molecules (not shown). Our results agree with those reported previously where MT1-MMP and CD44 were colocalized at the adherent edge of migrating cells and may be functionally related.[6, 44]

To further study the expression, enzyme activity and regulation of MMPs, we analyzed MMP expression in melanoma cell lines and dermal fibroblasts. Both types of cells 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 RT-PCR and zymography. Interestingly, the CML-6M cell line, which proceeds from a metastases,[32] was the higher expresser of MMP-9. In mice, it has been described that MMP-9 is especially important in the early steps of lung metastasis formation by melanoma cells,[45] and it has been suggested that the in vivo selection process for metastatic subclones favors those expressing MMP-9.[46]

It has become increasingly evident that the stromal cells and the microenvironment play a key role in tumor progression.[37, 47] Our results show that canine melanoma cells and normal fibroblasts may crosstalk leading to a higher, synergic MMP-2 activity in co-cultured cells. This synergic effect is more important in CML-1 and CML-10c2 cells, whereas is hardly visible in CML-6M cells, possibly due to their high basal MMP-2 activity. Similar results have been described in oral epithelial cancer cells[48] and human melanoma cells co-cultured in the presence of dermal fibroblasts.[49] An explanation to why both the tumor cells and the adjacent stromal cells express MT1-MMP, MMP-2 and/or MMP-9 may be that both cellular components contribute to the different part of the metastatic cascade. The tumor cell MMPs may contribute to the invasive growth of the tumor while the stromal elements contribute to the remodeling process and the desmoplastic reaction that occurs in the tissue adjacent to the tumor.[1]

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Finally, cell culture on top of type I collagen films is also able to modulate MMP expression in canine melanoma cells. A change in the ratio between the proactive and the active form of MT1-MMP protein visualized by western blot is observed in CML-1 cells. This effect seems to be cell type-specific, since it more noticeable in CML-1 than in CML-10c2 or CML-6M cells. Regulation of MMP activity by collagen has been described in several tumor types, but the effects are different depending on the MMP (MMP-2, MT1-MMP), collagen type or collagen-coating preparation procedure.[34, 50, 51] These results suggest that, not only the cell component of the tumor stroma may have a role in melanoma tumor progression, but also some specific extracellular matrix components may modulate the matrix degradative abilities of melanoma cells.

Altogether, the results described in the present work suggest 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 (HA, CD44), that would contribute to tumor progression in canine melanoma. The elucidation of critical steps in the regulation of MMPs may allow the identification of new potential targets for the development of specific antitumor drugs.[52]

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Footnotes

[a] Calbiochem, San Diego, CA.

[b] Santa Cruz Biotechnology, Santa Cruz, CA.

[c] Biotinylated goat anti-mouse immunoglobulin, Dako, Glostrup, Denkmark.

[d] Immunopure ABC peroxidase staining kit, Pierce, Rockford, Ill.

[e] Vector NovaRED sustrate kit, Vector laboratories Inc, Burlingame, CA.

[f] Sigma, St. Louis, MO.

[g] Leica DM-6000 microscope, Leica Microsystems, Wetzlar, Germany.

[h] Leica DFC480 integrated digital camera system, Leica Microsystems, Wetzlar, Germany.

[i] PASW for Windows, version 17.0, SPSS Inc, Chicago, IL

[j] Courtesy of Dr. E.G. MacEwen, University of Wisconsin, WI.

[k] Courtesy of Santi Cerrato, UNIVET, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain.

[l] All from Gibco BRL/Life Technologies, Rockville, MD.

[m] Nunc, Roskilde, Denmark.

[n] Type I collagen from calf skin and type IV collagen from human placenta. Sigma. St. Louis, MO.

[o] LAS-3000 imaging system with integrated CCD camera. Fujifilm, Tokyo, Japan.

[p] Multi Gauge software. Fujifilm, Tokyo, Japan.

[q] Bio-Rad protein assay. Bio-Rad, Munich, Germany.

[r] Immobilon-P membranes. Millipore Corp., Bedford, MA.

[s] GE Healthcare, Buckinhamshire, UK.

[t] ECL system, GE Healthcare, Buckinhamshire, UK.

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449 [u] Southern Biotech, Birmingham.

450 [v] Sigma, St. Louis, MO.

451 [x] Nikon Eclipse E800 epifluorescence microscope. Nikon. Japan.

452 [y] ORCA-ER c4742-80 with the image acquisition Wasabi software. Hamamatsu

453 Photonics, Hamamatsu, Japan.

454 [z] RNeasy isolation kit, Qiagen, Hilden, Germany.

455 [α] Expand Reverse Transcriptase, Roche, Mannheim, Germany.

456 [β] Expand High Fidelity PCR polymerase, Roche, Mannheim, Germany.

457 [χ] Gel Doc 2000 gel documentation system and Quantity One software. Bio-Rad,

458 Hercules, CA.

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Legends to the figures

Figure 1: Photomicrographs of sections of melanocytic tumors of dogs in which MMP-9 (a, b, c and d), MMP-2 (e and f) and MT1-MMP (g and h) were analyzed immunohistochemically. Melanocytomas (a and b) showing moderate diffuse MMP-9 expression. Cutaneous (c) and oral (d) malignant melanoma showed, respectively, intense and diffuse cytoplasmic expression of MMP-9 in tumor cells arranged in nests (c and d) and strands (c). Notice the presence of positive inflammatory cells (arrow; c). Cutaneous (e) and oral (f) malignant melanoma with moderate cytoplasmic expression of MMP-2 in tumor cells. Notice the presence of strongly positive stromal cells mainly located in the septa between tumor cell nests (arrows; e). Intensity of the signal may be heterogeneous inside the lesion (f). Cutaneous malignant melanoma with moderate diffuse cytoplasmic expression of MT1-MMP in tumor cells. Notice the presence of strongly positive peritumoral stromal cells indicated (arrow; g). Oral malignant melanoma with weak cytoplasmic expression of MT1-MMP in tumor cells. Notice the presence of positive endothelial cells in intratumoral blood vessels (arrow; h). Avidin-biotin peroxidase staining with Mayer's hematoxylin counterstain). Bar = 100 μm.

Figure 2. Expression patterns of MMPs in canine dermal fibroblasts and canine melanoma cell lines analyzed by RT-PCR (A), immunocytochemistry (B), zymography (C) and western blot (D). Total RNA was extracted from subconfluent cultured cells and subjected to RT-PCR with primers for canine MMP-2, MMP-9 and MT1-MMP. As control, amplification of a GAPDH fragment was used. MMP-2 and MT1-MMP were expressed by melanoma cell lines and dermal fibroblasts, whereas MMP-9 was only detected in melanoma cell lines (A). Detection of MMP-2 by immunofluorescence in

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canine dermal fibroblasts and canine melanoma cell lines using a polyclonal anti-MMP2 antibody. Nuclei were counterstained with Hoetsch 33342 (B). Conditioned media from canine melanoma cell lines and dermal fibroblasts were subjected to zymography as described in Materials and Methods. The mouse mammary carcinoma MXT cell line was used as positive control. MMP-2 was present in all four cell lines, whereas MMP-9 was only detected in melanoma cell lines. (C) Detection of MT1-MMP in cell extracts by western blot. Two bands corresponding to the latent and active forms of MT1-MMP are visualized. β-actin was used as loading control (D).

Figure 3. MMP- 2 activity in co-cultures of canine melanoma cell lines and canine dermal fibroblasts. After 48 h, conditioned media was collected and subjected to gelatin gel zymography. F = canine dermal fibroblasts. Mel = indicated canine melanoma cell lines. Notice that the active form of MMP-2 increased under co-culture conditions for CML-1 and CML-10c2 melanoma cell lines.

Figure 4. MT1-MMP levels analyzed by western blot in canine melanoma cell lines cultured on collagen-coated wells. Cells were cultured on type I collagen (A) and type IV collagen (B) for 48 h. Notice the switch between the latent and active form of MT1-MMP occurring in cells cultured on type I collagen, specially in CML-1 cells. β-actin was used as loading control.

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642 **Table 1.** Primer sets used for RT-PCR assay.
643

Target gene	GenBank accession No.	Primer sequence (5'-3')	Product size (bp)
MMP2	XM_535300*	Forward:GAGACCGCCATGTCCACTAT Reverse: TTGTTCCGTGGTGTCACTGT	506
MMP9	NM_001003219	Forward: GCAGCTGGCAGAGGAATATC Reverse: TGCCCAGAGTCCATAACTCC	515
MT1-MMP	XM_843664* (transcript variant 1) XM_851854* (transcript variant 2)	Forward: CCTACTTCTTCCGGGGAAAC Reverse: TTCAGAGGCAAAGTCCTCGT **	521
GAPDH	AB038240	Forward: TGAAGGTCGGAGTCAACGG Reverse: CCAGGAAATGAGCTTGACAAA	929

644 *Predicted canine sequence.

645 **Primers amplify a fragment common to both variants.

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Table 2. Immunohistochemical expression of MMP-9, MMP-2 and MT1-MMP in melanocytic lesions of dogs.

Tumors		MMP-9				MMP-2				MT1-MMP			
		0	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Melanocytoma (n = 14)		0	3	9	2	4	3	4	3	3	8	2	1
Malignant melanoma	Oral (n=14)	1	3	2	8	1	3	2	8	6	7	1	0
	Cutaneous (n=7)	0	1	2	4	1	0	3	3	1	3	2	1

0: Negative; 1: Weak; 2: Moderate; 3: Intense (see Materials and Methods).

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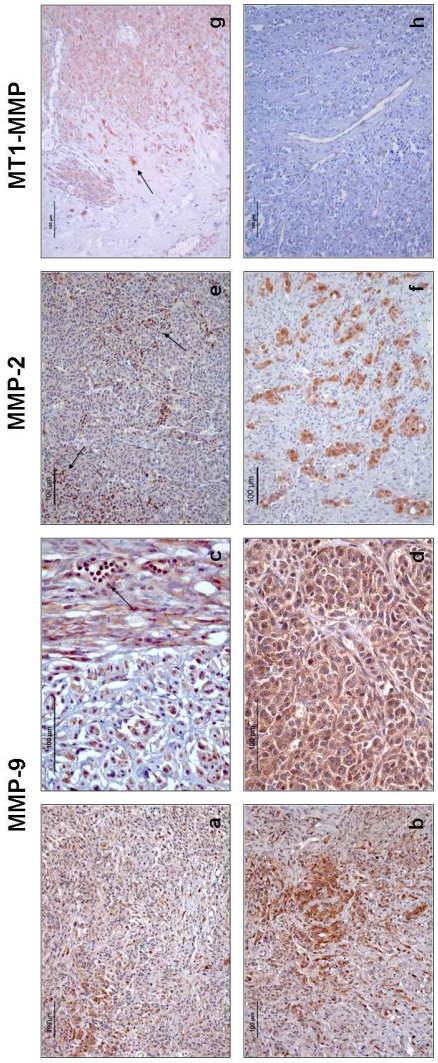


Figure 1

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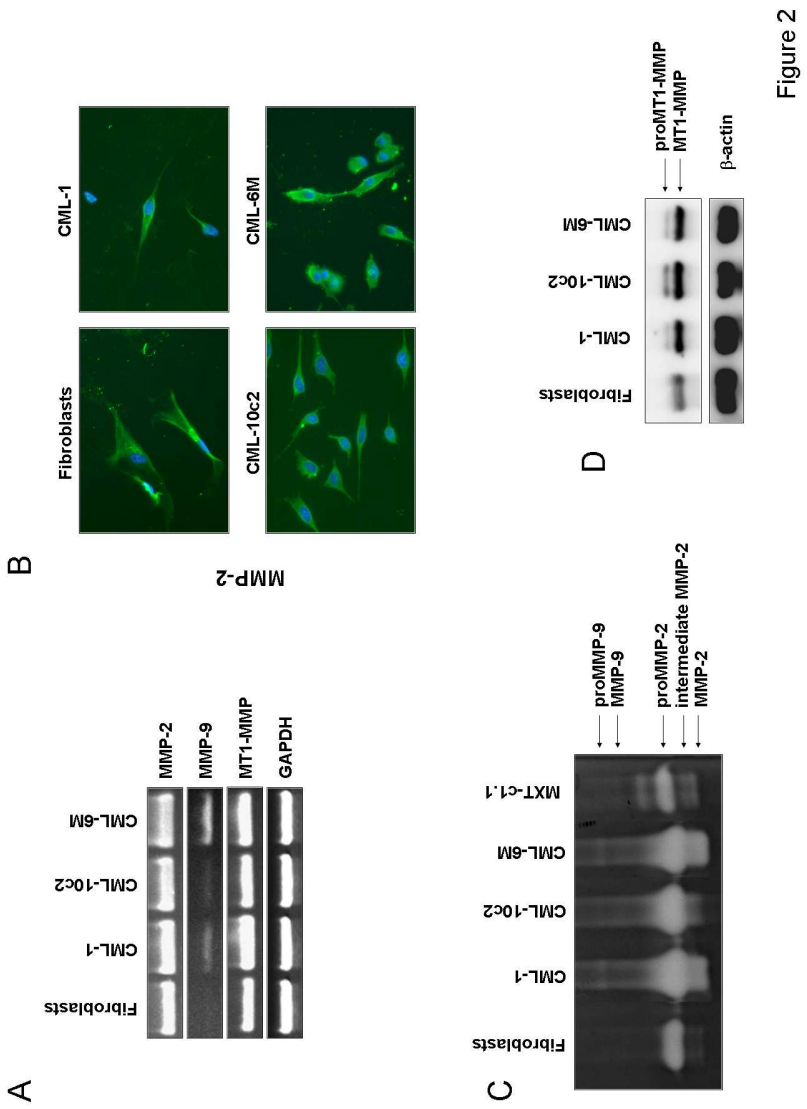


Figure 2

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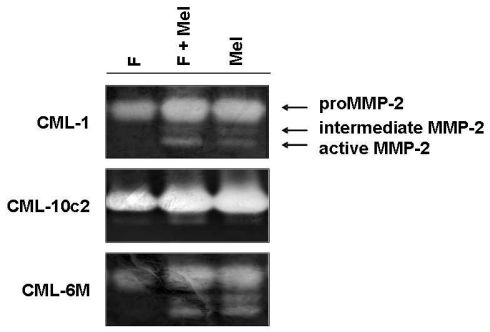


Figure 3

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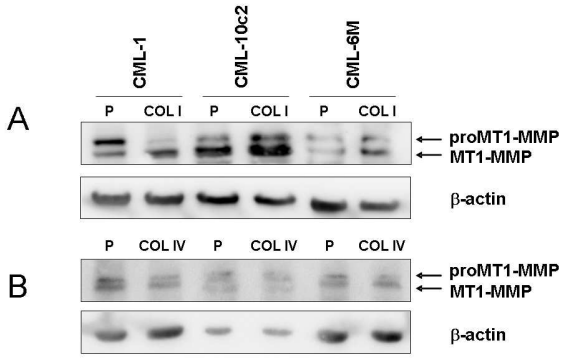


Figure 4

379x509mm (96 x 96 DPI)