Immunohistochemical analysis of matrix metalloproteinase-1, -3, and -13 in naturally occurring cartilaginous tumors of dogs

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Objective—To determine immunoreactivity of matrix metalloproteinase (MMP)-1, -3, and -13 in cartilaginous tumors of dogs, correlate expression of MMP with histologic grade of tumors and clinical outcome of dogs, and compare MMP immunoreactivity between chondrosarcomas and chondromas.

Sample Population—Formalin-fixed, paraffin-embedded tissues obtained from samples of naturally occurring chondrosarcomas (n = 31) and chondromas (8) of dogs that were submitted to our veterinary medical diagnostic laboratory.

Procedure—Histologic sections from each sample were stained with H&E and monoclonal antibody to MMP-1, -3, and -13 by use of an avidin-peroxidase immunohistochemical technique. For each section, histologic grade (I, II, or III) and immunohistochemical expression (0, 1, 2, or 3) were evaluated. Clinical outcome was obtained from medical records or interviews with referring veterinarians and scored as a good outcome, moderate outcome, or poor outcome. Correlations among variables and differences between chondrosarcomas and chondromas were analyzed.

Results—Samples from chondrosarcomas had significantly higher immunoreactivity of MMP-1 and -13, compared with immunoreactivity in samples from chondromas. In chondrosarcomas, a significant positive correlation (*r,* 0.386) was found between MMP-1 and -13 immunoreactivities, and a significant negative correlation (*r,* –0.390) was detected between MMP-3 and -13 immunoreactivities.

Conclusions and Clinical Relevance—A significant increase in expression of collagenases (MMP-1 and -13) in chondrosarcomas, compared with expression in chondromas, suggests that collagenases may play an important role in tumor progression, and possibly metastasis, in chondrosarcomas of dogs. (*Am J Vet Res* 2002;63:1285–1291)

Matrix metalloproteinases (MMP) are zinc-containing proteases that have a central role in turnover of **extracellular matrix (ECM)** during various conditions including skeletal development[1]; growth, invasion, and metastasis of tumors[1-3]; atherosclerosis[4]; wound healing[1,5,6]; and osteoarthritis.[7,8] Degradation of ECM in neoplastic tissues and surrounding tissues is a crucial event during the processes of tumor invasion and metastasis. More than 20 MMP have been identified and investigated with respect to disease mechanisms, diagnosis, prevention, and treatment.[9,10] Interstitial collagenase (ie, MMP-1), stromelysin-1 (ie, MMP-3), and collagenase-3 (ie, MMP-13) have received attention from arthrologists, because they are produced by chondrocytes and are capable of hydrolyzing cartilage ECM components, including collagen fibrils and proteoglycans.[8,9,11-13] In addition, these MMP play an important role during progression of several tumors in humans.[14-19] In humans, there is an increase in expression of MMP-1 and -13 and a decrease in expression of MMP-3 in chondrosarcomas, compared with expression in benign cartilaginous lesions.[18,19]

Chondrosarcomas are malignant cartilage-forming tumors. They are the second most-common bone tumor in dogs and comprise approximately 10% of all bone tumors in dogs.[20] In dogs, chondrosarcomas reportedly have a better prognosis than osteosarcomas.[21] In 1 study,[21] median survival time after surgical treatment of dogs with chondrosarcomas was 410 days, whereas in another study,[22] median survival time after surgical treatment for dogs with osteosarcomas was 126 days. However, chondrosarcomas are associated with highly variable clinical outcome, and it can be difficult to reliably predict a prognosis on the basis of histologic analysis alone.[23]

Results of immunohistochemical analysis and reverse-transcription polymerase chain reaction analysis for MMP in cartilage tumors of humans suggest that measurement of MMP expression in a tumor may be useful for predicting its biological behavior.[18,19,24-26] However, little data has been reported concerning the roles of MMP in any neoplasm of dogs.[27] To our knowledge, information regarding immunohistochemical analysis of MMP-1, -3, or -13 in naturally occurring tumors of dogs has not been reported. Expression of these MMP in chondrosarcomas obtained from dogs may contribute to the establishment of reliable diagnostic techniques and aid in predicting the clinical outcome for dogs with these tumors. Furthermore, establishment of MMP

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immunohistochemical analysis of canine tissues may be valuable for use in future studies.

In the study reported here, immunohistochemical expression of MMP-1, -3, and -13 was analyzed in samples of naturally occurring chondrosarcomas from dogs that were submitted to our veterinary medical diagnostic laboratory between 1993 and 2000. Expression of MMP was correlated with histologic grade of the tumors and clinical outcome for the dogs to determine whether any associations existed among these variables. In addition, immunohistochemical expression of these MMP was evaluated in samples of chondromas from dogs that were submitted to our diagnostic laboratory.

Materials and Methods

Sample population—Cartilaginous neoplasms of dogs were identified by searching the database of our veterinary medical diagnostic laboratory. We identified 31 chondrosarcomas and 8 chondromas that were submitted between 1993 and 2000.

Procedure—Data regarding signalment, location of tumor, mode of treatment, and prognosis were obtained from examination of medical records and interviews with referring veterinarians. Formalin-fixed, paraffin-embedded tissues were prepared for analysis by use of H&E and immunohistochemical stains.

Histologic examination—Sections of H&E-stained chondrosarcomas were subjectively classified into categories of grade I, II, or III. Two investigators (KK, JMK) performed the examinations and assigned histologic grades, using criteria published elsewhere.[23,28] Grades were assigned as follows: grade I (low grade), uniform cells with low cellularity, small densely staining nuclei, disorganized cartilaginous matrix, and lack of mitoses; grade II (intermediate grade), pleomorphic cells, nuclei with loose chromatin pattern, and a mild number of mitotic figures (< 2 mitotic figures/10 hpf); and grade III (high grade), severe pleomorphism with high cellularity, high invasiveness, and a marked number of mitotic figures (≥ 2 mitotic figures/10 hpf).

Immunohistochemical analysis—Immunohistochemical analysis was performed by use of mouse monoclonal antibodies against human MMP-1,[a] MMP-3,[b] and MMP-13.[c] Tissue sections were cut at a thickness of 5 µm, deparaffinized in xylene, and rehydrated in a graded series of ethanol solutions, which was followed by washing with buffer (50 m*M* Tris HCl and 0.15*M* NaCl). Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in methanol for 20 minutes, followed by rinses in buffer. Slides were then incubated in 0.1% trypsin solution with 0.1% $CaCl_2$ for 60 minutes at 37 C to unmask antigens. Nonspecific binding was blocked by incubation with 100% normal horse serum (30 minutes at 37 C). Slides were incubated overnight at 4 C with each MMP monoclonal antibody (concentration of 1.0 mg/ml for MMP-1, 10.0 mg/ml for MMP-3, and 2.0 µg/ml for MMP-13). The next day, slides were rinsed twice in washing buffer (50 m*M* Tris HCl, 0.15*M* NaCl, 0.05% Tween-20) and then incubated with biotinylated secondary antibody.[d] Bound primary antibody was detected by use of a streptavidin-horse radish peroxidase method with substrate-chromogen solution.[e] Sections were counterstained with Mayer hematoxylin solution.[f] Growth plates and articular cartilage obtained from a 6-week-old puppy were stained and served as a control sample of normal tissue. Mouse immunoglobulin isotypes identical to the primary antibody (IgG_{2b}[g] for MMP-1, IgG_1[h] for MMP-3 and -13) at similar protein concentrations were used as negative-control samples.

Intratumoral staining patterns were assessed. Sections were subjectively evaluated and scored by 2 investigators (KK, JMK) on the basis of the amount and intensity of staining for the respective MMP by use of the following scale: 0, staining not evident in the tissue section; 1, low-intensity staining of < 25% of the tissue section; 2, low-to-moderate intensity staining of > 25% of the tissue section; and 3, high-intensity staining of > 50% of the tissue section.

Clinical outcome—Information regarding clinical outcome for dogs and biological behavior of the chondrosarcomas and chondromas was obtained from examination of medical records and interviews with referring veterinarians. A scale for categorizing clinical outcome was developed as follows: good outcome, survival of > 2 years without recurrence; moderate outcome, survival of > 1 year with or without recurrence or metastasis; and poor outcome, survival of < 1 year. Effects of various therapeutic interventions among dogs were not taken into account for determination of prognosis.

Statistical analysis—All statistical analyses were performed by use of a computer software program.[i] A Mann-Whitney rank-sum test was used to determine significant differences between chondrosarcoma and chondroma samples for each variable. Case correlation coefficients between the variables were determined by use of a Spearman rank-order correlation. Significance was established at values of $P < 0.05$. In correlation analysis, a significance test was performed to determine whether given values of r indicated a significant correlation that was not obvious. Multiple regression analysis was applied to determine whether a combination of immunohistochemical variables would increase the correlation with histologic grade and whether such a combination would yield further information about clinical outcome of dogs with chondrosarcomas.

Results

Samples of 31 chondrosarcomas and 8 chondromas were analyzed. Mean ± SD age of dogs with chondrosarcomas was 7.9 ± 2.9 years and for dogs with chondromas was 7.3 ± 3.1 years. There was not a significant difference in age of dogs between the 2 groups. Most dogs with chondrosarcomas were medium- or large-breed types, and only 1 was a miniature breed (Yorkshire Terrier). The major breeds represented in the chondrosarcoma population included mixed-breed dogs (6/31; 18.9%), Golden Retrievers (4/31; 12.9%), and Rottweilers (4/31; 12.9%). Of all canine tissues submitted to our veterinary diagnostic laboratory for histopathologic diagnosis during the study period, 20.2% were from mixed-breed dogs, 5.1%, were from Golden Retrievers, and 2.3% were from Rottweilers. Seventeen of 31 (54.8%) chondrosarcomas developed from flat bones, with ribs (9 dogs) and scapula (4) being the most commonly involved sites. Eight of 31 (25.8%) chondrosarcomas originated in long bones, with tibia (5 dogs) being the most commonly involved site.

Among dogs with chondrosarcomas for which mode of treatment was confirmed, 15 of 30 dogs underwent surgical excision alone, 3 received surgical excision followed by adjunctive chemotherapy, and 2 received radiation therapy. Follow-up data were suc-

cessfully obtained for 23 of 31 (74.2%) dogs with chondrosarcomas and 5 of 8 (62.5%) dogs with chondromas. Of the 23 dogs with chondrosarcomas for which follow-up data was obtained, 3 (13.0%) survived > 2 years without recurrence or metastasis (ie, good outcome), 4 (17.4%) survived > 1 year with or without recurrence or metastasis (ie, moderate outcome), and 16 (69.6%) died or were euthanatized within 1 year for reasons related to the neoplasia (ie, poor outcome). Of the 5 dogs with chondroma for which follow-up data was obtained, all 5 survived > 2 years without recurrence or metastasis or were doing well at the time of this study.

Histologic evaluation—Samples of chondrosarcomas were graded on the basis of the aforementioned grading scale developed for this study. Chondrosarcomas were classified as grade I (n = 1, 3.1%), II (4; 12.9%), or III (26, 83.9%; **Fig 1**). There was not a significant correlation between histologic grade and clinical outcome.

Immunohistochemical analysis—Formalin-fixed, paraffin-embedded chondrosarcoma and chondroma tissues that had been obtained from dogs were successfully stained with MMP-1, -3, and -13 mouse monoclonal antibodies. In growth plate and articular cartilage obtained from the 6-week-old puppy, intense MMP-1 staining was evident in the cytoplasm of chondrocytes and interterritorial region of the ECM in zones of proliferation and hypertrophy. Expression of MMP-13 was detected in chondrocytes in the cytoplasm of zones of proliferation and hypertrophy with occasional nuclear staining, and MMP-3 staining was evident in the cytoplasm of proliferating and hypertrophic chondrocytes, but it was less intense than for MMP-1 or -13. Immunohistochemical staining of the negative-control sample did not reveal visible staining (data not shown). Staining patterns in chondrosarcoma tissues of dogs were similar to those described for chondrosarcomas of humans.[18,19,29] Positive expression was detected in cytoplasm of tumor cells (**Fig 2**).

Mean ± SD immunohistochemical staining scores for MMP-1, -3, and -13 in chondrosarcoma samples were 2.3 ± 0.9, 1.2 ± 0.9, and 2.1 ± 0.9, respectively. Scores for MMP-1, -3, and -13 in chondroma samples were 1.4 ± 0.5, 2.0 ± 1.0, and 1.3 ± 0.7, respectively. Immunoreactivities of MMP-1 and -13 in chondrosarcoma samples were significantly (P = 0.009 and 0.032, respectively) higher than immunoreactivities of those MMP in chondroma samples. There was not a significant difference in MMP-3 immunoreactivity between the 2 tumor types. For chondrosarcoma samples, there was a significant positive correlation (r, 0.386; P = 0.032) between immunoreactivities of MMP-1 and -13 and a significant negative correlation (r, 0.390; P = 0.030) between immunoreactivities of MMP-3 and -13. In other words, when an increase in MMP-13 staining was evident in chondrosarcoma tissues, MMP-1 expression was likely to be increased and MMP-3 expression was likely to be decreased.

None of the combinations of MMP immunoreactivity variables could be used to predict histologic grade or clinical outcome in this study. We did not detect a significant correlation between MMP immunostaining and clinical outcome or histologic grade. In chondroma samples, low-to-moderate intensity staining for MMP-1, -3, or -13 was evident in the cytoplasm of chondrocytes with occasional nuclear staining. The intensity and location of staining for MMP-1, -3, and -13 were essentially identical in all chondromas examined in this study.

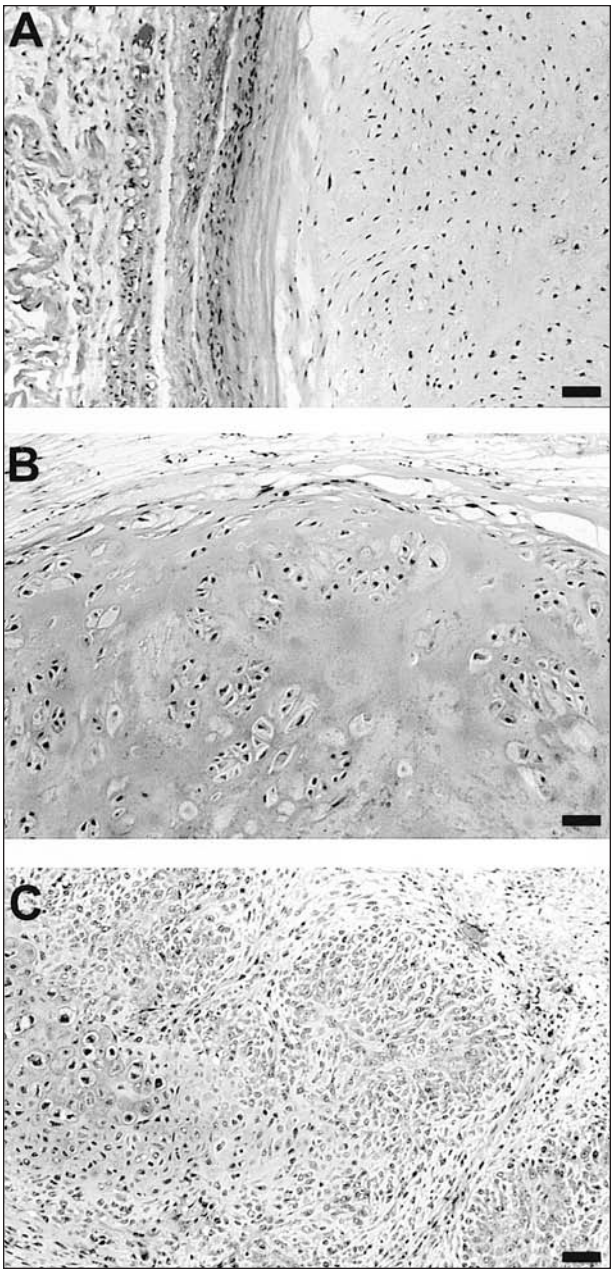

Figure 1—Photomicrographs of samples of chondrosarcomas of dogs that were classified as histologic grade I (A), grade II (B), and grade III (C). Panel A is tissue from a rib of an 8-year-old Labrador Retriever. Notice the uniform cells with small, densely staining nuclei and lack of mitosis. Panel B is tissue from a rib of a 7-year-old Australian Blue Heeler. Notice the moderate amounts of anisocytosis and anisokaryosis and minimum amount of mitosis. Panel C is tissue from the nasal cavity of a 10-year-old Labrador Retriever. Notice the severe pleomorphism with high cellularity and marked number of mitotic figures. H&E stain; bar = 200 μm.

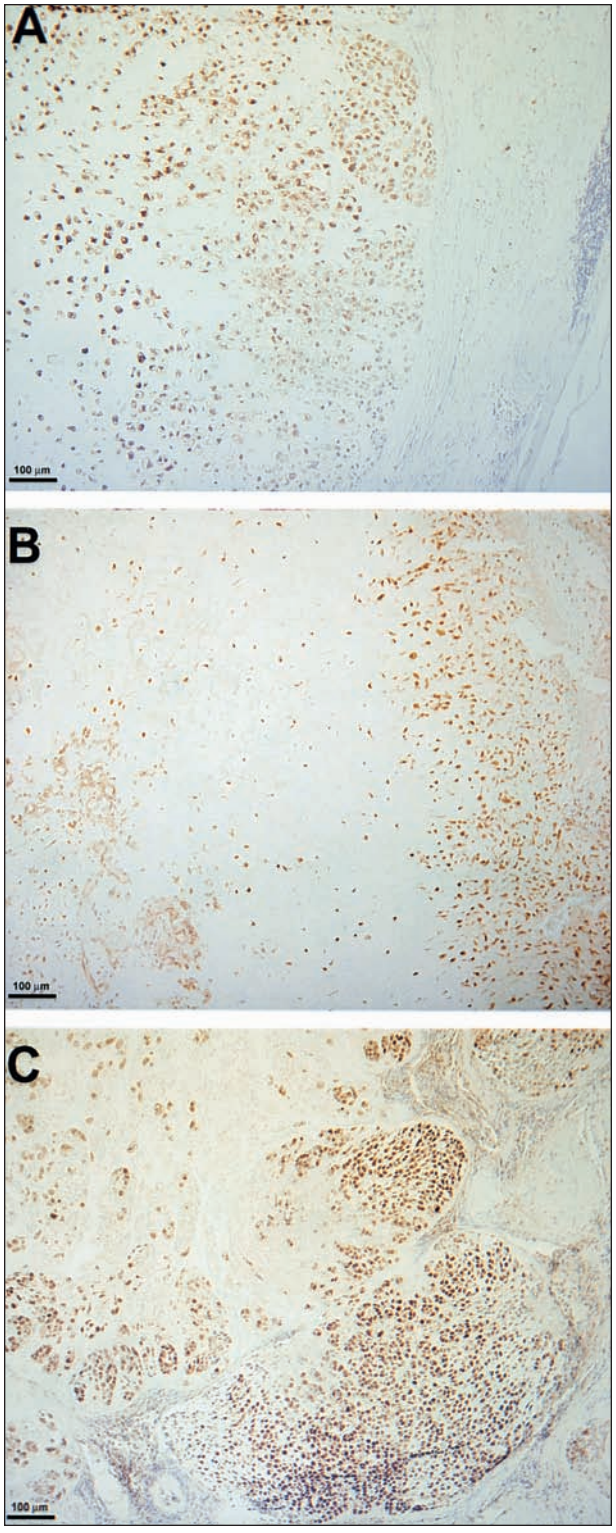

Figure 2—Photomicrographs of immunohistochemical analysis for matrix metalloproteinase (MMP)-1 (A), MMP-3 (B), and MMP-13 (C) in samples of chondrosarcomas of dogs. Panel A is tissue from a rib of a 14-year-old mixed-breed dog. Notice the MMP-1 staining of an area of cytoplasm and pericellular matrix. Panel B is tissue from the tibia of a Golden Retriever. Notice the MMP-3 staining associated with tumor chondrocytes. Panel C is tissue from the proximal portion of the left radius of an 8-year-old German Shepherd Dog. Notice the MMP-13 staining of an area of cytoplasm and an occasional nucleus. Avidin-biotin-peroxidase stain; bar = 100 µm.

Discussion

Signalment of dogs affected by chondrosarcoma in the study reported here is similar to that of other studies.[20,23,28] Mean age of dogs with chondrosarcoma (7.9 years) in our study is within the range of 6 to 8.7 years reported in other studies.[20,23,28] Most dogs with chondrosarcoma in our study were medium- or large-breed dogs without a predisposition on the basis of sex, which also is similar to other reports.[20,23,28] The major breeds of dogs that developed chondrosarcomas in our study were mixed-breed dogs, Golden Retrievers, and Rottweilers, whereas mixed-breed dogs, Boxers, Golden Retrievers, Irish Setters, and Doberman Pinschers were overrepresented in other studies.[20,23,28] For mixed-breed dogs, the frequency of chondrosarcomas (18.9%) was similar to the frequency for all submissions (20.2%) during the study period. However, for tissues submitted to our veterinary medical diagnostic laboratory for histopathologic diagnosis, a higher percentage of chondrosarcomas came from Golden Retrievers (12.9%) and Rottweilers (12.9%), compared with all tissues submitted from these 2 breeds (5.1 and 2.3%, respectively). This may represent a predilection for development of chondrosarcomas in these 2 breeds. However, a definitive predilection cannot be determined on the basis of these data.

Flat bones (54.8%) were the most frequent site of chondrosarcoma in the study reported here, which is similar to the frequency for flat bones (61 to 91%) reported in other studies.[20,23,28] However, there were considerable differences between our study and those other studies[20,23,28] with respect to tumor location. In those other studies, chondrosarcomas affected the nasal turbinates in 26 to 36% of dogs. However, only 2 (6.5%) dogs with chondrosarcomas had involvement of the nasal turbinates in our study. The appendicular skeleton was the primary site of involvement (14/31 [45.2%] dogs) in the study reported here, compared with < 28% in other studies.[20,23,28] Long bones were involved in 8 of 31 (25.8%) dogs in our study, compared with < 20% reported in other studies.[20,23,28] In addition, data reported in those other studies indicating that involvement of the appendicular skeleton was associated with a more favorable prognosis and surgical intervention resulted in prolonged survival times could not be substantiated in our study on the basis of the data obtained.

Histologic grading of tumors is often useful for providing a prognosis and determining therapeutic options. However, it is difficult to predict a reliable prognosis for dogs with chondrosarcomas on the basis of histologic appearance alone. Sylvestre et al[23] reported that histologic grade did not correlate well with prognosis, and this finding was consistent with the results of our study. Low-grade chondrosarcomas are often difficult to differentiate from benign chondromas. Although the validity of histologic analysis in cartilaginous tumors of dogs was supported by the finding of a good prognosis for dogs with chondromas and relatively poor prognosis for dogs with chondrosarcomas, development of other diagnostic methods that can provide reliable information for prediciting clinical outcome of dogs or biological behavior of chondrosarcomas is critical.

The study reported here documented that MMP-1, -3, and -13 are immunohistochemically detectable in formalin-fixed, paraffin-embedded canine cartilaginous tissues. These data help to validate cross-reactivity of anti-human MMP antibodies in canine tissues. Matrix metaloproteinase-1 is capable of degrading fibrillar collagens (types I, II, and III), type-VII collagen, type-X collagen, and proteoglycans,[13,30] and MMP-1 expression has been identified in many malignant neoplastic conditions of humans including breast cancer, esophageal cancer, oral squamous cell carcinomas, melanomas, and chondrosarcomas.[18,24,25,31-35] In cartilaginous tumors of humans, MMP-1 immunoreactivity is higher in chondrosarcomas than chondromas.[18] Data from the study reported here is consistent with data for cartilaginous neoplasms of humans as indicated by the increase in MMP-1 immunoreactivity in chondrosarcomas of dogs, compared with immunoreactivity for benign chondromas of dogs.

Matrix metalloproteinase-3 can cleave several cartilage matrix components, including proteoglycans, link proteins, and collagen types II, IX, X, and XI.[11,12] Increased activity of MMP-3 has been reported in neoplastic conditions of humans such as breast cancer, oral squamous cell carcinomas, colorectal cancer, and pulmonary carcinomas.[31,33,34,36,37] In a model that involved the use of mice, MMP-3 was expressed in stromal cells surrounding tumors, and conversion of tumors to highly malignant forms was associated with the expression of MMP-3 mRNA in tumor cells.[29] Similar amounts of MMP-3 immunoreactivity in chondrosarcomas and chondromas of dogs found in the study reported here indicated that MMP-3 may not be involved in invasion and metastasis. Interestingly, Kawashima et al[18] reported that chondrosarcomas express MMP-3 at lower amounts, compared with benign chondroid lesions in humans. Although there was not a significant difference, the lower MMP-3 expression in chondrosarcomas of dogs in our study may suggest that there are similarities in the disease process between humans and dogs.

Matrix metalloproteinase-13 efficiently degrades proteoglycans and the native helix of fibrillar collagens, cleaving ftype-II collagen at least 10 times faster than cleavage of MMP-1.[24,38,39] Because of its strong ability to hydrolyze type-II collagen and its origin in chondrocytes, MMP-13 has been targeted for diagnostic and therapeutic modalities in various arthropathies, including osteoarthritis.[9] Several studies[16-18,36,40,41] have revealed that MMP-13 expression is associated with invasive and metastatic tumors, including carcinomas of the breast, head, and neck, malignant melanomas, and chondrosarcomas. Those studies highlighted a role for MMP-13 in malignancy of tumors. In our study, increased immunoreactivity of MMP-13 in chondrosarcomas of dogs, compared with benign chondromas of dogs, is consistent with a study[18] conducted in humans and indicates that MMP-13 may play an important role in progression of chondrosarcomas in dogs.

In chondrosarcomas of the study reported here, MMP immunoreactivity was not significantly correlated with histologic grade. A correlation between histologic appearance of an intestinal tumor in humans and collagenolytic activity in that tumor has been reported.[42] It is possible that the histologic grading criteria used in our study did not reflect the aggressiveness of matrix-degrading activity by proteinases, resulting in lack of a significant correlation between MMP expression and histologic grade. It may be possible that the grading criteria of preceding studies were developed on the basis of too few animals to be truly meaningful. It also may be possible that MMP immunoreactivity is variable among variants of chondrosarcomas such as mesenchymal chondrosarcomas, dedifferentiated chondrosarcomas, clear-cell chondrosarcomas, or extraosseous chondrosarcomas, and lack of consideration of this classification in our study may have contributed to failure to correlate MMP expression and histologic grade. Another possible reason for lack of correlation between MMP immunostaining and histologic grade is the effect of necrosis. Necrosis, a typical component of highly progressive tumors, may cause less MMP to be produced in higher-grade tumor tissues.

In addition, limitations associated with the retrospective nature of the study reported here should be considered. The point during disease progression at which a definitive diagnosis was made for each case is not known. The timing of examinations and procurement of tissue for histologic assessment could have had major ramifications on histologic appearance of the tumors. This may be the reason that the majority (25/31; 80.6%) of chondrosarcomas in our study were grade-III tumors. This distribution of histologic grades, coupled with the relatively low numbers of grade-I and -II tumors, may have contributed to the lack of correlation.

The disease mechanism of tumor progression is a complex process associated with genetic alteration of cells and subsequent phenotypic changes. Genetic changes in cell-surface components influence detachment of cells from the primary tumor, migration, and invasion of new tissues.[43] During invasion and metastasis, degradation of ECM in neoplastic tissues and surrounding tissues is a crucial event. For physiologic conditions, MMP and **tissue inhibitors of metalloproteinases (TIMP)** bind in 1:1 (molar ratio) stoichiometry and are stringently controlled.[44] When the amount of MMP exceeds the locally available TIMP, there is excessive ECM degradation.[45] Therefore, the ratio between MMP and TIMP in the tumor site must more accurately reflect biological behavior of the tumor than MMP activity alone. This could be a reason for lack of any correlation between clinical outcome and MMP immunoreactivity in the study reported here, because TIMP activity was not assessed in our study. In fact, considerable positive correlations between an increase in MMP-1:TIMP-1 and tumor aggressiveness and an increase in MMP-1:TIMP-1 and a poor prognosis have been reported for chondrosarcomas of humans in studies[24,26] in which this ratio has been evaluated.

Participation of multiple proteinases other than these 3 types of MMPs in degradation of ECM during chondrosarcoma progression could be another reason for the lack of significant correlations between prognosis and MMP immunoreactivities. The importance of

other proteolytic enzymes, including cathepsin B and urokinase plasminogen activator in progression of chondrosarcomas in humans, has been reported.[46] In addition, the effects of therapeutic interventions must be considered to comprehensively evaluate these results. Various therapeutic interventions including surgical excision, chemotherapy, radiation therapy, and no treatment, as well as euthanasia, may have contributed to the lack of prognostic value of MMP immunostaining in our study. However, differences in treatment did not alter the finding that there was a lack of correlation among MMP immunoreactivity, histologic grade, and clinical outcome in the study reported here when categories of treatment were analyzed separately or as a group. A similar prospectively designed study could lead to definitive conclusions regarding whether MMP-1, -3, and -13 immunohistochemical analyses have prognostic values for dogs with chondrosarcomas. Although the study reported here did not provide immediate clinically applicable information, significant increases in the amounts of MMP-1 and -13 in chondrosarcomas, compared with amounts in chondromas, suggested involvement of these proteinases in ECM degradation during progression of malignant cartilaginous tumors of dogs. In addition, similarity of MMP activities in chondrosarcomas of dogs and humans suggests that chondrosarcomas share at least some similarities in biological behavior and that naturally occurring chondrosarcomas of dogs may be an appropriate model for use in development of diagnostic and therapeutic strategies for humans. Further elucidation of the role of MMP in chondrosarcomas may contribute to optimizing diagnostic strategies and therapeutic interventions.

[a]MMP-1 (Ab-1), Oncogene Research Products, Cambridge, Mass.
[b]MMP-3 (Ab-5), Oncogene Research Products, Cambridge, Mass.
[c]MMP-13 (Ab-1), Oncogene Research Products, Cambridge, Mass.
[d]LSAB2, Dako Corp, Carpinteria, Calif.
[e]Liquid DAB, Dako Corp, Carpinteria, Calif.
[f]Harris Hematoxylyn Solution Modified, Sigma Chemical Co, St Louis, Mo.
[g]Mouse IgG2b Negative Control, Dako Corp, Carpinteria, Calif.
[h]Mouse IgG1 Negative Control, Dako Corp, Carpinteria, Calif.
[i]Sigma Stat, Jandel Scientific, San Rafael, Calif.

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