Identification of matrix metalloproteinases in canine neoplastic tissue

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**Objective**—To identify matrix metalloproteinases (MMP) 2 and 9 in canine tumor tissue and to compare the amount of activity to that in unaffected stromal tissue.

**Animals**—30 dogs with spontaneously developing, high-grade osteosarcoma.

**Procedure**—Tumor and nearby stromal tissue (muscle) were obtained at the time of surgery. Specimens were homogenized, and supernatants were assayed, using gelatin zymography. Human derived standards were run concurrently. Densitometry was done to obtain a semiquantitative arbitrary unit value for each specimen. The amount of activity in tumor tissue was compared with the amount in stromal tissue.

**Results**—Gelatinolytic bands were observed from the analysis of all tumor tissues and in most stromal tissues. These bands migrated in the same molecular weight area as the human MMP 2 and 9 standards. Gelatinolytic activity could be quenched by the addition of 50 mM EDTA and 1 µg of synthetic tissue inhibitor of metalloproteinase (TIMP) 2 per 100 ml. There was significantly more gelatinolytic activity in tumor tissue than in stromal tissue.

**Conclusions and Clinical Relevance**—MMP 2 and 9 are detectable in canine neoplastic tissue. Matrix metalloproteinases activity in tumor tissue is higher than in unaffected stromal tissue, indicating that canine MMP may be involved in the pathogenesis of tumor growth and metastasis. (Am J Vet Res 2000;61:111–114)

**Materials and Methods**

Tumor and nearby stromal tissue was obtained at the time of surgery from 30 dogs with spontaneously developing, high-grade osteosarcoma. A piece of tumor approximately...
2 cm³ was taken from a grossly homogeneous area and divided, with one portion fixed in zinc-buffered 10% formalin and the second flash frozen in liquid nitrogen and stored at −70 C until further processing. Samples were homogenized, using a mechanical homogenizer with a 3 ml generator in 4 ml of cold 50 mM Tris-buffered saline (0.9% NaCl) solution, pH 8.0, with 0.25% (vol/vol) detergent.² Samples were then centrifuged for 10 minutes at 1500 × g at 4 C, and the supernatants harvested and stored at −70 C. Grossly unaffected nearby stromal tissue to be used as a control tissue was also harvested and processed in the same manner. Protein content of the homogenized specimens was measured using a protein-dye binding, detergent compatible assay.² With the homogenate specimen and loaded into the sample wells of a precast gel system, as described by Huessen and Dowdle.² Briefly, 10 μg of total protein was taken from each homogenate specimen and loaded into the sample wells with a 2 × sample buffer containing 0.5 M Tris-HCl (pH 7.0), 20% glycerol, 4% sodium dodecyl sulfate (wt/vol), and 0.005% bromophenol blue. Samples were electrophoresed on a 10% Tris-glycine acrylamide gel, with 0.1% gelatin incorporated as a substrate under nonreducing conditions at 125 V for approximately 90 minutes at 25 C. Gels were then washed in renaturing buffer (2.5% vol/vol in water) for 30 minutes followed by overnight incubation at 37 C while in developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl₂, 0.02% Brij 35 [wt/vol], pH 7.6). Bands of gelatinolytic activity were observed after staining the gels with 0.5% (wt/vol) Coomassie blue R250 in 40% ethanol/10% glacial acetic acid solution for 3 to 6 hours. Gels were then destained in deionized water. Human MMP 2 and 9 standards were run on each gel as positive controls. Inhibition of gelatinolytic activity was achieved by adding 50 mM EDTA to the developing buffer or TIMP 2 at a concentration of 1 μg/100 ml to the developing buffer.

Densitometry—All of the gels were analyzed wet by use of a densitometer and associated software. To obtain a semi-quantitative value for each specimen, the optical assessment value of each unknown band was compared with the optical assessment value of the human MMP standard band (5 ng loaded per gel). A ratio of unknown to standard was then calculated. By doing this, an arbitrary unit (AU) value was assigned to each specimen. When two bands indicative of proenzyme and active enzyme forms were found, the total amount of MMP was determined.

Statistical analysis—A 2-tailed, paired Student t-test was done to compare MMP activity in tumor verses stromal tissue. Significance was set at P < 0.05.

Results

Osteosarcoma tumor tissues from 30 dogs were analyzed using gelatin zymography. Unaffected stromal tissue from the same dog was also assayed in each instance. Gelatinolytic bands were observed from the analysis of all tumor tissues and in most stromal tissues. These bands migrated in the same molecular weight area as the human MMP 2 and MMP 9 standards (Fig 1). Subjectively, the amount of gelatinolytic activity was variable among specimens from different dogs, and the amount of activity in a tumor tissue was higher than in the corresponding stromal tissue from the same dog. When 50 mM EDTA was added to the developing buffer, gelatinolytic activity was quenched, indicating that the observed bands were the result of a zinc-dependant metal MMP (data not shown).

To further characterize the bands, gelatinolytic activity was partially quenched with TIMP 2 added to the developing buffer at 1 μg/100 ml. As expected, the TIMP 2 treated gel had less gelatinolytic activity in the MMP 2 bands than the untreated gel (Fig 2). Two bands were visible in the MMP 2 standard, representing proenzyme and active enzyme forms. These bands were also observed in canine tissues. The MMP 9 standard had 1 large band, representing proenzyme and active enzyme forms. Several other bands were observed in some tissues and probably represent protein complexes or cleavage products, which would alter molecular weight and migration.

Figure 1—Results of gelatin zymography on canine stromal and canine tumor tissues from 3 dogs (No. 28, 26, and 27) with osteosarcoma. Notice that canine tumor and corresponding stromal tissues have matrix metalloproteinase (MMP) 2 and 9 activities. Human standards appear on the far right. Bands from canine tissues migrate to the same molecular weight area as the human MMP 2 and MMP 9 standards. ProMMP = Proenzyme. Active MMP = Active enzyme.
During electrophoresis, these bands were not evaluated further.

Densitometry was done to obtain a semiquantitative value or AU for MMP 2 and MMP 9 so that the amount of gelatinolytic activity could be compared among dogs. When 2 bands were observed in the area of MMP 2, total MMP 2 amount was considered. For MMP 2, (Fig 3) mean (± SD) AU value for tumor tissues was significantly greater than for stromal tissues (0.674 ± 0.283 and 0.087 ± 0.074 AU, respectively; P < 0.001). For MMP 9, (Fig 4) mean AU value for tumor tissues was also significantly greater than for stromal tissues (0.228 ± 0.204 and 0.047 ± 0.056 AU, respectively; P < 0.001).

Discussion
Results of our study indicate that canine tumor and stromal tissues have MMP 2 and 9 activity. There are, however, limitations to our study. The first is the lack of canine derived standards. At this time there are no canine standards available. Sequence homology between other species and human MMP are high. Matrix metalloproteinase 2 has 96 to 98% sequence homology among rats, mice, and humans, whereas MMP 9 has 75 to 85% sequence homology among rats, mice, rabbits, humans, and cattle. It is not unreasonable to assume that canine MMP would also have high sequence homology with human MMP and would therefore migrate on a zymographic gel in a similar molecular weight region.
sequence canine MMP 2 has revealed high sequence homology with human, rat, and mouse sequences. 6 Coughlan et al. 7–9 have shown that the N-terminal amino acid sequence of MMP 2 has 87% homology with the human sequence. Because gelatinolytic bands were observed in the canine tissues and because they migrated at the same molecular weight level as the human MMP 2 and 9 standard, it is likely that these bands represent canine MMP 2 and 9. Standards used in our study were derived from cell culture supernatants and were not purified. Because of this, 2 distinct bands representing proenzyme and active MMP 2, 9 were not visible on the gels. The large band seen likely represents both forms of this enzyme. Quenching of the gelatinolytic activity seen by EDTA and by TIMP 2 also supports this assumption.

Another limitation of our study is that by using gelatin zymography and densitometry as assay methods, only semiquantitative values could be easily obtained. Results of our study indicate that there is a great deal of variability in gelatinolytic activity among the canine tissues assayed, which may reflect in vivo differences in enzymatic activities. Tumor tissues used in our study were not purified to remove enzyme-TIMP or artificially activated complexes, and either could alter the results of zymography. As with human tumor types, it is possible that greater MMP activity may translate into a more invasive and metastatic tumor. If this is true, then the amounts of MMP activity may serve as prognostic factors or predictors of outcome.

When comparing tumor and stromal tissues for MMP activity, our results indicate that there is greater activity within the tumor itself. This does not necessarily mean that the tumor cells are producing the enzymes. Most tumors are made up of a heterogeneous population of cells that contains some unaffected stromal cells, as well as inflammatory cells. Tumor tissues assayed in our study were homogenized as whole pieces of tissue and no attempts were made to purify the specimens. Stromal tissues were collected from muscle tissue several centimeters away from the primary tumor and were grossly and histologically normal in appearance. Results of our study do allow us to conclude that unaffected distant tissues have less enzymatic activity than that in the immediate tumor environment.

Another issue not addressed in our study is the use of frozen versus fresh tissue. It is possible that enzyme degradation could develop over time or with repeated freeze and thaw cycles. Samples used in our study were subjected to minimal freeze and thaw cycles (2) and were assayed within 4 months of obtaining them.

References