Interpreting Gelatinase Activity in Tumor Tissue and Serum as a Prognostic Marker of Naturally Developing Canine Tumors

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ABSTRACT. To evaluate the clinical usefulness of tissue and serum gelatinase activity as a prognostic marker of canine tumors, tissue samples from 60 tumors and corresponding serum samples from the same animals were collected at the time of biopsy and surgery. On the basis of histopathology and clinical aggressiveness of metastasis and recurrence (MR), the cases were divided into 6 categories: non-inflammatory (Inf(–)) and inflammatory (Inf(+) benign, and the Inf(–) MR(–), Inf(–) MR(+), Inf(+) MR(–), and Inf(+) MR(+) malignant. Gelatinase activity was determined semi-quantitatively using gelatin zymogram with a gelatinase standard from cultured canine peripheral blood mononuclear cells stimulated with lipopolysaccharide. No significant difference in gelatinase activities in tissue extracts was evident between the benign and malignant tumors. Inf(+) benign tumors, as well as Inf(–) MR(+), Inf(+) MR(–) and Inf(+) MR(+) malignant tumors, showed significantly higher tissue gelatinase activity than Inf(–) benign. The tissue activity in Inf(–) MR(–) malignant was significantly lower than in Inf(+) MR(–) and Inf(+) MR(+) malignant. The serum activity was significantly higher in the malignant cases than in the control and the benign. Inf(–) MR(+), Inf(+) MR(–) and Inf(+) MR(+) malignant tumors induced significantly higher gelatinase activity in serum than Inf(–) benign tumors. Gelatinase activity in serum was positively correlated with that in tumor extracts. Increased gelatinase in tumor tissue and serum may be correlated with inflammation as well as tumor aggressiveness, and thus should be used in combination with histopathology for predicting tumor metastasis or recurrence.

KEY WORDS: gelatinase, MMP, prognosis, tumor, zymography.


Matrix metalloproteinases (MMPs) are a family of enzymes with unique structural characteristics: a pro-peptide domain that is cleaved at the time of activation in the extracellular matrix (ECM), and an active domain containing zinc and calcium-binding sites. MMPs, which target collagens, gelatin, elastin, fibronectin, and proteoglycans in the ECM, are regulated constitutively or inductively at different stages of tissue remodeling such as angiogenesis, ossification and wound healing, as well as in progressive diseases including rheumatoid arthritis [2], osteoarthritis [18], and neoplasia. At the boundary between malignant tumors and surrounding normal tissues, MMPs destroy the ECM and the basement membrane, while they sometimes play a reconstructive role, or induce growth factors that play crucial roles in angiogenesis and fibrogenesis at different stages of tumor enlargement [1, 22].

MMP-2 and 9, which are also referred to as gelatinase A and B, respectively, have strong catabolic activity to type IV and V collagens as well as gelatin; consequently they may play a role in degrading the basement membrane at the onset of tumor invasion into the surrounding normal tissues. Gelatinolytic metalloproteinase in canine tumors was first detected by gelatin zymography as a 92-kDa gelatinase secreted from cultured mastocytoma cells [3]. In a semi-quantitative study using gelatin zymography and densitometry, the gelatinase activity in supernatants of homogenized osteosarcomas was significantly higher than that in canine stromal tissues [10]. Subsequently, in different canine tumors, such as mast cell tumors [11], mammary gland tumors [6] or carcinomas [15, 20], and other malignancies [12], evident correlations between the production of pro- and active gelatinases and the degree of histological malignancy have been demonstrated. One study suggested that serum MMP-9 plays an important role in the progression of canine mammary tumors and that assay of serum MMP-9 is helpful for early diagnosis of progressive adenocarcinoma, in view of its higher level in dogs with mammary adenocarcinoma than in those with benign tumors [20]. Our aim in the present study was to determine the gelatinase activity in assorted canine tumors, and to evaluate the usefulness of tissue and serum gelatinase activity as a prognostic marker of canine tumors.

MATERIALS AND METHODS

Samples: Serum samples were obtained from 60 dogs with neoplasia brought to our veterinary hospital at Kagoshima University (18 breeds aged 2 to 15 years, body weight 1.7 to 55 kg). Thirty-one of the tumors were benign and 29 were malignant, as shown in Table 1. Control serum samples were obtained from 24 healthy dogs. At the time of biopsy and surgery, small pieces of the tumors were collected to determine the histopathological diagnosis and to quantify their gelatinase activity. The pieces for pathological examination were divided into two parts; one part was fixed with 10% formalin and the other was frozen in liquid
nitrogen and then stored at –80°C until analysis. Tumor histopathology was determined by one pathologist (Dr. Miyoshi) using hematoxylin and eosin stain and the specific procedures as appropriate. One hundred milligrams of fresh tumor tissue was chopped and then homogenized with 500 µl of cold extraction solution containing 154 mM KCl and 5 mM ethylenediamine tetraacetic acid according to a protocol reported previously [5]. After centrifugation (105,000 x g for 30 min at 4°C), aliquots of the supernatant were stored at –80°C until assay.

Preparation of gelatinase from cultured mononuclear cells: Canine peripheral blood mononuclear cells (PBMC) were isolated by centrifugation (400 x g) on a discontinuous density gradient (62.5% (v/v) Percoll in 1.5 M NaCl and distilled water (DW)). PBMC at the Percoll/plasma interface was washed 3 times with phosphate-buffered saline (PBS). The washed cells were resuspended in RPMI1640 containing L-glutamine (300 mg/l), penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% fetal calf serum at a concentration of 5 x 10⁵ cells/ml and incubated for 4 hr at 37°C in 5% CO₂. The non-adherent cells were removed by washing in RPMI medium and adherent cells were allowed to spread out and then incubated for 24 hr in the presence of 5 µg/ml lipopolysaccharide (LPS). The culture media supernatants were stored in aliquots at –80°C until enzyme assay by gelatin zymography as a canine gelatinase standard.

Gelatin zymography and densitometry: Gelatin zymography was performed using a protocol described previously [5] with some modifications. Two microliters of supernatant from the tumor extracts and canine gelatinase standard (canine PBMC culture medium supernatant) was added to 8 µl of zymography sample buffer containing 7.5% sodium dodecyl sulfate (SDS), 2.5% glycerol, 1% bromophenol blue, and 1 M Tris-HCl (pH 6.8). Then 10 µl of the mixture was incubated once for 1 hr, poured into each well of a 10% polyacrylamide gel containing 2.5 mg/ml gelatin, and then electrophoresed at room temperature. The gel was placed in 2.5% Triton X-100 dissolved in DW for 1 hr in order to remove SDS, and then rinsed repeatedly in DW to remove the Triton. The gelatinolytic reaction was allowed to proceed at 37°C for 18 hr in the gelatin refolding buffer including 50 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 50 mM CaCl₂, and 0.067% of Brij35. After staining the gels with 1% Coomassie brilliant blue for 30 min and immersion in DW overnight, gelatinases were visible in the gels as fine decolorized bands on a blue background.

Serum gelatinase activity was analyzed on 7.5% polyacrylamide gels copolymerized with 1 mg/ml gelatin [17]. After electrophoresis of a mixture of 5 µl of serum (diluted 3:100 with DW) plus 5 µl zymography buffer including 4% SDS, 20% glycerol, 1% bromophenol blue, and 2.5 M Tris-HCl (pH 6.8), the SDS was removed from the gels by several washes with 2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5. The gel was then rinsed with DW and subsequently the zymograms were developed for 18 hr at 37°C in the refolding buffer, including 50 mM Tris-HCl, pH 7.5, plus 0.15 M NaCl, 10 mM CaCl₂ and 0.02% NaN₃. The gels were stained with 1% Coomassie brilliant blue for 30 min and then destained in distilled water overnight. A mixture of 5 µl of canine gelatinase standard (diluted 3:10 with DW) from LPS-stimulated canine PBMC culture plus 5 µl of zymography buffer was loaded into one well per plate as the internal standard for serum gelatinase activity.

The enzyme activity of the samples was quantified as the net intensity of the gelatinolytic activity including the bands on a box plot, we expressed the data as mean ± standard deviation (SD) and carried out statistical testing using analysis of variance; Scheff’s method was used for simultaneous multiple comparisons. As the 6 categories did not show a normal data distribution for each category, we compared the data using the non-parametric Kruskal-Wallis and Mann-Whitney U tests. Differences at p<0.05 were considered statistically significant. Correlation of gelatinase levels between tumor extracts and serum samples was determined by regression analysis.
RESULTS

Gelatin zymography: Gelatin zymograms of tumor extracts (Fig. 1) showed gelatinolytic bands of various sizes that included not only the pro- and active types of MMP-2 and -9 but also tissue inhibitors of metalloproteinases-bound MMPs and the other gelatinolytic MMPs. Most of the extracts of MR-positive malignant tumors had more intense bands of the pro- and active types of MMP-2 and -9 than benign tumors. However, a few benign tumors including adipose, mammary mixed tumor, and complex mastadenoxy showed intense bands indicative of higher gelatinase activity, while some samples of malignant tumors including mast cell tumor and thyroid carcinoma showed weak or faint signals. Although no data are presented here in the form of figures, the positive signals of gelatinase activity on the zymograms were increased in inflammatory tumors as well as in metastatic/recurrent malignancies. Gelatinase activity in tumor extracts (Fig. 2): No significant difference of gelatinase activity in the extracts was evident between benign (2.09 ± 2.23) and malignant tumors (3.09 ± 2.67). Inf(+) benign tumors (3.40 ± 2.83) as well as Inf(−) MR(+) (5.87 ± 5.32), Inf(+) MR(−) (3.23 ± 1.93) and Inf(+) MR(+). 3.74 ± 1.75) malignant tumors, had significantly higher tissue gelatinase activities than Inf(−) benign tumors (1.15 ± 0.98). The activity in Inf(−) MR(−) malignancy (1.37 ± 0.83) was significantly lower than that in Inf(+) MR(−) and Inf(+) MR(+) malignancy.

Gelatinase activity in sera: Gelatin zymography (Fig. 3) showed that most tumor samples formed two major bands: the pro-forms of MMP-2 and -9. Dogs with inflamed or metastatic/recurrent malignancies had more intense MMP-2 and -9 bands in their serum than dogs with benign tumors. The results of densitometry, presented in graph form in Fig. 4, showed that serum gelatinase activities were significantly increased in dogs with malignant tumors (1.51 ± 0.69) than in controls (1.12 ± 0.30) or dogs with benign tumors (1.03 ± 0.51). Dogs with Inf(−) MR(+) (1.65 ± 0.12), Inf(+) MR(−) (1.83 ± 0.81) and Inf(+) MR(+) (1.57 ± 0.53) malignancies had significantly higher serum gelatinase activity than dogs with Inf(−) benign tumors (0.90 ± 0.43). Inf(+) benign (1.18 ± 0.55) and Inf(−) MR(−) malignant tumors (1.19 ± 0.79) produced no significant increase of serum gelatinase. Gelatinase activity in serum was positively correlated (r=0.34, p=0.0115) with that in tumor extracts (Fig. 5).

DISCUSSION

To quantify the gelatinase activity in canine tumors and serum, we previously performed preliminary studies using gelatin degradation enzyme-linked immunosorbent assay (ELISA) [2] as well as immunoblot densitometry [6]. The ELISA (data not shown) was developed with canine gelatinase from LPS-stimulated PBMC and a polyclonal antibody raised against porcine gelatin. However, we were unable to obtain reproducible ELISA data because of the higher...
degree of non-specific reaction. When we tried to quantify the positive signals on immunoblots with antibodies specific to human MMP-9 by densitometry, it was even harder to detect the signals from MMP-2 and -9 activities because of the non-specific reaction due to the lower affinity of the antibody for canine gelatinase in both the tumor extracts and serum samples (data not shown). The assay system for human MMP-2 and -9 in practical use, which is designed to
detect efficiently the gelatinase activity in plasma, serum and culture medium by specific antibodies for the MMPs [21], is not available for quantification of canine samples because the antibody does not cross-bind to canine MMP-2 and -9. Currently, therefore, the best way to quantify gelatinase activity in canine samples is gelatin zymogram densitometry using a gel scanner. The intra- and inter-assay variability for serum gelatinase activity (percentage of SD by the mean value) for this system were calculated to be 3.2% (mean ± SD; 1.26 ± 0.04) and 5.4% (1.11 ± 0.06), respectively, indicating that the measurements from our assay would be highly reproducible.

Our assay system determined the gelatinase activity in various kinds of canine benign as well as malignant tumors, as reported previously [12], but the results indicated that malignant tumors did not necessarily show higher tissue gelatinase activity than benign tumors. With regard to other potential factors affecting gelatinase activity in tumor tissue, we found that benign tumors with inflammatory cell infiltration, which accounted for 47% of the canine tumors we examined (42% of benign and 52% of malignant tumors) showed higher gelatinase activity. Although we have no quantitative data on coexisting inflammation in human tumors, canine tumors may show a higher frequency of inflammation because they may be subject to more frequent irritation or injury, or may be infected at the time of initial presentation to a veterinary clinic. To analyze the relationship between gelatinase activity and inflammation in tumors, and to test the hypothesis that gelatinase activity might be a promising prognostic marker of metastasis/recurrence in canine tumors, the gelatinase activity from the tumor cases were divided into 6 categories, as described in Materials and Methods.

In comparison with Inf(−) benign tumors, Inf(+) benign and Inf(−) MR(+) malignant tumors had significantly higher gelatinase activity than dogs with Inf(−) benign tumors. *; p<0.05, **; p<0.01.

![Graph showing significant correlation between gelatinase activity in tumor extracts and that in serum.](image)
tissue gelatinase activity. This result suggests two possibilities. First, although canine benign tumors might have less gelatinase than malignant ones, as discussed in previous papers [12, 20], benign tumors with inflammation may have higher gelatinase activity than silent benign tumors. The induction of MMP-2 and the expression of MMP-9 in monocytes are up-regulated primarily at the transcription level by inflammatory cytokines (tumor necrosis factor-alpha and interleukin-1) and adhesion molecules on the surface of activated T-cells [14]. MMP-9 is produced by polymorphonuclear neutrophils during the inflammation reaction and facilitates their migration through the endothelial basement membrane [7]. In this way, the inflammatory response could increase tissue gelatinase activity in canine tumors even if they are benign. The second possibility is that the higher gelatinolytic activity in malignant tissue could be positively correlated with tumor aggressiveness or poor prognosis (metastasis and/or recurrence). A previous paper has suggested that activated MMP-9 from tumor cells enhances the invasive phenotype of cultured cells, as their ability to degrade both the ECM and basement membrane is significantly increased following zymogen activation [4, 16]. MMP-2 could play a constitutive role in ECM turnover in tumors, while the inductive expression of MMP-9 via cytokines, growth factors (epidermal growth factor) and cell/stroma interactions could be associated with tumor invasion into the surrounding tissues. The higher gelatinase activity in Inf(−) MR(+) malignant tumors suggests that more aggressive malignancies have higher gelatinase activity, and thus gelatinase activity could be a useful marker for predicting tumor metastasis and recurrence. It is also interesting to note that the lower gelatinase activity in Inf(−) MR(−) malignant tumors was not significantly different from that in Inf(−) benign tumors. If the source of active gelatinase was the inflammatory cells infiltrating the tumor, as well as the tumor cells with an invasive phenotype, then a low level of gelatinolysis in Inf(−) MR(−) malignancy could be indicative of a good prognosis.

This is the first paper to have presented data on serum gelatinase activity in dogs with various kinds of tumors. Previously, Yokota et al., who first developed an assay system with a specific antibody for canine MMP-9, reported an increased level of MMP-9 in serum samples from dogs with canine mammary adenocarcinoma [20]. In agreement with their data, the serum gelatinase activities in our dogs with malignant tumors were significantly higher than those in dogs with benign tumors and also healthy dogs. Furthermore, there was a positive correlation between gelatinase activity in tissue extracts and that in serum, suggesting that overproduction of gelatinase from tumor tissue could significantly increase the level in serum. A previous study has demonstrated that densitometric measurements of MMP-2 and -9 in sera are significantly correlated with oncogene (c-erbB-2) overexpression, estrogen receptor expression, and nuclear grade in human breast cancer, and also suggested that production of gelatinase may be higher in c-erbB-2-positive breast cancers with higher metastatic potential [8]. Increased serum levels of MMP-9 are also correlated with earlier upstaging and shorter progression-free survival in human patients with early B-cell chronic lymphocytic leukemia [13]. Among patients with lung cancer, those with a serum MMP-9 level of <1,293 ng/ml before treatment had a significantly longer median survival (1,218 days) than those with higher serum levels (421 days) [9]. Another study has shown that high serum levels of MMP-9 as well as tissue inhibitor of metalloproteinases-1 are correlated with poor cumulative survival in lung cancer patients [19]. Therefore, we hypothesize that the serum level of MMP-9 has potential as a new powerful prognostic marker that could help to stratify patients with different stages of cancer into low- and high-risk groups. We have demonstrated in this study that dogs with metastatic and/or recurrent malignancies have significantly higher serum gelatinase activity, suggesting that the serum gelatinase level could become a reliable prognostic marker in dogs with malignancies.

The higher serum gelatinase level in dogs with Inf(+) MR(−) malignant tumors was of interest. In order to use serum gelatinase activity clinically as a prognostic marker in veterinary cases of tumor, it will be necessary to clarify the extent by which gelatinase from inflammatory tissue in tumors raises the serum level, or to rule out the contribution that is due to tumor-unrelated inflammation. On the basis of the present findings, we conclude that (1) gelatin zymogram densitometry gives a reproducible estimate of gelatinase activity in canine tumors and serum, (2) the level of gelatinase activity in the tumor is correlated with both inflammation and tumor aggressiveness, and (3) estimation of serum gelatinase activity would be helpful for predicting tumor prognosis, if combined with histopathology.

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