FULL PAPER *Pathology*

The Activity of Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs) in Mammary Tumors of Dogs and Rats

Kouji KAWAI[1)], Koji UETSUKA[1)], Kunio DOI[1)] and Hiroyuki NAKAYAMA[1)]*

[1)]*Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan*

ABSTRACT. We conducted zymography for detecting the activity of matrix metalloproteinases (MMPs) and reverse zymography for the activity of tissue inhibitors of metalloproteinases (TIMPs) in canine spontaneous and rat 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumor tissues. The activities of MMPs of canine mammary tumors were quite higher than those of the rat chemically induced tumors. The activities of MMPs were significantly higher in malignant tissues than in benign ones of canine tumors, whereas the activity of only MMP-2 was higher in both benign and malignant rat tumors compared to normal tissues. There were no differences of MMPs activities between benign and malignant rat tumors. The results of reverse zymography indicated that the activities of TIMP-1, -2 and -3 were strikingly higher in rat tumors than in canine tumors. The activities were higher in malignant tissues than in benign ones of dogs, and higher in tumor tissues than in normal mammary tissues of rats. The results of film *in situ* zymography for tissue localization of gelatinolytic activity showed that the digested area was more extended in malignant tumors than in benign ones of dogs. However, the area was similarly extended in both benign and malignant rat tumors. These results may indicate that the canine spontaneous malignant mammary tumors possess more aggressive nature than the rat chemically induced counterpart, resulting from the high level of MMPs and low level of TIMPs activities of the tumor tissues.
KEY WORDS: canine, mammary tumor, MMP, rat, TIMP.

Malignant tumors often metastasize to other organs such as lymph nodes and lungs, which often influences the prognosis of the patients. Destruction of the basement membrane is said to be one of the most important steps for tumor metastasis [11]. Matrix metalloproteinases (MMPs) belong to a family of zinc-dependent proteases and are capable of degrading basement membrane as well as extracellular matrix surrounding cells. It is widely known that MMPs are important for tumor invasion and metastasis [25]. Of MMPs, MMP-2 and MMP-9 have gelatinase activity, and are also known as type IV collagenases. The basement membrane consisting of type IV collagen functions as a barrier against carcinoma invasion and metastasis. A part of MMPs activities are controlled by tissue inhibitors of metalloproteinases (TIMPs). TIMPs are known not only to inhibit MMPs activity but to play a role for activation of some MMPs [10]. The total enzymatic activities of MMPs are determined by the activity of both latent and active forms of MMPs, and by the inhibition of the activity by TIMPs as well [15]. The unbalanced activities of MMPs and TIMPs are involved in tumor progression [12]. Tissue activities of MMP-2 and -9 are reported to correlate well with malignancy and prognosis in human mammary tumors [2, 6, 13, 20, 21, 28].

Mammary tumors are the most common tumor in dogs as well as in human. Malignant cases may occupy approximately a half of canine mammary tumors [1]. Activities of MMP-2 and -9 correlate well with malignancy and prognosis in canine mammary tumors [13, 28] as in human ones. However, there are no reports regarding TIMPs activities in canine mammary tumors. In the present study, we examined both MMPs and TIMPs activities in spontaneous canine mammary tumors using zymography and reverse zymography, and also performed film *in situ* zymography (FIZ) method to detect gelatinolytic activity on a tissue section. FIZ method was applied to several human cancers, and reported to correlate with their malignancy [7, 8, 24, 29]. Localization of MMP-2 expression in canine mammary tumors is recently reported [19], but that of the enzymatic activity *in situ* is still unknown.

We also examined the MMPs and TIMPs activities of chemically induced mammary tumors in Sprage-Dawley (SD) rats by administering 7, 12-dimethylbenz(a)anthracene (DMBA). Tumor invasion and metastasis are very rare in the rat model though they are common in canine malignant tumors [22]. Therefore, some different mechanisms may determine the tumor progression between spontaneous canine tumors and chemically induced rat tumors. The balance of MMPs/TIMPs activities is compared in the tumors of the species.

MATERIALS AND METHODS

Canine mammary tumors: All the samples of canine mammary tumors used in the present study were collected from biopsy cases at the Department of Veterinary Pathology, the University of Tokyo. A half of each sample was fixed in 10% neutral buffered formalin, and paraffin sec-

* CORRESPONDENCE TO: NAKAYAMA, H., Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan.

tions of 4 μm thick were stained with hematoxylin and eosin (HE). The other half of the sample was stored at   –80°C and used for making frozen sections and for performing protein and RNA extraction. According to the histopathological classification proposed by WHO [16], 10 adenocarcinomas and 10 adenomas were chosen for zymography and reverse zymography. FIZ was performed using 9 samples of the mammary tumors.

DMBA-induced mammary tumors of SD rats: Female SD rats were purchased from Japan SLC (Hamamatsu, Japan). Animals were kept in isolator cages (Niki Shoji, Tokyo, Japan) under a controlled condition (23 ± 2°C with 55 ± 5% humidity and a 14 hr light/10 hr dark cycle) and fed commercial diet pellets (MF; Oriental Yeast, Tokyo, Japan) and water *ad libitum* during experiments. The tumor induction was conducted according to the report by Huggins *et al.* [5]. The animals received a single intragastric administration of DMBA (20 mg/rat) dissolved in sesame oil at 50 days of age. The number and location of induced tumors were recorded each week until the termination of the experiment (13 weeks). After the animals were humanly sacrificed with ethyl ether, mammary tumors were excised and weighed. A half of the tissue was fixed in 10% neutral buffered formalin, and paraffin sections of 4 μm thick were stained with HE for histopathological examination. The other half was stored at –80°C and used for making frozen sections and performing protein extraction.

Protein extraction: Frozen tumor samples were minced and homogenized in three volumes of extraction buffer (1% NP-40, 50 mM Tris-HCl, pH 7.4, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM phenylmethane sulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). The homogenate was centrifuged at 10,500 × g for 20 min and the supernatant was used for zymography and reverse zymography. The protein concentration of the supernatant was measured by the Lowry's method using the DC protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

Zymography and reverse zymography: Gelatin zymography was performed to detect MMP-2 and MMP-9 activities in the extracted samples from mammary gland tumors using the methods described previously [9] with a minor modification. Proteins in a sample were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels containing 1 mg/ml gelatin. MMP-2 and MMP-9 protein standards (Oncogene, San Diego, CA, U.S.A.) were used as positive controls. Samples were electrophoresed at 20 mA for 70 min. The gels were washed in a washing buffer (50 mM Tris, 0.2 M NaCl, and 2.5% Triton X-100) for 1 hr, and then incubated in a digestion buffer (50 mM Tris, 0.2 M NaCl, 0.02% Brij-35, and 5 mM CaCl$_2$) at 37°C for 20 hr. The gels were stained with 2.5% Coomassie Blue R-250 in 50% methanol and 10% acetic acid for 20 min, and destained in 30% methanol and 10% acetic acid solution, until active bands become clear. Reverse zymography was performed by almost the same procedure, except that the concentration of gel was 15% without SDS, and that the gel contained 0.15 μg/ml recombinant human (rh) Pro-MMP-2 [18]. Control proteins (TIMP-1 and -2 from human fibroblasts, rhTIMP-3 and rhTIMP-4) for reverse zymography were purchased from Oncogene (Cambridge, MA, U.S.A.).

Film in situ zymography: Detection of *in situ* gelatinolytic activity was performed using FIZ method (FIZ-GN). Films which are coated with a 7 μm-thick layer of cross-linked gelatin (FIZ-GN film), and those coated with 1,10-phenanthrolin (an MMP inhibitor) as negative controls (FIZ-GI film), were commercially provided by Wako Pure Chemical, Tokyo, Japan. Six-μm frozen tissue sections were mounted with the films. The film-mounted sections were then incubated at 37°C for 7.5 hr in a moisture chamber.

Table 1. Canine mammary gland tumors used in the study

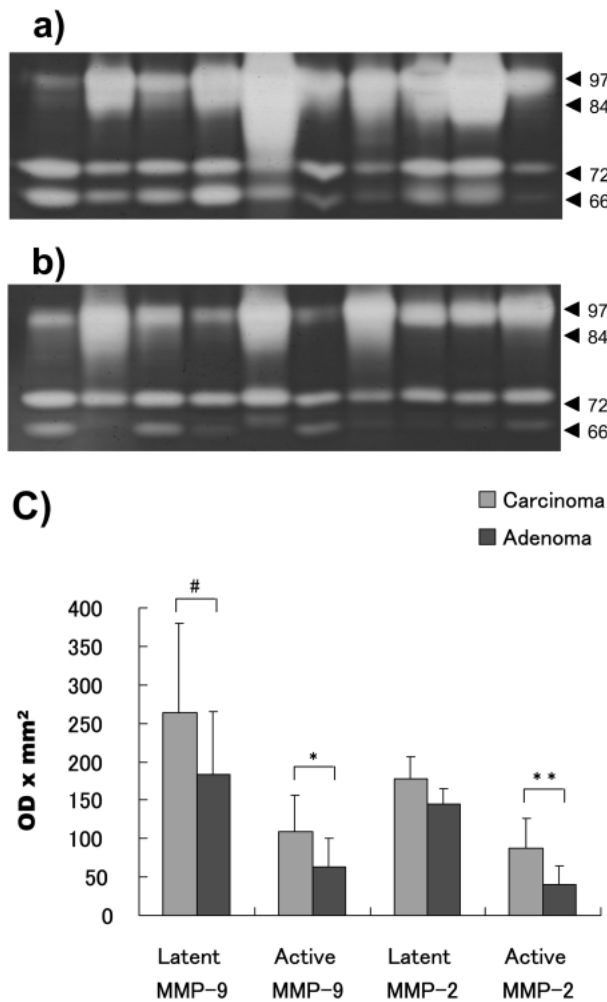|	Case	Breed	Sex	Age	Diagnosis
Carcinoma	1	Chihuahua	Female	9y9m	Tubulopapillary carcinoma
	2	Golden retriever	Female	11y0m	Tubulopapillary carcinoma
	3	Shih Tzu	Female	15y0m	Tubulopapillary carcinoma
	4	Golden retriever	Female	9y0m	Solid carcinoma
	5	Mix	Spayed female	11y0m	Solid carcinoma
	6	Beagle	Spayed female	11y5m	Solid carcinoma
	7	Mix	Spayed female	12y0m	Solid carcinoma
	8	Mix	Female	12y10m	Solid carcinoma
	9	Shiba	Spayed female	5y0m	Complex carcinoma
	10	Golden retriever	Female	8y5m	Anaplastic carcinoma
Adenoma	11	Pomeranian	Female	5y3m	Simple adenoma
	12	Yorkshire terrier	Female	10y11m	Simple adenoma
	13	Maltese	Female	11y1m	Simple adenoma
	14	Shih Tzu	Female	12y0m	Simple adenoma
	15	Mix	Spayed female	15y10m	Simple adenoma
	16	Miniature Duchshund	Female	5y8m	Complex adenoma
	17	American cocker spaniel	Female	5y9m	Complex adenoma
	18	Miniature Duchshund	Female	6y0m	Complex adenoma
	19	Yorkshire terrier	Female	10y3m	Complex adenoma
	20	Shih Tzu	Female	13y4m	Complex adenoma

Fig. 1. Activities of MMP-2 and MMP-9 in canine mammary tumors. Gelatin zymography of mammary carcinoma (a) and adenoma (b). Bands of 97, 84, 72 and 66 kDa indicate latent MMP-9, active MMP-9, latent MMP-2 and active MMP-2, respectively. Each lane represents individual case. From left to right; Cases 1 to 10 in (a) and Cases 11 to 20 in (b). Panel (c) is a quantitative representation of the activities. *: $P<0.05$, **: $P<0.01$ (Student's t-test), and #: $P<0.05$ (Welch's t-test).
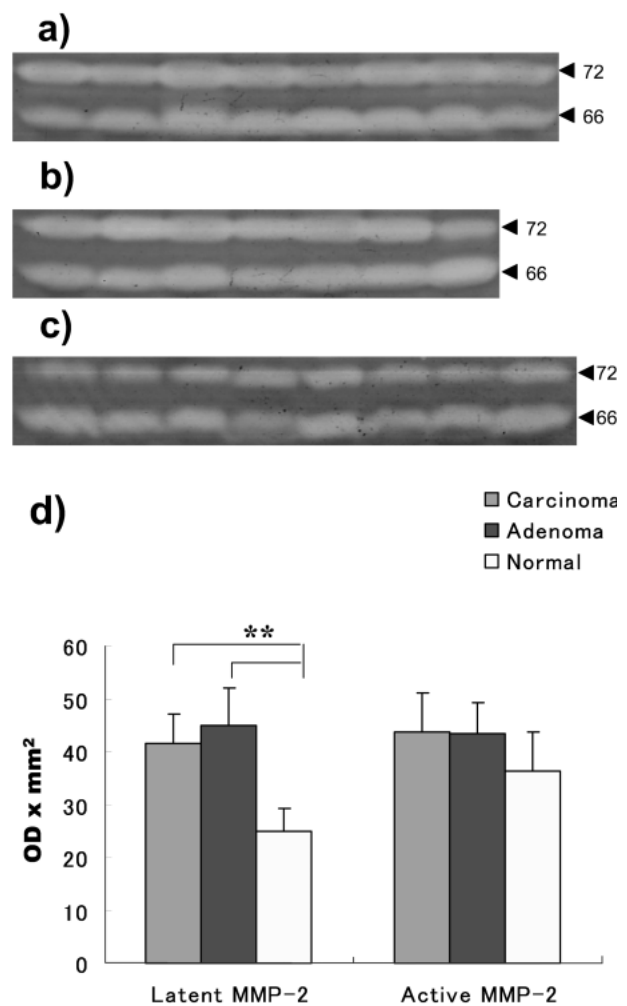

Fig. 2. Activities of MMP-2 in chemically induced mammary tumors in rats. Gelatin zymography of mammary carcinoma (a), adenoma (b) and normal mammary tissue (c). Bands of 72 and 66 kDa indicate latent MMP-2 and active MMP-2, respectively. Each lane represents individual case randomly selected. Panel (d) is a quantitative representation of the activities. **: $P<0.01$ (One-way ANOVA).

After incubation, the films were air-dried for 30 min and stained with Biebrich-Scaret Red (Wako Pure Chemical) for 4 min.

Statistical analyses: Statistical significance was determined by Student's t-test or Welch's t-test for the canine data, following the determination of homogeneity of variance by F-test. One-way ANOVA was applied for rats' data. Pearson's correlation test was used to determinate the significance for correlation. A significance level of $P<0.01$ or $P<0.05$ was considered acceptable.

RESULTS

Canine mammary tumors: Details and histopathological diagnoses of 20 canine mammary tumor cases examined in the study are listed in Table 1. The diagnoses were 10 malignant tumors including 5 solid carcinomas, 3 tubulo-papillary carcinomas, 1 anaplastic carcinoma and 1 complex carcinoma, and 10 benign tumors including 5 simple adenomas and 5 complex adenomas.

DMBA-induced mammary tumors in SD rats: Mammary tumors were detected by palpation at 8 weeks or later after DMBA administration. Tumors were observed in 42 of 45 rats which survived until the end of the experiment. The tumors were histologically diagnosed according to the pre-
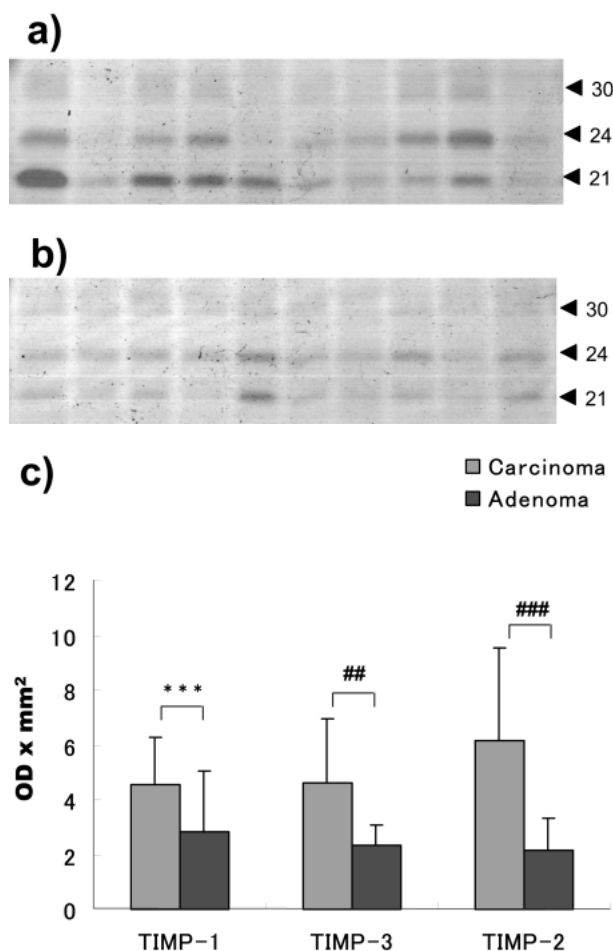
Fig. 3. Activities of TIMP-1, -2 and -3 in canine mammary tumors. Gelatin reverse zymography of mammary carcinoma (a) and adenoma (b). Bands of 30, 24 and 21 kDa indicate TIMP-1, -3 and -2, respectively. Each lane represents individual case. From left to right; Cases 1 to 10 in (a) and Cases 11 to 20 in (b). Panel (c) is a quantitative representation of the activities. ***: $P<0.001$ (Student's t-test), ##: $P<0.01$, and ###: $P<0.001$(Welch's t-test).
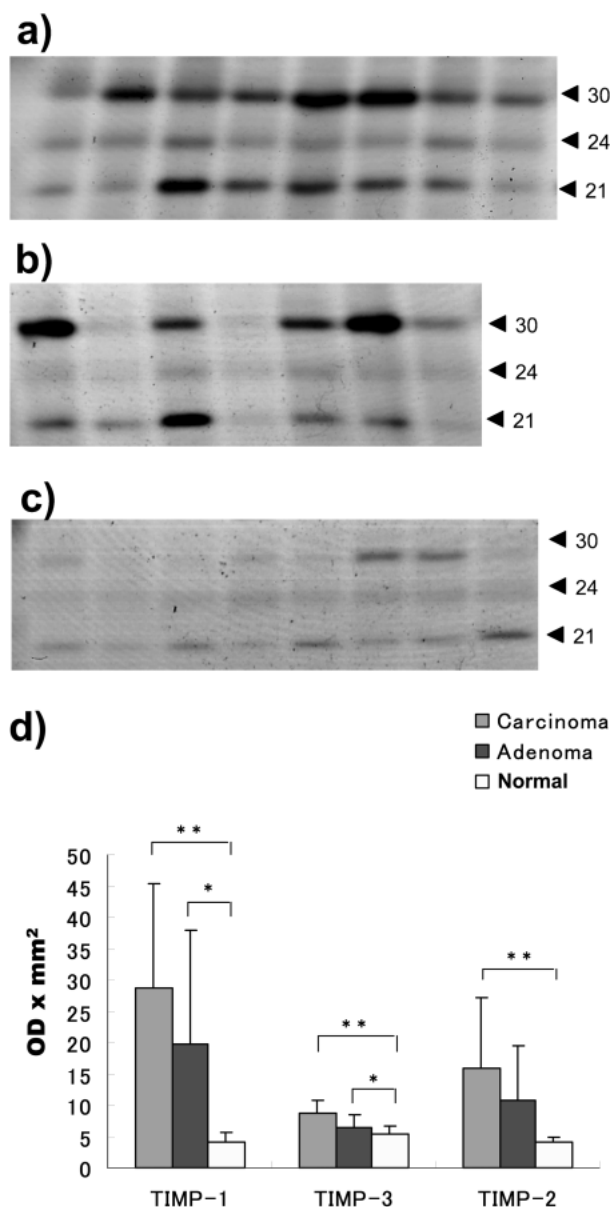

Fig. 4. Activities of TIMP-1, -2, and -3 in chemically induced mammary tumors of rats. Gelatin reverse zymography of mammary carcinoma (a), adenoma (b) and normal mammary tissue (c). Bands of 30, 24 and 21 kDa are TIMP-1, -3 and -2, respectively. Each lane represents individual case randomly selected. Panel (d) is a quantitative representation of the activities. *: $P<0.05$, and **: $P<0.01$ (One-way ANOVA).

vious report [23]. Of 211 mammary gland tumors, 202 were malignant including 42 papillary carcinomas, 99 cribriform carcinomas, 3 comedo carcinomas, and 58 tubular carcinoma, whereas only 9 were benign including 7 lactating adenomas, and 2 tubular adenomas. A precancerous lesion of ductal carcinoma *in situ* was observed in one rat treated with DMBA and without any other mammary tumors. Normal mammary glands were scattered in the ventral subcutaneous fat tissue.

Zymography: The zymographic band patterns of protein extracts from canine mammary carcinomas and adenomas are shown in Fig. 1. Bands of latent MMP-9 (97 kDa) and MMP-2 (72 kDa) were clearly detected in all the benign and malignant tumor samples examined, whereas active forms of MMP-9 (84 kDa) and MMP-2 (66 kDa) were less expressed in some samples. Enzymatic activities of latent

MMP-9 as well as active forms of MMP-2 and MMP-9 in carcinomas were significantly higher than those in adenomas, which is consistent with previous reports. The zymographic band patterns of mammary tumors and normal mammary glands from rats are shown in Fig. 2. Bands of latent (72 kDa) and active (66 kDa) forms of MMP-2 were
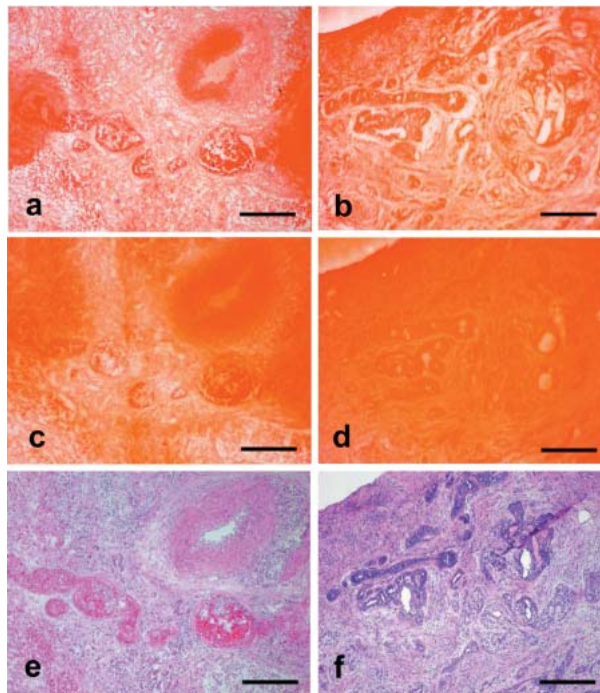
Fig. 5. *In situ* gelatinolytic activities in the canine mammary tumor by film *in situ* zymography (FIZ). Mammary carcinoma (a, c and e) and adenoma (b, d and f). Sections coated with cross-linked gelatin alone (FIZ-GN) (a and b), and those coated with cross-linked gelatin including an MMP inhibitor, 1,10-phenanthorolin (FIZ-GI) (c and d). HE stain sections of the same areas (e and f). A strong gelatinolytic activity was observed in carcinoma samples but not in adenoma. The gelatinolytic activity was weakened by using FIZ-GI films. Bar = 500 μm (a-f).
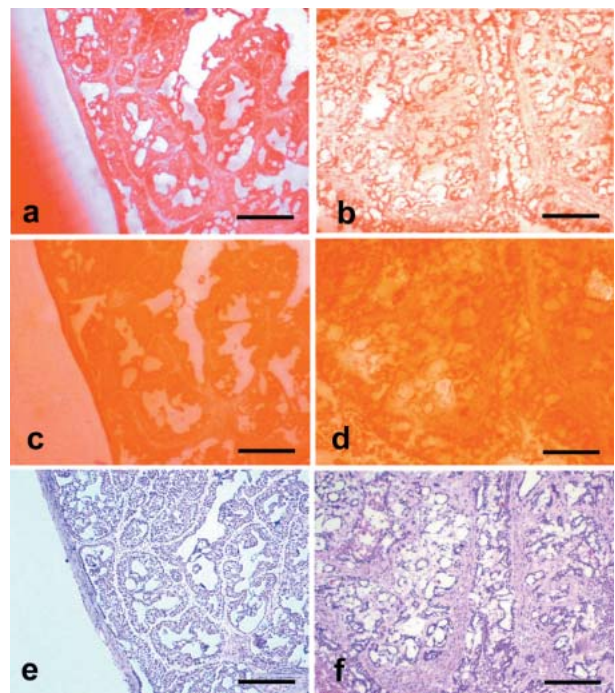
Fig. 6. *In sit*u gelatinolytic activities in the rat mammary tumor by FIZ. Mammary carcinoma (a, c and e) and adenoma (b, d and f). Sections coated with cross-linked gelatin alone (FIZ-GN) (a and b), and those coated with cross-linked gelatin including an MMP inhibitor, 1,10-phenanthorolin (FIZ-GI) (c and d). HE stain sections of the same areas (e and f). A strong gelatinolytic activity was observed both in carcinoma and adenoma samples. The gelatinolytic activity was weakened by using FIZ-GI films. Bar = 500 μm (a- f).

detected in all the samples examined, although the densities of the bands were strikingly lower than those of canine samples. MMP-9 activity was not clear in rat cases (data not shown). Gelatinolytic activity of latent MMP-2 in tumor samples was higher than that in normal mammary tissues, while there were no differences between carcinoma and adenoma. Activity of active form of MMP-2 seemed to be at the same level among carcinoma, adenoma, and normal mammary tissues.

Reverse zymography: The reverse zymographic band patterns of protein extracts from canine mammary carcinoma and adenoma are shown in Fig. 3. Activities of TIMP-1, -3 and -2 were detected as bands of 30 kDa, 24 kDa, and 21 kDa, respectively, whereas those were extremely low when compared with those of rat tumors. Activities of all the TIMPs examined in canine carcinomas were significantly higher than those in adenomas. The same results were obtained in the experiment using rat tissues (Fig. 4) except for higher activity levels of the enzymes. The highest TIMPs activity was observed in carcinomas, followed by adenomas and normal mammary tissues. TIMP-4 activity was not detectable in the present study.

Film in situ zymography: The FIZ-GN film method

revealed *in situ* gelatinolytic activity in mammary tumor samples from dogs and rats. In canine cases, a strong gelatinolytic activity was observed on tumor lesions in carcinoma samples, but not in adenoma (Fig. 5). In contrast, such strong activity was exhibited in both carcinoma and adenoma in rat samples (Fig. 6). The gelatinolytic activity was weakened by using FIZ-GI films in both species (Figs. 5, 6).

DISCUSSION

In canine mammary tumors, activities of active forms of MMP-2 and MMP-9 have been reported to correlate with malignancy of tumors [13, 28]. The results of the present study were consistent with the previous observation. Gelatinolytic activity of canine mammary tumors detected by FIZ was stronger in carcinoma samples than in adenoma, supporting the results of zymography. Such *in situ* gelatinolytic activity was significantly inhibited in the experiment using gelatin films including an MMP inhibitor (FIZ-GI film), indicating that the majority of the gelatinolytic activity may be due to MMPs activity. However, other proteases such as trypsin may be involved in the activity, because trypsin is

known to be produced in several kinds of human cancer tissues [4, 26]. Reverse zymography conducted in the present study showed that the activities of TIMP-1, TIMP-2, and TIMP-3 in canine mammary tumor samples were quite low, but significantly correlated with the malignancy of the tumors.

In cases of rat mammary tumors, the activity of only latent form of MMP-2 was higher in tumor tissues than in normal mammary tissues, but the activities of active MMP-2 was at the same level in both tumors and normal tissue. Activities of latent or active forms of MMP-9 were undetectable in the present study. The present rat mammary tumors did not show any invasive and/or metastatic lesions (data not shown). The lack of invasion and metastasis in this animal model may be due to lower levels of activities of active MMP-2 and to the lack of MMP-9 activity. In addition, the higher activity of TIMPs may explain the characters of the rat tumor. In the present study, diffuse *in situ* gelatinolytic activities were detected in both malignant and benign rat tumor cases, in spite of low MMPs activities, indicating that other gelatinolytic enzymes might play a role. TIMPs are known to have an activity to promote cell growth [3, 14, 17, 27], as well as to inhibit MMPs activity. Therefore, higher activities of TIMPs detected in the rat malignant mammary tumors may contribute to both MMP inhibition and cell growth.

In the present study, the difference of MMPs activities between malignant and benign chemically induced rat mammary tumors was extremely less than those between malignant and benign canine mammary tumors. However, the difference of TIMPs activities was larger in rat cases than in canine cases. These results may indicate that the canine spontaneous malignant mammary tumors possess more aggressive nature such as metastatic and invasive behaviors than the rat chemically induced counterpart, resulting from the high level of MMPs and low level of TIMPs activities as well.

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