

Activation of matrix metalloproteinase (MMP)-2 by membrane type 1-MMP and abnormal immunolocalization of the basement membrane components laminin and type IV collagen in canine spontaneous hemangiosarcomas

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Summary. We performed immunohistochemical investigation of the basement membrane (BM) components, namely, type IV collagen and laminin, in 83 canine hemangiosarcomas (HSAs), 22 hemangiomas, and some granulation tissues (GTs). Additionally, we analyzed the expression and activities of matrix metalloproteinase (MMP)-2, MMP-9, and membrane type 1-MMP (MT1-MMP) using the same samples by immunohistochemistry and gelatin zymography to investigate whether MMPs were associated with the BM degradation. In immunohistochemistry for the BM components, many HSAs showed discontinuous linear/negative immunoreactivity in the BM (type IV collagen: 49.4%/14.5%, laminin: 60.3%/10.8%, respectively). In contrast, almost all hemangiomas showed continuous staining in the BM (type IV collagen: 90.9%, laminin: 95.5%, respectively). Interestingly, positive cytoplasmic immunoreactivity for type IV collagen and laminin was observed in 97.6% and 91.6% HSA, respectively. Although MMP-9 immunoreactivity wasn't detected in neoplastic and active angiogenic endothelial cells (ECs), MMP-2 was detected in all ECs of GTs and in neoplastic cells of both vascular tumors. A strong immunoreactivity for MT1-MMP was observed in active angiogenic ECs in GTs and in neoplastic ECs in HSAs. However, almost all hemangiomas showed weak/negative immunoreactivity. In gelatin zymography, significantly strong activity of active MMP-2 was observed in HSAs, similar to that in

active angiogenesis in GTs; however, weak/no activity of active MMP-2 was detected in hemangiomas. In canine HSA, neoplastic cells had active MMP-2, possibly activated by MT1-MMP, and discontinuous status of BM might be associated with activity of active MMP-2.

Key words: Dog, Hemangiosarcoma, Basement membrane, MMP

Introduction

Hemangiosarcoma (HSA) is a malignant neoplasm of vascular endothelial cells (ECs) that is microscopically characterized by the formation of irregular vascular clefts or channels. In humans, although HSA is rare, it has been reported to occur in various organs, such as the skin, liver, and spleen (Meis-Kindblom and Kindblom, 1998; Fedok et al., 1999; Maluf et al., 2005; Thompson et al., 2005). In contrast, spontaneous HSA in dogs is a relatively common neoplasm and occurs in the spleen, heart, skin, and lungs (Brown et al., 1985). Canine spontaneous HSA, similar to human cases, has a very aggressive nature; therefore, the prognosis is dismal. Local infiltration and systemic metastases are common, and metastatic sites are widespread. Various therapeutic regimens, including surgery and intensive chemotherapy, have been recommended for dogs with HSA; however, the median survival period is 6 months (Prymak et al., 1988; Hammer et al., 1991; Clifford et al., 2000; Sorenmo et al., 2000).

By taking advantage of relatively easy collection of

canine spontaneous HSA cases, some studies have reported on the mechanisms of malignant growth of canine HSA as a model for human HSA, and new aspects of origin or malignant growth conditions were proposed based on the findings of these studies (Fosmire et al., 2004; Dickerson et al., 2005; Lamerato-Kozicki et al., 2006). Previously, we reported that the malignant proliferating ECs of canine HSA were similar to those in the active phase of angiogenesis from the viewpoint of expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and their receptors (Yonemaru et al., 2006). The same findings were also reported in human HSA by Itakura et al. (2008). Angiogenesis, which is controlled strictly by various growth factors, is an important phenomenon during tissue repair (Garcia-Barros et al., 2003), as well as during various pathological processes. The first step of angiogenesis is sprouting of ECs from mature blood vessels. This requires the degradation of both the interstitial matrix and the underlying basement membrane (BM)—a dynamic and self-assembled layer consisting of a network of molecules, mainly type IV collagen and laminin (Iivanainen et al., 2003). Matrix metalloproteinases (MMPs) can degrade extracellular matrix (ECM) components (Sternlicht and Werb, 2001; Egeblad and Werb, 2002). MMP-2 and MMP-9 are particularly well known for their ability to degrade type IV collagen (Murphy and Crabbe, 1995). MMP-2 has been extensively studied for its contribution to angiogenesis (Iivanainen et al., 2003). Additionally, mice lacking membrane type 1-MMP (MT1-MMP), which is an activator of latent MMP-2, die approximately 3 weeks after birth and are reported to suffer from defects in skeletal development (Holmbeck et al., 1999) and angiogenesis (Zhou et al., 2000). Therefore, MMPs contribute to the endothelial migration and extension in angiogenesis. Because the role of growth factors in HSA is similar to that in the active angiogenic phase, the invasion of malignant ECs in HSA may also be ascribed to the expression and activities of angiogenesis-associated MMPs.

In this study, we investigated immunohistochemical expression of laminin and type IV collagen for the analysis of BM formation as well as that of MMP-2, MMP-9, and MT1-MMP in canine spontaneous HSA and benign hemangioma. Additionally, the activities of

MMP-2 and MMP-9 were investigated by gelatin zymography.

Material and methods

Samples

This study was based on archival samples collected between 1998 and 2007 from the Veterinary Teaching Hospital of Gifu University and from private animal hospitals. A total of 83 HSAs (spleen, 55; subcutis, 15; liver, 6; heart, 1; lung, 1; kidney, 3; nictitating membrane, 1; oral cavity, 1) and 22 hemangiomas (subcutis, 20; perimetrium, 1; oral cavity, 1) were examined. Additionally, 10 samples of canine granulation tissues (8, 1, and 1 from the skin, tongue, and ileum, respectively) containing active ECs were investigated as proangiogenic ECs in an active phase of angiogenesis (positive controls for angiogenesis). The samples had been surgically removed, and a part of each sample was immediately fixed in 10% neutral buffered formalin, embedded in paraffin wax, and sectioned for either hematoxylin and eosin (HE) staining or immunohistochemistry. For gelatin zymography, snap frozen samples of 25 HSAs, 8 hemangiomas, and 2 granulation tissues used in immunohistochemistry were stored at -80°C until use. The diagnosis of each tumor was confirmed by reviewing the HE-stained slides and by analyzing serial sections of all the putative vascular neoplasms for von Willebrand factor and CD31 by immunostaining with specific antibodies (anti-von Willebrand factor rabbit antibody and CD31 mouse monoclonal antibody; DakoCytomation, Glostrup, Denmark). All the samples were reviewed by the certified veterinary pathologists.

Immunohistochemistry for laminin, type IV collagen, and MMPs

The polymer-immuno complex method was used on selected slides from all cases by employing the antibodies listed in Table 1. Three-micrometer-thick serial sections were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval pretreatment was carried out either by heating the tissue sections in target retrieval solutions (pH 6.0 or high pH

Table 1. Details of antibodies used in the present study.

Antigen	Source	Clone	Dilution	Pretreatment*	Manufacturer
Type IV Collagen	Human	CIV22	1:300	Proteinase K (0.4 mg/ml)	DakoCytomation, Glostrup, Denmark
Laminin	Rat	Rabbit, polyclonal	1:500	Proteinase K (0.1 mg/ml)	LabVision, Fremont, USA
MMP-2	Human	75-7F7	1:200	HIAR**, pH 9.9	Daiichi Fine Chemical, Takaoka, Japan
MMP-9	Human	Sheep, polyclonal	1:500	HIAR, pH 6.0	Serotec, Kidlington, UK
MT1-MMP	Human	113-5B7	1:75	Proteinase K (0.1 mg/ml)	Daiichi Fine Chemical, Takaoka, Japan

*Pretreatment. For details, refer to Materials and Methods. **HIAR, heat-induced antigen retrieval treatment.

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[pH 9.9], DakoCytomation) in an autoclave for 15 minutes, or 1 minute at 121°C, or by enzymatic digestion with proteinase K (Takara Bio Inc., Shiga, Japan) in 0.05 M Tris-HCl buffer (pH 7.6) for 5 minutes at 37°C. Antibodies for MMP-2 and MMP-9 used in this study recognize both latent (72 and 92 kDa, respectively) and active (62 and 82 kDa, respectively) forms of these proteins. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol for 20 minutes at room temperature, except in the case of MMP-2, where the sections were immersed for 40 minutes. To prevent the binding of nonspecific proteins to the primary antibodies, the sections were treated with Protein Block Serum-free (DakoCytomation) for 30 minutes and then incubated overnight at 4°C. The sections were then incubated with the appropriate secondary antibodies (EnVision+™ System HRP for type IV collagen, laminin, MMP-9, and MT1-MMP; HRP-labeled anti-sheep immunoglobulin rabbit polyclonal antibody [1:400] for MMP-9; DakoCytomation) for 30 minutes at room temperature. The sections were rinsed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) between each step. After signal detection by using a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate-Chromogen System; DakoCytomation), the sections were washed in distilled water, counterstained with Mayer's hematoxylin, and dehydrated. For the negative control, the primary antibody was replaced with PBS. For positive control, preexisting vessels were used on the laminin and type IV collagen, mammary carcinomas were used for MMP-2, -9 and MT1-MMP as described before (Hirayama et al., 2002; Papparella et al., 2002).

Analysis of immunohistochemical reactivity

For the evaluation of staining pattern of laminin and type IV collagen in the BM region and to determine stroma deposition and cellular localization, 10 different non-necrotic randomly chosen neoplastic areas were screened in a high power field (HPF, 400X magnification). The staining pattern of laminin and type IV collagen in the BM region was microscopically classified as follows: continuous, continuous linear staining of more than 90% of the neoplastic cell-stroma interface; negative, staining of less than 10% of the neoplastic cell-stroma interface; and discontinuous, a staining pattern between continuous and negative. When positive cytoplasmic immunoreactivity was obtained in >10% of neoplastic cells in 10 HPFs, it was considered as positive cytoplasmic staining.

The immunoreactivity of MMP-2, MMP-9, and MT1-MMP was assessed using a grading system based on the percentage of neoplastic cells with positive staining in the cytoplasm or cell membrane. The immunohistochemical staining results were divided into the following 3 categories based on the total percentage

of neoplastic cells staining positively and the staining intensity; negative (-) = nonstained neoplastic cells or <20% neoplastic cells with weak staining; weakly positive (+) = >20%, <60% neoplastic cells with weak to moderate staining, or showed heterogeneous positive staining; strongly positive (++) = >60% neoplastic cells with intense staining.

Gelatin zymography

To prepare tissue lysates, frozen samples of tumor tissue and granulation tissue were minced and homogenized on ice in 3 volumes of extraction buffer (360 mM Tris-HCl, pH 6.8). Tissue homogenates were sonicated for 5 minutes, followed by centrifugation at 1,500 rpm for 10 minutes at 4°C to remove debris. Protein concentration of the supernatant was measured by Lowry's method using the DC protein assay kit (Bio-Rad, Hercules, CA, USA) (Lowry et al., 1951).

Gelatinase activities of MMP-2 and MMP-9 in the supernatant from tissue lysates were assayed by gelatin zymography. The supernatants (10 µg protein/lane) were mixed with a sample buffer (1.86 times in volume) and then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel impregnated with 1 mg/ml gelatin (Sigma). The gels were run under nonreducing conditions at 90 V for 150 minutes. After electrophoresis, the gels were incubated in a renaturing solution (2.5% triton X-100 in distilled water) at room temperature for 2 x 30 minutes and then incubated in an incubation buffer (50 mM Tris-HCl [pH 7.6], 10 mM CaCl₂, 50 mM NaCl, and 0.05% Brij 35) for 30 minutes. Finally, the gels were incubated in a fresh incubation buffer at 37°C for 18 hours. The gels were prefixed with freshly prepared 20% methanol and 7.5% acetic acid, stained with 2.5% coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid for 30 minutes, and destained in 5% methanol and 7% acetic acid solution until bands of gelatinolytic activity were visualized. Subsequently, the gels were scanned to perform semi-quantitative digital image analysis to detect the expression of MMPs by densitometry analysis using Scion Image software (Scion Corporation, Frederick, MD, USA). The results of the densitometry analysis were expressed as ratios of active MMP-2 to standard and active MMP-2 to total MMP-2. A mixture of purified human MMP-2 and human MMP-9 (3.5 µg/lane; Chemicon International, Temecula, CA, USA) was run in a separate lane and distilled water was used as a negative control.

Statistical analysis

The following statistical analyses were performed: 1) chi-square test for independence for cytoplasmic immunoreactivity of laminin and type IV collagen and 2) Mann-Whitney U test for immunohistochemical results of MMPs and semi-quantitative data of gelatin zymography for HSA and hemangioma. P<0.05 was

considered as statistically significant.

Results

Distribution of type IV collagen and laminin

In immunohistochemical staining, both type IV collagen and laminin showed strong positive immunoreactivities in the basal side of mature blood vessels showing continuous linear staining in the granulation tissue. In addition, both type IV collagen and laminin showed positive cytoplasmic staining in the ECs of the proliferative capillary vessels (Fig. 1a,d).

Immunostaining of laminin and type IV collagen revealed the presence of basement membrane in both HSAs and hemangioma. Table 2 summarizes the results of the staining pattern of laminin and type IV collagen in HSAs and hemangiomas. In 21 (95.5%) and 20 (90.9%) cases of hemangioma, laminin and type IV collagen, respectively, showed continuous linear staining pattern similar to that observed in mature vessels of the granulation tissue (Fig. 1b,e). In contrast, laminin and type IV collagen showed discontinuous staining pattern in 50 (60.3%) and 41 (49.4%) cases of HSA. Only 9 and 12 HSAs cases showed negative staining in the BM region for laminin and type IV collagen, respectively. It is noteworthy that positive cytoplasmic staining in neoplastic ECs was detected in most cases of HSA (laminin: 91.6%, type IV collagen: 97.6%) (Fig. 1c,f),

whereas most cases of hemangioma showed negative cytoplasmic staining in ECs for both these components (laminin: 90.9%, type IV collagen: 95.5%).

Table 2. Immunohistochemical results of laminin and type IV collagen in HSA and hemangioma.

	HSA (n = 83) (%)	Hemangioma (n = 22) (%)
Type IV Collagen		
BM staining pattern*		
Continuous	30 (36.1)	20 (90.9)
Discontinuous	41 (49.4)	2 (9.1)
Negative	12 (14.5)	0 (0)
Cytoplasmic staining		
Positive	81 (97.6)	1 (4.5)
Negative	2 (2.4)	21 (95.5)
Laminin		
BM staining pattern*		
Continuous	24 (28.9)	21 (95.5)
Discontinuous	50 (60.3)	1 (4.5)
Negative	9 (10.8)	0 (0)
Cytoplasmic staining		
Positive	76 (91.6)	2 (9.1)
Negative	7 (8.4)	20 (90.9)

*BM staining pattern: Continuous, more than 90% of the tumor-stroma interface showed linear staining; Negative, less than 10% of the tumor-stroma interface showed linear staining; Discontinuous, a staining pattern between "Continuous" and "Negative"

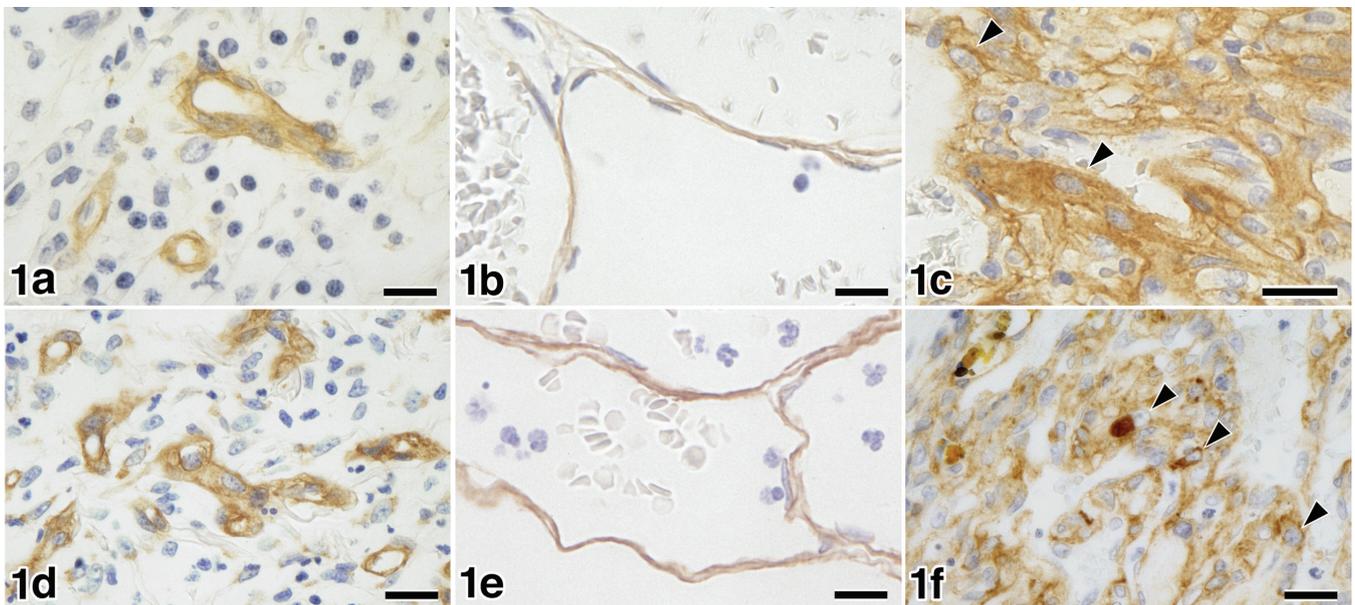


Fig. 1. Immunoreactivity of type IV collagen (a–c) and laminin (d–f) in the granulation tissue (a and d), hemangioma (b and e), and HSA (c and f). Immunohistochemical staining of laminin and type IV collagen showed a continuous linear pattern, which stained lumen formation, surrounding the mature blood vessels in vessels of granulation tissue and hemangioma (a, b, d, and e). In addition, some immature ECs showed cytoplasmic immunoreactivities in the granulation tissue (a and d). In HSA, some neoplastic cells showed positive cytoplasmic immunoreactivity; distinct distribution (c and f, arrowheads) was also observed in the neoplastic cells. Bar: 20 μ m.

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Expression of MMP-2, MMP-9, and MT1-MMP

The immunohistochemical expression of MMP-2, MMP-9, and MT1-MMP was investigated to evaluate their localization and staining intensity.

In the granulation tissue, a high staining intensity of MMP-2 and MT1-MMP was detected mainly on the cell surface and in the cytoplasm of newly formed microvascular ECs, neutrophils, macrophages, lymphocytes, and fibroblasts (Fig. 2a,c). ECs in the mature blood vessels also showed immunoreactivity for both MMP-2 and MT1-MMP. In contrast, neither ECs of newly formed microvascular network nor ECs of mature vessels showed immunoreactivity for MMP-9, although neutrophils and macrophages showed positive immunoreactivity for MMP-9 (Fig. 2b).

Similar to the observation in the granulation tissue, a high staining intensity of MMP-2, MMP-9, and MT1-MMP was detected in the fibroblasts and inflammatory cells (mainly neutrophils and macrophages) that had infiltrated into both HSAs and hemangiomas. The immunoreactivities of MMP-2, MMP-9, and MT1-MMP in HSAs and hemangiomas are shown in Table 3. MMP-2 was immunolocalized on the cell surface and in the cytoplasm of the neoplastic cells in HSAs and hemangiomas (Fig. 2d,g). Of the 83 HSA cases, 60 (72.3%) and 23 (27.7%) cases showed ++ and + MMP-2 immunoreactivity, respectively. Similarly, of the 22 hemangioma cases, 19 (86.4%) and 3 (13.6%) cases showed ++ and + MMP-2 immunoreactivity, respectively. Thus, there were no negatively stained samples for MMP-2 in both HSA and hemangioma

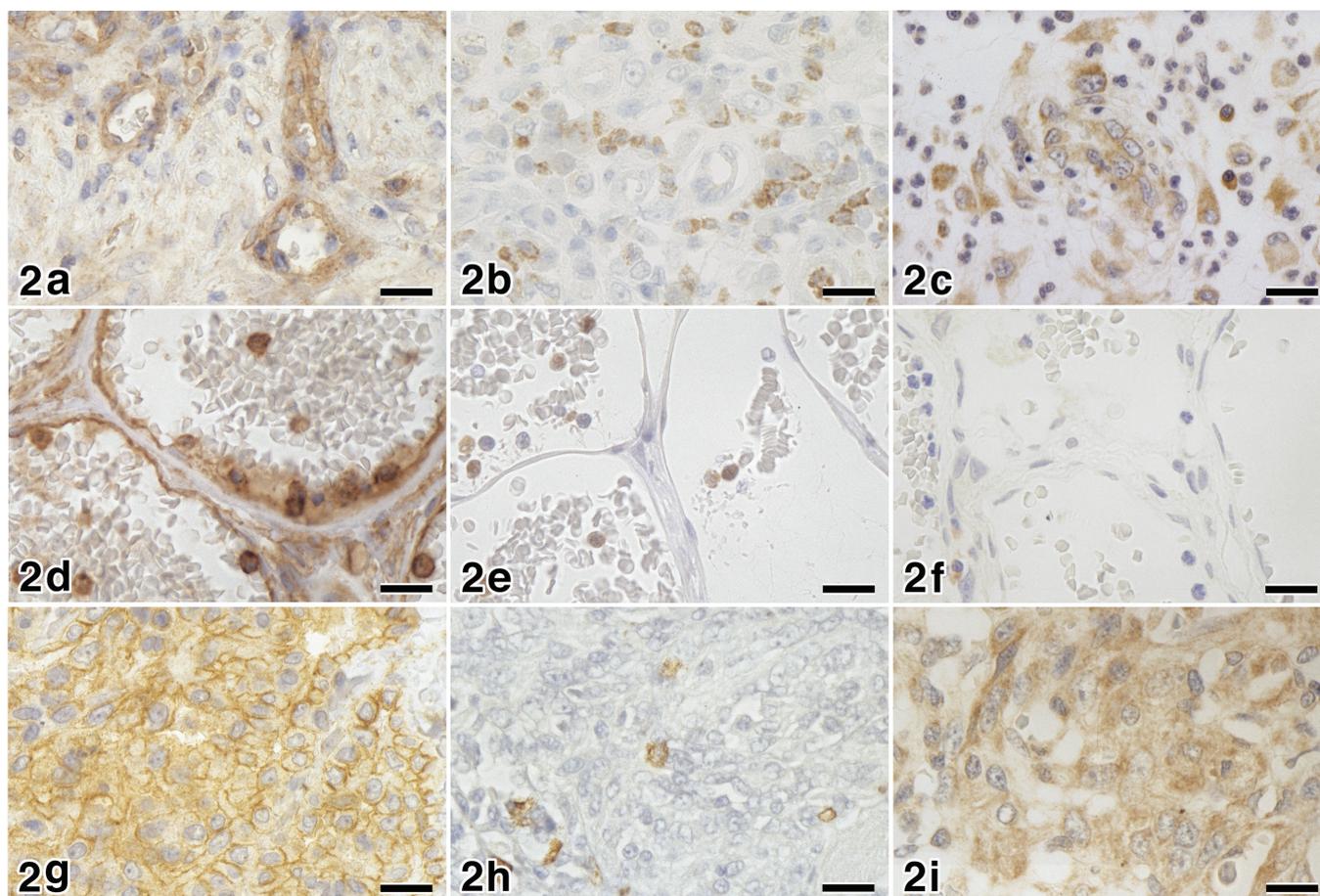


Fig. 2 Immunoreactivity of MMP-2 (**a, d, and g**), MMP-9 (**b, e, and h**), and MT1-MMP (**c, f, and i**) in the granulation tissue (**a-c**), hemangioma (**d-f**), and HSA (**g-i**). A high staining intensity of MMP-2 (**a**) and MT1-MMP (**c**) was detected in the cytoplasm of newly formed vascular endothelial cells and fibroblasts. MMP-9 was not detected in angiogenic endothelial cells, although neutrophils and lymphocytes showed strong positive immunoreactivity. In hemangioma, MMP-2 was detected in the cytoplasm of neoplastic endothelial cells (**d**); however, MMP-9 (**e**) and MT1-MMP (**f**) were negative. In HSA, a strong positive staining of MMP-2 was detected on the cell surface and in the cytoplasm of the neoplastic cells (**g**). Similar to MMP-2, a positive staining of MT1-MMP was noted in the cytoplasm of the neoplastic cells and in inflammatory cells, including neutrophils. Although HSA cells were negative for MMP-9, a high staining intensity was detected in lymphocytes and neutrophils. Bar: 20 μ m.

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Table 3. Immunohistochemical results of MMP-2, MMP-9, and MT1-MMP in HSA and hemangioma.

	HSA (n = 83) (%)	Hemangioma (n = 22) (%)
MMP-2		
-	0 (0)	0 (0)
+	23 (27.7)	3 (13.6)
++	60 (72.3)	19 (86.4)
MMP-9		
-	83 (100)	22 (100)
+	0 (0)	0 (0)
++	0 (0)	0 (0)
MT1-MMP*		
-	0 (0)	12 (54.5)
+	29 (34.9)	9 (40.9)
++	54 (65.1)	1 (4.6)

*MT1-MMP: A significant difference was observed in immunohistochemical results between HSA and hemangioma by Mann-Whitney U test ($P < 0.01$)

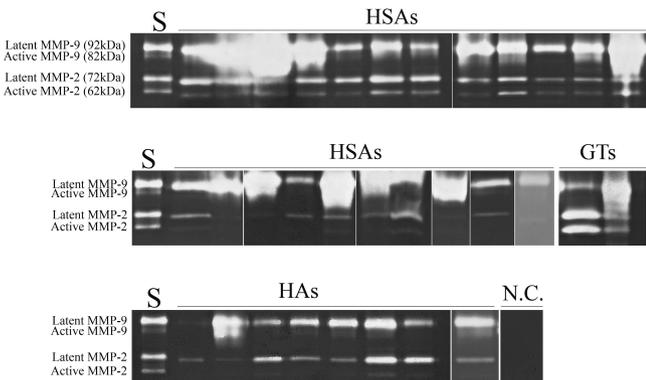


Fig. 3. Gelatin zymography of HSA, hemangioma, and granulation tissue (GT). A total of 25, 8, and 2 cases of HSA, hemangioma, and granulation tissue, respectively, were examined. "S" indicates a control and "N.C." indicates negative control.

cases. MT1-MMP was immunolocalized mainly in the cytoplasm of neoplastic cells in HSAs and hemangiomas. Of the 83 HSA cases, 54 (65.1%) and 29 (34.9%) cases showed ++ and + MT1-MMP immunoreactivity, respectively (Fig. 2i); thus, none of the HSA cases showed negative staining. Of the 22 hemangioma cases, only 1 (4.6%) case showed ++ MT1-MMP immunoreactivity, while 9 (40.9%) and 12 (54.5%) cases showed + and negative immunoreactivity, respectively (Fig. 2f). The number of MT1-MMP-positive cases (++ and +) was significantly higher in HSA than in hemangiomas ($P < 0.01$). In contrast to the immunoreactivity of MMP-2 and MT1-MMP, MMP-9 showed no immunoreactivity in the neoplastic cells in both HSA and hemangioma cases (Fig. 2e,h).

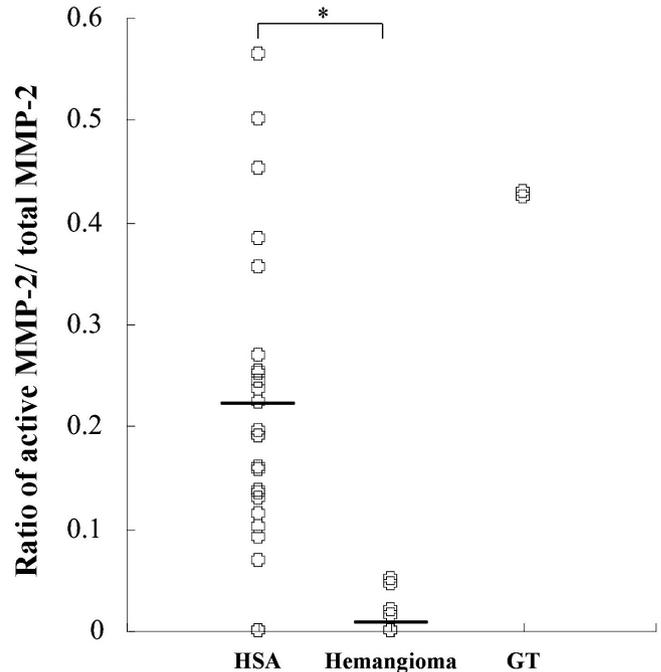


Fig. 4. The ratios of active MMP-2 activity to total MMP-2 activity in HSA, hemangioma, and granulation tissue. The activation ratios were calculated as described in Materials and Methods. The ratios of active MMP-2 to total MMP-2 in 2 granulation tissues were 0.429 and 0.426. The ratio of active MMP-2 to total MMP-2 was significantly higher in HSA (mean \pm SD, 0.22 ± 0.14) than in hemangioma (mean \pm SD, 0.017 ± 0.021). * $P < 0.01$ in Mann-Whitney U test.

Gelatin zymography

A total of 25, 8, and 2 samples of HSA, hemangioma, and granulation tissue, respectively, were subjected to gelatin zymography. Bands of both precursor and active form of MMP-9 (92 and 82 kDa, respectively) and MMP-2 (72, and 62 kDa, respectively) were observed in the gelatinolytic pattern of the standard used in the study. In both HSA and hemangioma cases, the latent and active forms of MMP-2 were found, while the active form of MMP-9 was rarely observed (Fig. 3). The ratios of active MMP-2 to total MMP-2 in 2 granulation tissues were 0.429 and 0.426.

In gelatin zymography, the latent form of MMP-2 was observed in all cases of HSA and hemangioma. In contrast to latent MMP-2, the distinct active form of MMP-2 (62 kDa) was frequently present but some were faint in HSAs, although the distinct active form of MMP-2 was rarely observed in hemangiomas (Fig. 3). A definite gelatinolytic band for pro-MMP9 was detected in both HSA and hemangioma cases; however, the active form of MMP-9 was rarely detected in either HSA or hemangioma cases. In many cases, the MMP-9 activity was inconsistent (Fig. 3). The ratio of the active form to all forms of MMP-2 was significantly higher in HSA

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(mean \pm SD, 0.22 ± 0.14) than in hemangioma (0.017 ± 0.021) ($P<0.01$) (Fig. 4).

Discussion

Canine vascular tumors are classified as hemangioma, lymphangioma, HSA, and lymphangiosarcoma (Hendrick et al., 1998). Although hemangioma and HSA can be distinguished from lymphangioma and lymphangiosarcoma based on the presence of erythrocytes in the lumen (Goldschmidt et al., 2002), in humans, the malignant neoplastic growth of endothelial cells is diagnosed as angiosarcoma, irrespective of the presence of erythrocytes in the lumen. Therefore, the term angiosarcoma currently encompasses cases that are diagnosed as angiosarcoma and lymphangiosarcoma (Neuhauser et al., 2000). Except for the accumulation of erythrocytes in the lumen, canine hemangiosarcoma and human angiosarcoma share very similar histological features.

In the present study, the results were not correlated with the affected organs; thus, the results of this study were considered as common features of malignant ECs independent of the affected organ.

The mechanism of development of HSA has remained unclear because of few studies on HSA. However, inactivation of some tumor suppressor genes is thought to contribute to the carcinogenesis of HSA. In human HSA, a high incidence of p53 mutation is reported (Naka et al., 1997); however, in canine HSA, we could not detect p53 mutation in the promoter region and DNA binding sites (Yonemaru et al., 2007). In contrast, the overexpression of cyclin D1 and upregulation of phosphorylation of the Rb protein that results in a high proliferation of neoplastic cells were reported previously (Yonemaru et al., 2007). Mutations of phosphatase and tensin homolog that result in deletion from chromosome 10 were detected as a common alteration in the tumor suppressor gene in human and canine hemangiosarcoma (Dickerson et al., 2005; Tate et al., 2007).

In addition to genetic alteration, microenvironmental status, for example, growth factors and ECM, plays an important role in the proliferation and/or survival of neoplastic cells. In this study, we focused on the similarity between malignant growth of HSA and active angiogenesis, because the proliferation of HSA is regulated by the autocrine and/or paracrine pathways of VEGF and bFGF (Yonemaru et al., 2006; Itakura et al., 2008), which resembles the process of active angiogenesis. In fact, malignant ECs also exhibit invasive proliferation and form irregular clefts or channels. Thus, their behavior resembles that of active angiogenic ECs. In the first step of angiogenesis, ECs migrate from the original mature blood vessels following the degradation of ECM components, including BM, by MMPs depending on a high concentration of VEGF and bFGF. Subsequently or simultaneously, ECs vigorously

proliferate and form immature vascular tubes. Toward the end stage of angiogenesis, BM is reconstructed, and unnecessary ECs are pruned with a decrease in the level of growth factors (Keshet and Ben-Sasson, 1999). Thus, ECs enter the active phase of angiogenesis as they degrade BM, and active ECs become quiescent after the reconstruction of BM (Iivanainen et al., 2003). In addition, it is well known that interaction between ECs and their surrounding ECM is necessary for a series of events during angiogenesis (Conway et al., 2001; Iivanainen et al., 2003). Although a continuous linear immunoreactivity for laminin and type IV collagen in the BM region was observed in more than 90% of hemangioma cases, only 28.9% and 36.1% of HSA cases, respectively, showed such immunoreactivity. Many HSA cases showed negative or discontinuous immunoreactivities for laminin and type IV collagen. A complete or partial loss of BM may lead to the contact of neoplastic ECs with the interstitial ECM. Angiogenic ECs communicate with the ECM via cellular receptors. Integrins are cellular receptors for ECM proteins expressed by all adhesive cells in multicellular organisms, and integrin-mediated cellular adhesion to the ECM leads to intracellular signaling events that are necessary for cell survival, proliferation, differentiation, and migration (Iivanainen et al., 2003). The ligation of integrin $\alpha v \beta 3$, which mediates the adhesion of cells to vitronectin, fibronectin, von Willebrand factor, osteopontin, tenascin, and thrombospondin (Van der Flier and Sonnenberg, 2001), has been particularly linked to signaling via the NF- κ B survival pathway (Malyankar et al., 2000), and agents that block integrin $\alpha v \beta 3$ binding to the ECM will also promote apoptosis of angiogenic ECs *in vitro* and *in vivo* (Brooks et al., 1994; Brassard et al., 1999). These results suggest that $\alpha v \beta 3$ integrin may be a critical regulator of EC survival. Fosmire et al. (2004) reported the expression of $\alpha v \beta 3$ integrin in canine HSA-derived cell lines, although there is no evidence that HSA cells express $\alpha v \beta 3$ integrin *in vivo*. Neoplastic HSA cells may easily communicate with the surrounding ECM due to lack of BM and receive survival signals via cellular receptors, for example, integrin. In contrast, hemangioma cells are separated from the surrounding ECM by the BM; therefore, they are likely quiescent. In a previous *in vitro* study, ECS were made quiescent by culturing on the BM (Boudreau et al., 1997). In addition, the existence of the BM induces capillary formation of ECs on three-dimensional culture (Myers et al., 2000). Hemangioma cells have very low proliferative activity and form well-differentiated vascular channels; thus, the quiescent characteristic would be caused by the existence of an intact BM.

Interestingly, together with discontinuous positive reaction in the tumor-basement membrane interface, immunoreactivities of both laminin and type IV collagen were detected in the cytoplasm of neoplastic cells in most HSA cases (91.6% and 97.6%, respectively). Meis-

Kindblom et al. reported that laminin was detected focally within the cytoplasm of some neoplastic ECs in more than half of the cases (n = 31); they also observed BM as the external lamina on ultrastructural examination in human HSAs (Meis-Kindblom and Kindblom, 1998). These findings indicate that neoplastic ECs are capable of laminin production. Active ECs in angiogenesis synthesize laminin and type IV collagen (Soini et al., 1994). In granulation tissues, positive staining of laminin and type IV collagen was observed in the BM of matured blood vessels, as well as in the cytoplasm of proliferating ECs. These findings indicate that proliferating ECs in the granulation tissue increase the production of BM proteins for the formation of new blood vessels. It is possible that the aberrant cytoplasmic positive reaction for laminin and type IV collagen observed in HSA cells may be due to the increase in the production of BM proteins following the degradation of the BM. However, there are other possibilities of the cytoplasmic positivity may be resulted from disabilities to excrete BM protein into extracellular or to form BM property.

The upregulation of MMPs was reported in a number of canine tumors (Loukopoulos et al., 2003) and human tumors (Kayano et al., 2004); it is also implicated in the degradation and remodeling of the ECM under physiological conditions and in tumor progression and invasiveness (McDonnell et al., 1999; Nguyen et al., 2001). MMP-2 and MMP-9 are well known for their ability to degrade type IV collagen, a major component in the BM (Murphy and Crabbe, 1995). In the present study, immunohistochemistry revealed that both HSA and hemangioma cells were positive for MMP-2. Since the primary antibody against MMP-2 recognizes both latent and active form of MMP-2, it became essential to confirm whether immunoreactivity was observed for latent or active form of MMP-2. In gelatin zymography, we detected significantly higher activities of active MMP-2 in HSAs than those in hemangiomas. Thus, MMP-2 immunoreactivity observed in HSAs turned out to be active MMP-2. In contrast, activities of active MMP-2 were rarely detected in hemangiomas. Thus, MMP-2 in hemangiomas was considered to be mainly the latent form. Neoplastic ECs in HSA also showed strong positive staining for MT1-MMP compared to those in hemangioma that showed negative or weakly positive staining. In addition, a high staining intensity of MMP-2 was detected on the cell surface of neoplastic cells in both HSAs and hemangiomas. In HSAs, MT1-MMP processes the latent form of MMP-2 into the active form on the surface of cytoplasmic membrane of neoplastic cells (Evans and Itoh, 2007) through the formation of the trimolecular complex pro-MMP/TIMP-2/MT1-MMP. Thus, pro-MMP-2 appears to be activated efficiently by MT1-MMP in HSAs.

MMP-9 has been demonstrated to play an important role in both normal and tumor angiogenesis. However in this study, MMP-9 showed positive immunoreactivity only in infiltrating inflammatory cells but showed no

immunoreactivity in neoplastic cells in malignant or benign endothelial tumors or even in ECs in the granulation tissues. Freitas et al. (1999) reported that positive reactivity for MMP-9 was observed at specific stages of the menstrual cycle. This suggested that neoplastic cells and ECs in neovascularization were possibly in the stage in which they did not synthesize MMP-9. Therefore, in gelatin zymography, the active and latent form of MMP-9 detected in some cases in HSAs and hemangiomas might be originated from infiltrating inflammatory cells. Moreover, the amount of inflammatory cells contained in the tissue may reflect the quantity of both active and latent forms of MMP-9.

MMP-2 and MT1-MMP expression is promoted by VEGF in ECs in vitro (Sounni et al., 2002; Wary, 2003). The production of VEGF and bFGF in HSA was stimulated in an autocrine manner similar to that observed in active angiogenesis in canine HSAs (Yonemaru et al., 2006) and human HSAs (Prymak et al., 1988; Hammer et al., 1991; Clifford et al., 2000; Sorenmo et al., 2000; Amo et al., 2001). It is likely that the upregulation of MMP-2 and MT1-MMP detected in this study were promoted by VEGF, which was produced by the neoplastic cells themselves.

In conclusion, although the significance of cytoplasmic positive for BM protein in HSA was unclear, the neoplastic cells in canine HSA had active MMP-2, possibly activated by MT1-MMP, and discontinuous status of BM might be associated with activity of active MMP-2.

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