

**Pathological Study on Mechanisms of Malignant
Proliferation of Canine Hemangiosarcomas**
(犬血管肉腫の悪性増殖機構に関する病理学的研究)

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General introduction

Endothelial cell (EC)-derived tumors range from benign hemangioma (HA) to aggressive malignant metastatic ones, which respond poorly to current treatments and have a very high mortality rate [1]. Canine hemangiosarcoma (HSA) is among the most challenging and mysterious diseases encountered in veterinary practice. It is an incurable malignant mesenchymal tumor arising from vascular ECs [2] and occurs more frequently in dogs than in any other species, with an incidence between 0.3 and 1.98% [3]. The first reports of canine HSA in the literature are dating to the middle of the nineteenth century [4-7]. It has been reported that there is a breed-associated risk for development of hematopoietic neoplasms, including HSA [8]. Breeds that represented the highest risk included Boxers, Basset hounds, and St. Bernards. Scottish terriers, Bulldogs, Airedales, Weimaraners, golden retrievers, Doberman pinschers, Labrador retrievers, English setters, and Great Danes were among other breeds at risk [9-11].

HSA can develop in any tissue or organ containing vascular structures, although the most frequently reported primary locations in dogs are the spleen (28–63%), right atrium and auricle of the heart (3–50%), skin or subcutaneous tissues (23.9%), and the liver [10, 11]. Visceral HSAs especially splenic ones are much more common than cutaneous HSAs, and they are associated with extremely poorer prognosis [12]. Histologically, the neoplasms are cellular with moderate to extensive areas of hemorrhage and necrosis. The main distinction feature is that neoplastic cells are observed lining irregular vascular spaces (capillaries or sinusoids) filled with blood [13]. They can have capillary, cavernous or solid appearance.

The morphology of neoplastic endothelial cells in animal HSAs is generally heterogeneous. Spindle-shaped cells with round, oval or pleomorphic nuclei, prominent nucleoli and a low nuclear to cytoplasmic ratio, as well as moderate to abundant, basophilic and usually vacuolated cytoplasm can be detected [14]. The malignant cells can be highly pleomorphic solid or poorly differentiated. HSAs can mimic other sarcomas and so immunohistochemistry (IHC) using antibodies specific for the endothelial markers CD31 and von Willebrand factor (vWF) are a useful diagnostic tool [15, 16]. However, canine HSAs are often characterized by a minimal or absent expression of vWF.

The histological appearance and the progression of HSA resemble those seen in human angiosarcoma (AS), Human ASs are uncommon soft tissue sarcomas that can arise in a variety of locations, including liver, spleen, skin, breast, and endocrine organs [17, 18]. The incidence of AS is approximately 2 % of soft-tissue sarcomas, and 5.4 % of cutaneous soft-tissue sarcomas [19]. Much of the published data on human AS are from case series, which causes difficulty to investigate the molecular biology of this rare endothelial malignancy. In a consequence, canine HSA is a useful comparative model for studying the biology of human AS. The prognosis of HSA and AS is very poor because of the low survival rate and frequent local recurrence and distant metastasis. For instance, the overall 5-year survival rate is approximately 43 to 60% with the median survival being 7 months in the case of AS [20]. However, the 1-year survival rate is less than 10% in case of HSA [21].

Although the majority of AS and HSA arise spontaneously, there are few reports of malignant transformation within pre-existing benign vascular lesions. The etiologic and cellular origins of HSA are incompletely understood. Breed predilection remains a consistent

finding, suggesting that heritable traits contribute to this disease [22-24]. It has been proposed that this is directly proportional to the error rates of DNA polymerases, adding an important stochastic component to the risk that can be attributed to heritable factors [25]. Environmental factors also are likely to play a role in the etiology of HSA, although risk factors to which a realistic contribution could be attributed based on exposure and disease have not been identified in pet dogs. Frequent exposure to high levels of ionizing radiation has the ability to promote the development of HSA in experimental dogs [26, 27].

Understanding the cellular origin of HSA has huge merit for both diagnoses for HSA, monitoring response to treatment, and the development of novel therapeutics. The precise cellular origins of HSAs remain incompletely understood. It is classically hypothesized that HSAs originated from transformed endothelial cells; this presumption was based mainly on the histological appearance and the expression of angiogenic factors and their receptors. Angiogenesis, the process of new blood vessel formation from the existing vasculature, is tightly regulated by a complex network of pro and anti-angiogenic growth factors. Angiogenesis is critical for tumor growth and metastasis [28] but it is dysregulated in malignancy, which results in tumor vasculature with abnormal form and function. There is intense interest in the role of angiogenesis in the pathobiology of vascular tumors. Stand on the aforementioned hypothesis, within the last ten years, expression of some angiogenic factors and its receptors have been reported in spontaneous canine hemangiosarcoma using clinical samples. For instance, overexpression of VEGF and its receptors (VEGFR), bFGF, PDGF-BB and its receptor (PDGFR) and their receptors have been reported [29, 30]. Moreover, increased activities of matrix metalloproteinase-2 and the expression of

homeobox proteins have been detected in canine HSA [29, 31, 32]. In addition to the importance of clinical sample on the progression of oncology field, tumor cell lines have made a substantial contribution to cancer research and biomedical discovery. Recently, several HSA cell lines from xenograft tumors have been newly established, they service as a gold standard model to understand the molecular pathophysiology of canine HSA [13, 33, 34]. Despite their vascular origin, even the addition of novel anti-angiogenic drugs has shown a minimal to absent response in HSA. Thus, very little therapeutic progress has been made over the past several decades to increase the progression-free survival or overall patient survival of individuals suffering from this sarcoma, and effective therapeutics against this disease are desperately needed. The majority of HSA rapidly metastasizes due to their vascular cell composition, and unlike many epithelioid sarcomas, HSA generally metastasizes directly to the internal organs via the vascular system. Treatment of HSA involves radiation, surgery, and neoadjuvant and/or adjuvant chemotherapy with doxorubicin. Evasion of apoptosis and metastasis are major enemies for HSA therapy. New treatment modalities are needed to treat cancer, especially ones that can effectively ablate tumors evasion of apoptosis and sustained angiogenesis and metastasis.

The present study aims to put a significant step forward to understand the mechanism of HSA. Moreover, identify new molecular targets for treatment of HSA. Urokinase-type plasminogen activator (uPA)/its receptor (uPAR), serine protease system, play a key role in the degradation of extracellular matrix and basement membranes and intensifying the tumor invasion. Apart from its protease activates, a large body of evidence has shown that the uPA system has a prevalent role in angiogenesis [35]. The urokinase plasminogen activator system

(uPA) has been demonstrated to be required for the movement of cells through a matrix. These observations have been extended to the migration of endothelial cells during the process of angiogenesis, and recent data suggest that the uPA system is central to this process. uPA is a serine protease that is capable of initiating an extracellular cascade of proteolysis that involves the activation of plasminogen and matrix metalloproteases [36]. These proteolytic cascades remodel extracellular matrix (ECM) and basement membrane (BM), allowing for the movement of cells across and through these barriers. In addition, these proteolytic cascades process and the release of various growth and differentiation factors that are sequestered on the cell surface or within the ECM contribute to the evolution of a migratory or invasive cell phenotype. uPA is also able to modulate signaling and cell adhesion through its specific cell surface receptor, uPAR. Recent data suggest that the nonproteolytic activities of the uPA system are coupled to adhesion, migration and signaling through various integrins [37].

The immunohistochemical expression of uPA and its receptor uPAR was investigated in canine splenic, and non-splenic HSA, cutaneous HA, and canine granulation tissues as a model for pathophysiological angiogenesis. Furthermore, the correlation between uPA/uPAR expression and the Ki-67 labelling index was estimated in HSAs and HAs.

Peroxiredoxin- 6 (PRDX6) is a member of the peroxiredoxin family of antioxidant proteins which have been reported to promote cancer development in many types of tumors [38]. As a protein with peroxidase activity, it has been shown to protect cells from oxidative damage, promote cell maintenance and tumor-supportive adaptation in cancerous states, and finally, contributes to carcinogenesis [39]. It has been reported that the expression of PRDX-6

contributes to the invasive and metastatic potential of breast cancer cells by stimulating the signaling pathways involving uPA, and uPAR [40, 41]. Based on the aforementioned reports, the expression of PRDX6 protein was investigated using surgically removed HSAs and HAs samples. Moreover, PRDX6 mRNA and protein were analysed in HSA cell lines compared to NED. Finally, to identify the functions of PRDX6, downregulation of PRDX6, using siR-PRDX6 has been done using HSA cell lines.

Chapter 1

Immunohistochemical detection of urokinase plasminogen activator and urokinase plasminogen activator receptor in canine vascular endothelial tumors

Introduction

Human angiosarcoma (AS) is a rare soft-tissue sarcoma of endothelial cell (EC) origin, which is associated with a poor prognosis [42]. Approximately 2% of soft-tissue sarcomas and 5.4% of cutaneous soft-tissue sarcomas are ASs [43, 44]. Although the incidence of this disease has risen over the past 30 years, this rise may be due to the increased use of radiotherapy during this time [45]. AS predominantly arises in skin, soft tissue, breast, liver, bone, and splenic tissues [46]. Meanwhile, hemangiosarcoma (HSA) is a common malignant neoplasm in dogs [47, 48]. Indeed, HSA occurs more frequently in dogs than in any other species, with an incidence between 0.3 and 2.0% [49]. While the disease can develop in any tissue, the most frequently reported primary sites in dogs are the spleen (28–63%), the right atrium and auricle (3–50%), and the skin or subcutaneous tissues (23.9%) [50, 51]. As with human AS, the prognosis of canine HSA is poor: the mean survival time is less than 6 months, even with surgical and chemotherapeutic interventions [2]. Because much of the published data on human AS were derived from case series, it has been difficult to investigate the pathogenesis of this endothelial malignancy. Therefore, spontaneous canine HSA is a useful model for studying the pathogenesis of human AS, as well as other endothelial cell malignancies.

The serine protease urokinase plasminogen activator (uPA) and the uPA membrane receptor (uPAR; also known as CD87) are involved in many physiological and pathological processes, including the immune response, tissue regeneration, angiogenesis, and the growth and spread of tumours [52]. In particular, there is a large body of evidence indicating that the uPA system plays a prevalent role in angiogenesis [53]. Specifically, uPA controls

extracellular matrix (ECM) degradation via the conversion of plasminogen into plasmin and is regarded as the critical trigger for plasmin generation during cell migration and invasion both in physiological processes, such as angiogenesis and in pathological conditions such as cancer metastasis. The proteolytic activity of uPA is responsible for the activation or release of several growth factors and cytokines and modulates the cell survival/apoptosis ratio through the dynamic control of cell-matrix contacts [54, 55]. In addition to its proteolytic activity, it has been reported that uPAR acts as a multifunctional polyvalent receptor. As uPAR reacts with neighbouring receptors, such as low-density lipoprotein (LDL) receptor-related protein, integrins, epidermal growth factor receptor, or platelet-derived growth factor receptor, it may also affect the proliferation and survival of angiogenic ECs [56].

The development of new blood vessels, termed angiogenesis, is a hallmark of cancer development that has long been considered an attractive therapeutic target [57]. As previous studies suggested similarities between HSA and pathophysiological angiogenesis, we chose to investigate the expression patterns of uPA and its receptor uPAR in canine splenic and non-splenic HSA, cutaneous HA, and canine granulation tissues as a model for pathophysiological angiogenesis. Furthermore, we examined the correlation between uPA/uPAR expression and the Ki-67 labelling index in both HSAs and HAs.

Material and methods

Samples

For this study, formalin-fixed, paraffin embedded blocks from canine splenic and non-splenic HSA, and cutaneous HA tissues were retrieved from an archive of surgical samples located at the Laboratory of Veterinary Pathology, Gifu University, Japan. All samples were collected between January 2012 and December 2014 and were characterised as primary tumours on the basis of clinical examination and surgical findings. The diagnosis for each case was made prior to the initiation of this study via examination of hematoxylin and eosin (H&E)-stained slides, and was confirmed by immunohistochemical analysis of the expression levels of von Willebrand factor (vWF) and CD31 (using a polyclonal rabbit anti-human vWF antibody and a monoclonal mouse anti-human CD31 antibody, respectively; Dako, Glostrup, Denmark).

Primary HSAs from a total of 57 dogs (35 males and 22 females; mean age, 10.5 years; age range, 6–15 years) were examined. The HSA tissues were divided into two groups according to their primary locations: splenic HSAs ($n = 41$) and non-splenic HSAs ($n = 16$). The primary sites of the non-splenic HSAs were the skin ($n = 8$), liver ($n = 3$), intraperitoneal masses of unknown origin ($n = 2$), and kidney, adrenal gland, and testis tissues ($n = 1$ each). In addition, we examined cutaneous HA tissues from 26 dogs (18 males and 8 females; mean age, 9.6 years; age range 6–16 years). Lastly, granulation tissues harvested from cutaneous ulcers of five dogs were evaluated by immunohistochemistry, as these samples are known to contain active angiogenic ECs [58]. The HSAs and HAs analysed in this study were harvested from various breeds of dogs.

Immunohistochemical staining

Serial sections were generated from the archival samples and subjected to immunostaining with goat polyclonal anti-uPA (M-20, sc-6831 Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at a 1: 2,000 dilutions, and a rabbit polyclonal anti-uPAR (Biorbyt, LLC., San Francisco, CA, USA) at a 1:500 dilution. Immunostaining was performed using the dextran polymer conjugated immunoperoxidase method, as previously described [59]. Briefly, consecutive, formalin-fixed, 3- μ m-thick sections were deparaffinized with xylene and then rehydrated via a graded ethanol series before undergoing heat-induced antigen retrieval by incubating with an antigen retrieval solution (Dako) at 121°C for 5 min. Endogenous peroxidase activity was quenched by immersing slides in 0.3% hydrogen peroxide in methanol for 20 min at room temperature (RT), and the tissue sections were blocked by incubating in a protein blocking serum-free reagent (Dako) for 45 min at RT. The sections were then incubated with primary antibodies overnight at 4°C, washed with phosphate-buffered saline (PBS), and incubated with the appropriate secondary antibody ImmPress HRP anti-goat immunoglobulin, peroxidase [Vector Laboratories Inc., Burlingame, CA, USA] for detection of uPA) and (EnVision + System, HRP labelled polymer anti-rabbit (Dako) for detection of uPAR for 30 min at RT. The sections were developed by treating with the Liquid DAB (3,3-diaminobenzidine solution) + Substrate Chromogen System (Dako), and counterstaining with Mayer's hematoxylin (Sakura Finetek Japan, Tokyo.Japan) Canine normal kidney and skin tissues were employed as positive controls, as normal renal tubular cells and epidermal keratinocytes are known to express both uPA and uPAR [60, 61]. Negative controls included sections that were treated with PBS instead of the primary

antibodies. To evaluate the cell growth fraction, immunolabelling of the Ki-67 antigen (clone MIB-1, M7240, Dako, 1:50 dilution) was performed, as described previously [62].

Scoring of immunohistochemistry

Immunoreactivity was evaluated by two independent observers using a light microscope (BH-2 Olympus, Tokyo, Japan). The percentage of neoplastic cells that were positive for uPA and uPAR was determined by analysing 1000 neoplastic cells in 10 high-power fields for each tissue section, representing the various levels of immunoreactivity per slide. Tumours were considered negative when less than 10% of the neoplastic cells were positive for both uPA and uPAR, as described in a previous study [63]. Positive labelling was scored as follows: 10–25% positive cells, 1+; 26–75% positive cells, 2+; and greater than 75% positive cells, 3+ [24]. The Ki-67 LI was determined as the percentage of positive cells in a minimum of 1000 neoplastic cells in 10 high-power fields.

Statistical analyses

The chi-square test was used to compare the proportions of uPA- and uPAR-positive cells in splenic HSAs to those in non-splenic HSAs, the proportions of uPA- and uPAR-positive cells in all HSAs to those in cutaneous HSAs, as well as the correlation between uPA and uPAR expression in HSA and cutaneous HA tissues. Average Ki-67 LI values for uPA+/uPAR+ splenic HSAs, and uPA+/uPAR+ non-splenic HSAs were compared using the Kruskal-Wallis test and the Steel-Dwass method. Likewise, the proportions of uPA+/uPAR+

HSAs, uPA-/uPAR+ HSAs, and HAs were compared using the Kruskal-Wallis test and the Steel-Dwass method. *P*-values < 0.05 were considered statistically significant for all tests.

Results

General histological features of the HSA and HA tissues

Histologically, the HSAs were well circumscribed and were composed of variably sized vascular spaces that were filled with blood and lined by a single layer of uniform ECs (Figure 1A). HSAs were infiltrative and did not exhibit capsules or clear borders to separate the tumour from normal tissue. The morphological features of HSA cells were variable both within and between cases, and ranged from a spindle shape to polygonal to ovoid in shape; areas of growth were recognisable as vascular clefts or channels in the tumor (Figure 1B). In some areas, a cell poor and brightly eosinophilic stroma was observed between the clefts or the channels. Meanwhile, there were also solid areas of neoplastic ECs that were indistinguishable from a fibrosarcoma or other poorly differentiated sarcomas (Figure 1C). Diagnosis of HSAs was confirmed by detection of CD31 and vWF expression in the neoplastic cells.

uPA and uPAR immunostaining

To the best of our knowledge, there have been no analyses of uPA/uPAR expression levels in canine tissues. As a result, we evaluated the expression of these proteins in normal canine dermal tissues and granulation tissues. In the normal cutaneous tissues, the macrophages, mast cells, leiomyocytes, and fibroblasts exhibited varying degrees of uPA expression (Figure 2A), and while uPAR immunoreactivity was also detected in macrophages and fibroblasts, the number of uPAR-positive cells was less than the number of uPA-positive cells. In vascular walls, the leiomyocytes were positive for both uPA and uPAR; however,

the quiescent ECs were negative for both proteins (Figure 2A and 2B). In contrast, positive staining for uPA and uPAR was detected in the cytoplasm of newly formed vascular ECs in granulation tissues, as well as in fibroblasts and inflammatory cells, including macrophages (Figure 2C and 2D).

Neoplastic ECs were positive for both uPA and uPAR and exhibited diffuse cytoplasmic staining (Figure 3A and 3B), with scores ranging from 1+ to 3+. The results of the uPA and uPAR staining, as well as the Ki-67 LIs for the HSAs and cutaneous HAs, are summarised in Table 1. uPAR expression was observed in all splenic and non-splenic HSAs, and in eight cutaneous HAs (30.7%). Furthermore, nuclear uPAR expression was detected in neoplastic ECs in eight HSA tissues (Figure 3C). While 30 splenic HSAs (73.2%) and 11 non-splenic HSAs (75%) were positive for uPA, only one cutaneous HA (3.8%) tissue exhibited uPA expression, albeit with a score of 1+ for uPA and uPAR. Immunoreactivity was detected, not only in neoplastic ECs, but also in the surrounding tumour stromal cells of both the HSA and HA tissues, and the staining pattern was similar to that observed in the granulation tissues.

Statistically, there were no significant differences in uPA and uPAR expression between splenic and non-splenic HSAs. Conversely, the proportion of HSAs that were positive for uPA and uPAR was significantly higher than that of the cutaneous HA samples ($P < 0.05$). Notably, all uPA-positive HSAs were also positive for uPAR. In addition, 15 uPA-negative HSAs (11 Splenic HSAs and 4 non-splenic HSAs) were positive for uPAR. In contrast, there was only one uPA/uPAR-double positive cutaneous HA sample, and 18 HAs (69.2%) were negative for both uPA and uPAR.

Ki-67 immunostaining

The Ki-67 LIs for uPA+/uPA+ splenic and non-splenic HSAs were 36.3 ± 13.9 and 23.9 ± 15.8 (mean \pm SD), respectively. Meanwhile, the Ki-67 LIs for uPA-/uPA+ splenic and non-splenic HSAs were 17.0 ± 6.5 and 10.4 ± 7.6 , respectively. There was no statistical difference between values; however, when the splenic and non-splenic HSAs were combined, the Ki-67 LI of the uPA+/uPAR+ HSAs (32.8 ± 15.3) was significantly higher than those of the uPA-/uPAR+ HSAs (15.2 ± 7.2) and the HAs (2.1 ± 0.7) ($P < 0.05$ for both; Figure 4, and 5).

Discussion

Human AS and canine HSA are deadly neoplasms characterised by the aggressive growth of malignant ECs, widespread metastases, and poor response to chemotherapy [64, 65]. Human AS can arise anywhere in the body, most commonly presenting as cutaneous disease. Although less frequent, the disease can also arise within various organs [66]. In dogs, non-splenic HSAs occur less commonly than splenic HSAs, but are still frequently detected and can affect a wide variety of organs. In contrast, cutaneous HAs are more common than visceral HAs. Indeed, the latter are particularly uncommon in dogs [12]. Therefore, splenic HSAs accounted for the majority of the samples analysed in the current study. However, there were no significant differences in the proportions of uPA- or uPAR-positive cases between the splenic and non-splenic HSAs. As a result, we propose that uPA/uPAR immunoreactivity is not dependent on the primary organ of HSAs.

Morphologically, there is a similarity between the malignant ECs present in HSA tissues and the pericyte-poor, thin-walled ‘mother vessels’ that are formed during angiogenesis [67]. Currently, there is growing evidence that dysregulation of the molecular pathways that control angiogenesis may also be important in the pathogenesis of malignancies of EC origin. For instance, malignant ECs secrete growth factors and cytokines that promote angiogenesis [30, 68-71]. Moreover, a previous study demonstrated that several homeobox proteins (Hox) that regulate the behaviour of ECs during angiogenesis play major roles in the pathogenesis of HSA, and that HoxA9, HoxD3, and pre-B-cell leukaemia homeobox-1 (Pbx1) are involved in the malignancy of canine HSA [72]. Likewise, the anti-apoptotic factors B-cell

lymphoma-2 (Bcl-2) and survivin, which control the pruning phase of angiogenesis, are thought to participate in HSA pathogenesis [73]. Furthermore, matrix metalloproteinase-2 (MMP-2) and membrane type 1-MMP, which facilitate the migration of active ECs during angiogenesis and cancerous cells during metastasis, have been reported to facilitate the pathogenesis of canine HSA [74]. Meanwhile, gene expression profiling indicated that inflammation and angiogenesis are distinct features of canine HSA; thus, it is crucial to investigate the role of the molecules associated with these processes to fully understand canine HSA malignancy. Regardless, the morphological and molecular evidence obtained from these studies support the notion that the characteristics of malignant ECs might be similar to those of active ECs in pathophysiological angiogenesis.

In the present study, we utilised immunohistochemistry to confirm that newly formed vascular ECs express both uPA and uPAR in granulation tissue. In contrast, quiescent ECs within normal dermal tissues were negative for these markers. Thus, the uPA and uPAR system is considered that it plays an important role in pathophysiological angiogenesis. While 76.3% and 75.0% of splenic and non-splenic HSA tissues were uPA+/uPAR+, respectively, there was only one uPA+/uPAR+ cutaneous HA sample, and this tissue was only weakly positive (1+). The average Ki-67 LI value for the HSA samples, which reflects the fraction of cells that are growing, was significantly higher than that for the HAs. Moreover, the uPA+/uPAR+ HSA group had a higher Ki-67 LI than the uPA-/uPAR+ HSA group. Therefore, the uPA system may be linked to the elevated proliferative activity observed in canine HSA. In addition, we detected no significant association between the primary location from which the HSAs were derived

and the uPA/uPAR proliferative activity. This observation is consistent previous findings in breast and urinary bladder neoplasms [75, 76].

Binding of uPA to its receptor promotes uPA activity, which then enhances the activation of plasminogen to plasmin. As a result, the detection of expression of both the ligand and the receptor within individual cancerous cells indicates that these HSA cells are likely capable of regulating plasminogen activation via uPA/uPAR production. In addition, plasmin activation leads to changes in cell signalling via increased availability of active basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), as a consequence of ECM degradation, as well as via the direct activation of latent transforming growth factors. Therefore, the role of plasmin extends beyond ECM cleavage to the control of cell growth and differentiation through the activation or release of growth factors from the ECM [77]. The effects of uPA/uPAR on growth factors and proteolytic activity might enhance the ability of HSA cells to migrate, invade normal tissue, and metastasise.

Notably, uPA and uPAR expression was detected, not only in neoplastic ECs, but also in the surrounding tumour stroma. Similar results have been reported in lung, breast, ovarian, and colorectal cancers [78, 79]. The concentrations of uPA present in the microenvironments formed by neoplastic tissue may contribute to the activation of the uPA/uPAR-pathway, as both uPA and uPAR were expressed in fibroblasts, and macrophages.

It was reported that VEGF-initiated angiogenesis requires both the coordinated proteolytic degradation of ECM, provided by the uPA/uPAR system, and the regulation

of cell migration via integrin-matrix interactions [55]. Meanwhile, a previous study reported an association between high levels of expression of VEGF and VEGF receptors (VEGFR) and enhanced proliferation in canine HSA [70]. These data, therefore, suggest that elevated expression of uPA and uPAR may be linked to the activation of the VEGF pathway. This same group also reported that there was cooperation among tissue PA, uPA, plasminogen activator inhibitor-1, and VEGF throughout the processes of tumour growth and progression to malignant gliomas [80]. Likewise, Zhang and co-workers (2006) reported that uPA and/or uPAR, in conjunction with VEGF, contribute synergistically to tumour invasion and angiogenesis in gastric cancer. Meanwhile, in a human melanoma cell line, inhibition of receptor-bound uPA by uPA-specific antibodies reduced cell proliferation rates, suggesting that cell growth is constantly stimulated in an autocrine fashion by uPAR occupancy [81].

Similar to the cytoplasmic distribution of uPAR expression, nuclear staining of neoplastic cells was detected in eight HSA tissues. Nuclear uPAR immunostaining has also been observed in breast and pancreatic cancer samples [82]. The possibility that uPAR undergoes nuclear translocation was emphasised by a recent study of an important signalling complex which contains uPAR, nucleolin, and casein kinase 2 proteins [83]. Although this complex has only been studied with regard to its role in mutagenesis [84], the average Ki-67 LIs of the nuclear-stained samples were in the same range as those of the cytoplasmic-only HSAs. Therefore, further study is required to clarify the role of the nuclear translocation of uPAR.

Recently, several therapeutic approaches designed to interfere with the expression and reactivity of uPA and/or uPAR at the gene or protein level were examined. These approaches include the use of selective inhibitors of uPA activity, antagonist peptides, monoclonal antibodies that inhibit the binding of uPA to uPAR and gene therapy techniques that silence uPA/uPAR expression [85]. The results of these analyses suggest that the use of specific proteinase inhibitors may represent a novel therapeutic strategy for the inhibition of neoplastic endothelial cell proliferation. In conclusion, while the levels of uPA and uPAR expression were similar in splenic and non-splenic HSAs, the levels of these proteins were significantly higher in canine HSAs than in canine cutaneous HAs. Moreover, the uPA+/uPAR+ HSAs exhibited significantly greater proliferative activity than the uPA-/uPAR+ HSAs. These results suggest that uPA and its receptor uPAR play a significant role in the malignant proliferation of canine HSA cells, regardless of their primary location. Thus, the uPA/uPAR system comprises a potential therapeutic target for canine HSA.

Table 1. Summary of immunohistochemical analyses of canine splenic and non-splenic hemangiosarcomas (HSA) and cutaneous hemangiomas (HA). Tissues were stained with urokinase plasminogen activator (uPA)-, uPA receptor (uPAR)-, and Ki-67 antigen-specific antibodies, and visualised by light microscopy. Table include the scoring values for uPA and uPAR staining as well as the Ki-67 labelling index.

Score [#]	HSA (n=57)				HA (n=26)	
	Splenic (n=41)		Non-splenic (n=16)		uPA	uPAR
	uPA	uPAR	uPA	uPAR		
-	11	0	4	0	25	18
1+	16	8	6	6	1	4
2+	7	15	4	5	0	2
3+	7	18	2	5	0	2
+ cases (%)	73.2 (n=30) *	100 (n=41) *	75.0 (n=12) *	100 (n=16) *	3.8 (n=1)	30.7 (n=8)
uPA/uPAR	Average of Ki-67 Labeling index (%±SD)					
+/+	36.3±13.9 [§]		23.9±15.8 [§]		2.1±0.7	
-/+	17.0±6.5		10.4±7.6			
+/-	0		0			
-/-	0		0			

[#]-, <10%; 1+, 10–25%; 2+, 25–75%; 3+, >75%.

*Significantly higher than that of HA ($P < 0.05$).

[§]Significantly higher than those of uPA-/uPAR+ HSA and HA ($P < 0.05$)

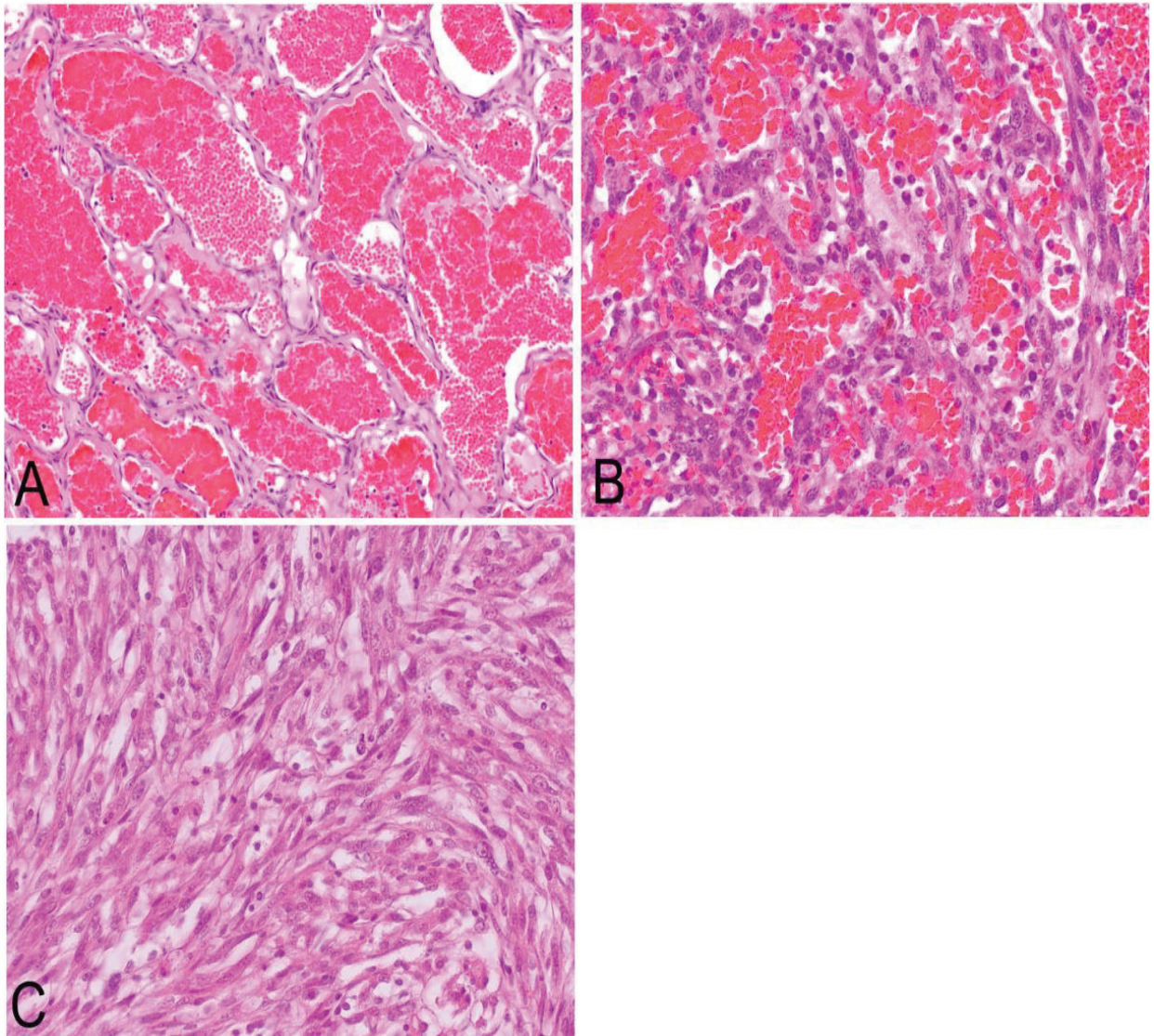


Figure 1. Histopathological features of hemangiosarcoma (HSA) and hemangioma (HA) tissues. Tissue sections were stained with hematoxylin and eosin (H&E) stain. (A) Cavernous HA exhibiting variably sized vascular channels filled with blood and lined by a single layer of uniform flattened ECs (100×). (B) Well differentiated HSA exhibiting polygonal and fusiform neoplastic endothelial cells with areas of vascular clefts and channels (200×). (C) Poorly differentiated HSA with solid areas of neoplastic endothelial cells (ECs) that were indistinguishable from a fibrosarcoma (200×)

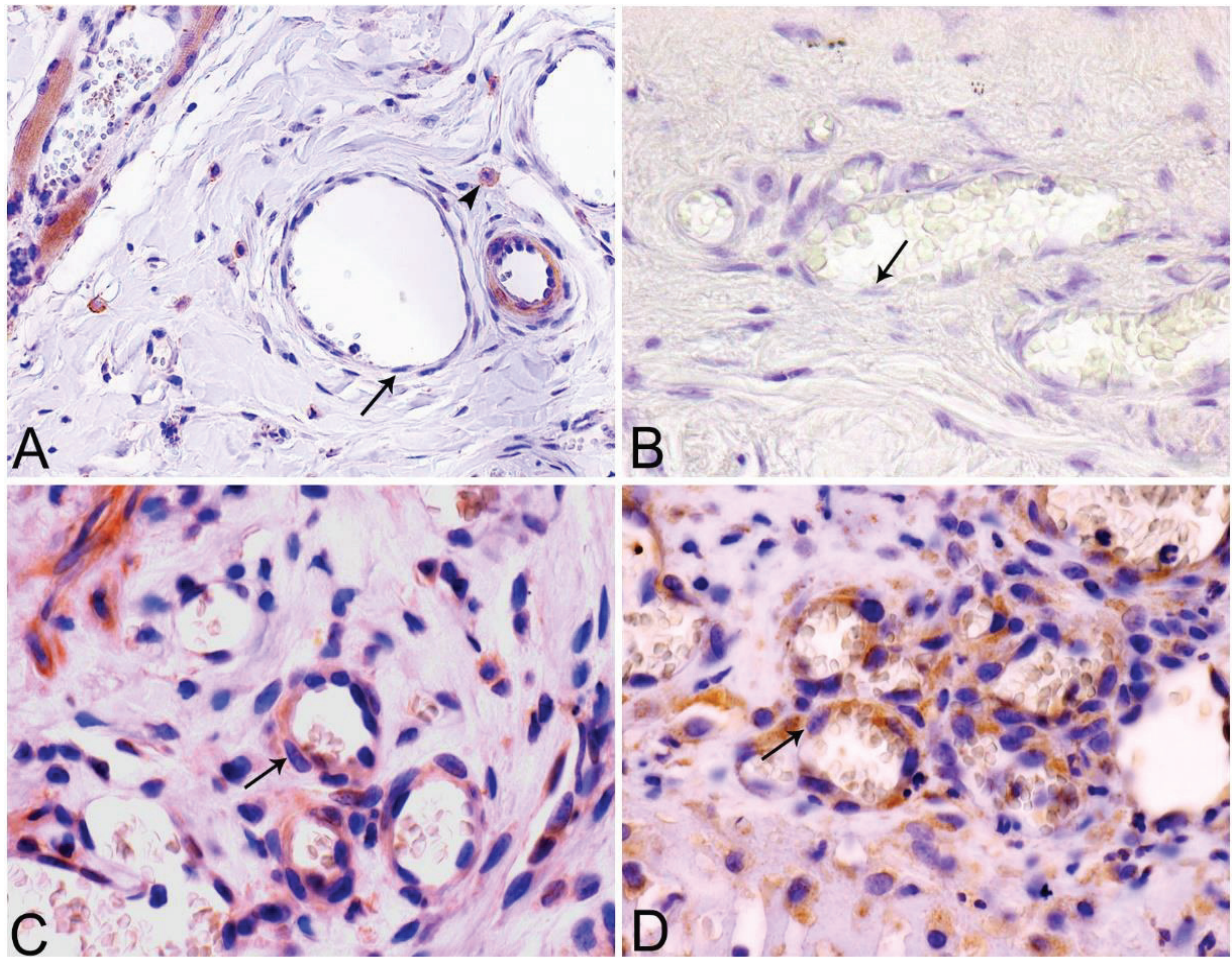


Figure 2. Detection of urokinase plasminogen activator (uPA) and uPA receptor (uPAR) in normal canine tissues and granulation tissue. Normal canine dermal (A and B) and granulation (C and D) tissues were stained using uPA- and uPAR-specific antibodies, and visualised by light microscopy. (A) The indicated mast cell (arrowhead) and the leiomyocytes are uPA positive. Conversely, the endothelial cells (arrow) are uPA-negative (200 \times). (B) Image depicting uPAR-negative endothelial cells (arrow) present in a capillary vessel (400 \times). (C) uPA expression was detected in the cytoplasm of newly-formed vascular endothelial cells (arrow), fibroblasts, and inflammatory cells (200 \times). (D) uPAR staining was detected in the cytoplasm of newly-formed vascular endothelial cells (arrow), fibroblasts, and macrophages (200 \times).

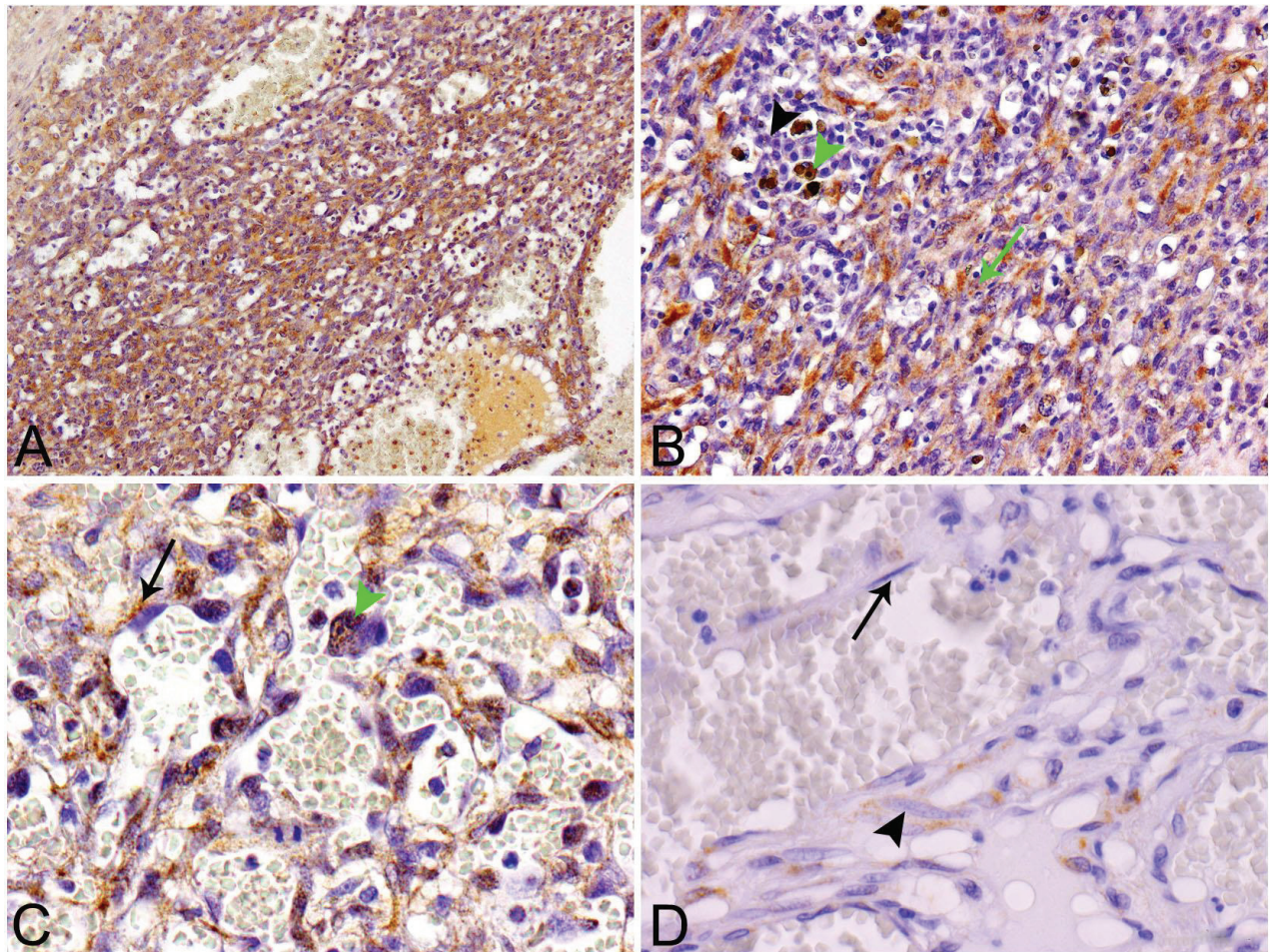


Figure 3. Detection of urokinase plasminogen activator (uPA) and uPA receptor (uPAR) in canine HSA and HA tissues. Canine splenic hemangiosarcoma (HSA) (A, B, and C) and cutaneous hemangioma (HA) (D) tissues were stained using uPA- and uPAR-specific antibodies, and visualised by light microscopy. (A) Image depicting splenic HSA tissue stained with the uPA-specific antibody that was scored as 3+. The cytoplasm of the neoplastic cells is intensely and diffusely positive for uPA (100 \times). (B) Image depicting splenic HSA tissue stained with the uPAR-specific antibody that was scored as 3+. While the neoplastic cells are intensely and diffusely positive for uPAR (arrow), the plasma cells (arrowhead) and hemosiderin-laden macrophages (green arrowhead) are negative for this protein (200 \times). (C) Image depicting canine splenic HSA, scored as 3+ for uPAR. In this tissue, there are high levels of uPAR expression in the nuclei (arrowhead) and cytoplasm (arrows) of neoplastic cells (200 \times). (D) Image depicting canine cutaneous HA tissue. There are a few uPAR-positive fibroblasts (arrowhead); however, the neoplastic endothelial cells (arrow) are negative for both proteins (200 \times).

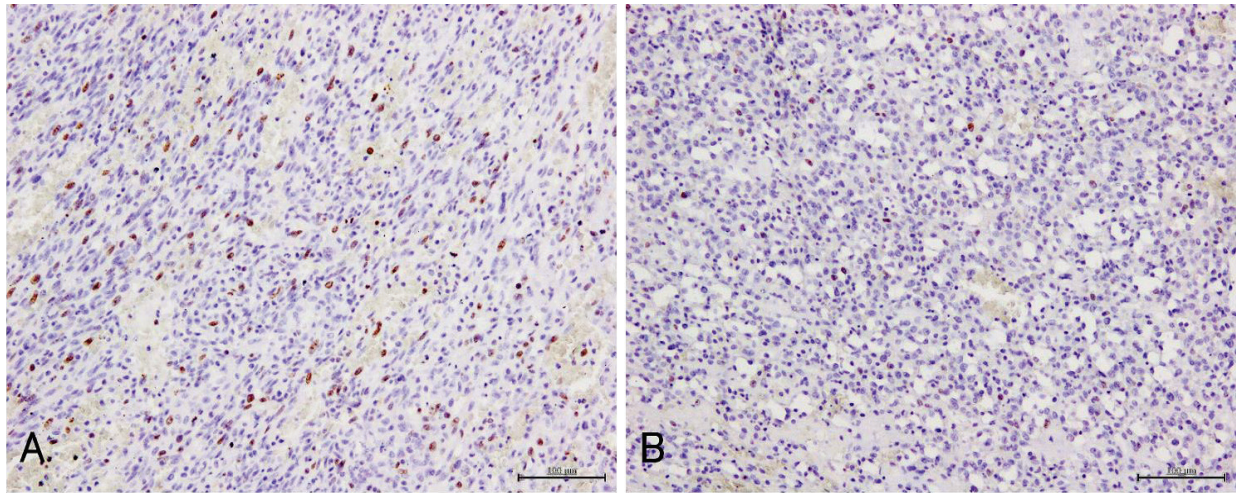


Figure 4. Immunohistochemical analysis of Ki-67 in canine hemangiosarcoma (HSA) and cutaneous hemangioma tissues (HA). Canine splenic HSA (A and B) and cutaneous HA tissues were stained using Ki-67 antigen-specific antibodies and visualised by light microscopy. (A) Image depicting canine splenic uPA+/uPAR+ HSA tissue; the majority of the neoplastic cells are positive for the Ki-67 antigen. The Ki-67 labelling index (LI) value for this tissue is 53.2% (200×). (B) Image depicting canine splenic uPA-/uPAR+ HSA tissue; there are a few neoplastic cells that are positive for the Ki-67 antigen. The Ki-67 LI for this tissue is 15.9% (200×).

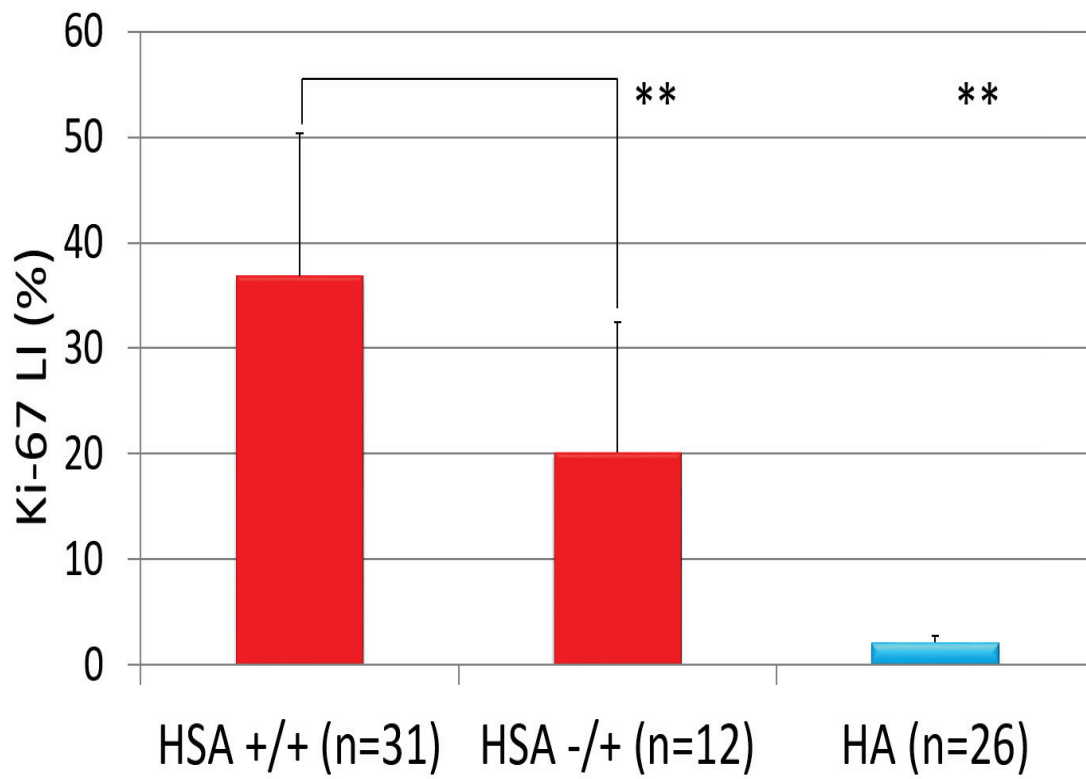


Figure 5. Summary of the average Ki-67 LIs for uPA+/uPAR+ HSAs, uPA-/uPAR+ HSAs, and HAs. Bars indicate standard deviations. *Significantly different ($P < 0.05$) from the uPA-/uPAR+ HSA and HA values (Kruskal-Wallis test and multiple comparison with the Steel-Dwass method).

Abstract

The expression levels of uPA and uPAR in 57 primary canine vascular malignant tumors and in 26 canine cutaneous hemangiomas (HAs) has been evaluated by immunohistochemical analysis. For comparative analysis of active angiogenic endothelial cells, I also stained canine cutaneous granulation tissues. Moreover, the correlation between uPA/uPAR expression and the Ki-67 labelling index (LI) were estimated in both HSA and HA tissues. While the proportion of splenic HSAs and non-splenic HSAs exhibiting positive immunoreactivity for uPA were 73.2% and 75.0%, respectively, all HSA tissues tested were positive for uPAR. These values were significantly higher than those obtained from the cutaneous HA tissues (3.8% and 30.7%, respectively). The average Ki-67 LI of the uPA+/uPAR+ HSAs was significantly higher than that of both the uPA-/uPAR+ HSA and the HA tissues (means \pm SDs: 32.8 ± 15.3 ; 15.2 ± 7.2 ; and 2.1 ± 0.7 , respectively, $P < 0.05$). These results suggest that uPA and uPAR play a significant role in the malignant proliferation of canine HSA, regardless of the primary organ of the tumor.

Chapter 2

Overexpression of peroxiredoxin-6 protects neoplastic cells against apoptosis in canine hemangiosarcoma

Introduction

Malignant endothelial neoplasms such human angiosarcoma (AS) and canine hemangiosarcoma (HSA) are devastating neoplasms. HSA is a malignant neoplasm that originates from vascular endothelial cells [86, 87]. It is widely considered in veterinary medicine as one of the most serious canine neoplastic diseases, because of its aggressive biological behavior and its one-year survival rate of less than 10%. Although surgery remains the first line of treatment for dogs with HSA, adjuvant chemotherapy is often indicated because distant metastasis is present at the time of diagnosis in most cases. However, no effective chemotherapy protocols with satisfactory outcomes are available for canine HSA [86, 88]. The discovery of new therapeutic approaches that are aimed at novel molecular targets is imperative in veterinary medicine.

Evasion of apoptosis is one of the hall marks of cancer [89]. The overwhelming ability of tumor cells to increase in number is influenced not only by un-limited replicative potential but also to a great degree by their ability to evade apoptosis. Apoptosis is the programmed cell death that ensures the removal of old and genetically altered cells that cannot be repaired. So the process is very important for the continued preservation of genetic information in all body cells [90]. Cancer cells acquire the overwhelming ability to evade apoptosis, thereby the genetic mutations necessary for carcinogenic phenotype start accumulating. The cancer cell thus becomes immortalized, this genetically mutated, immortalized cell then divides and reproduces genetically mutated daughter cells, and thus tumorigenesis develops [91, 92].

The mammalian peroxiredoxins (PRDXs) are a highly conserved family of thiol-containing peroxidases. Their major functions are considered to be protecting cells against

reactive oxygen species (ROS) through their peroxidase activity against hydrogen peroxide, peroxynitrite, and phospholipid hydroperoxides that reduces peroxides using conserved cysteine residues in the catalytic centre [93]. Six members (PRDX1-PRDX6) of this family have been identified in mammalian tissues. These proteins contain either one (1-Cys PRDX) or two (2-Cys PRDX) redox-active cysteine residues. The 2-Cys group includes PRDX1-PRDX5, whereas PRDX6 is the sole member of the 1-Cys group [94]. Despite this difference, the peroxidase activities of both 1-Cys and 2-Cys groups commonly contribute to cellular protection against oxidative stress [95].

PRDX6 in particular is a cytoprotective, bifunctional enzyme with both glutathione peroxidase and phospholipase A2 activities [96]. However, the functions of PRDX6 are complex and little is known regarding its role in human cancer [97], although current theories propose that it is involved in tumour cell protection, proliferation, and metastasis. Recently, it has been reported that PRDX6 expression is upregulated in a variety of human cancers, consequently providing resistance to radiation and chemotherapeutics [98]. To date, little data is available concerning the expression and functions of PRDX6 in angiogenesis and in endothelial cell malignancies. The aim of the current work was to investigate the expression and function of PRDX6 in canine spontaneous and cell-cultured HSAs.

Materials and Methods

Immunohistochemical Expression of PRDX6 Protein in Spontaneous HSAs and Hemangiomas (HAs)

Samples for immunohistochemistry

Routinely processed and diagnosed HSAs and HAs were selected randomly from dogs undergoing surgery between January 2012 and November 2014 and stored in the archive of surgical samples located at the Laboratory of Veterinary Pathology, Gifu University, Japan. All tumors were considered to be primary tumours on the basis of clinical examination and surgical findings. The diagnosis for each case was made previously via examination of the hematoxylin and eosin (H&E)-stained slides and confirmed by immunohistochemistry for the expression of von Willebrand factor and CD31 using a polyclonal rabbit anti-human von Willebrand factor antibody and a monoclonal mouse anti-human CD31 antibody, respectively (Dako, Glostrup, Denmark) [70]. Two experienced observers reviewed the H&E stained slides, after which one appropriate paraffin block for each sample was selected for this study.

Primary HSAs from a total of 54 dogs (34 males and 20 females; mean age, 10.5 years; age range, 6–15 years) were examined. The HSA tissues were divided into two groups according to their primary locations: splenic HSAs (n = 39) and non-splenic HSAs (n = 15). The primary sites of the non-splenic HSAs were the skin (n = 8), liver (n = 3), intra-peritoneal masses of unknown origin (n = 2), and the kidney and adrenal gland (n = 1 each). In addition, cutaneous HAs from 29 dogs (23 males and 16 females; mean age, 9.6 years; age range 6–

16 years) were examined. The expression of PRDX6 in granulation tissues from coetaneous ulcers in five dogs was also evaluated by immunohistochemistry, as these samples contained active angiogenic endothelial cells. The HSAs/HAs were obtained from various breeds of dogs.

Immunohistochemical staining

Serial sections from the archival samples were used for immunostaining with anti-PRDX6 (rabbit polyclonal, 1:2000 dilution; ab59543; Abcam, Cambridge, UK). Immunostaining was performed using the dextran-polymer conjugated immunoperoxidase method as previously described [59]. Briefly, consecutive, formalin-fixed, 3 μ m sections were initially deparaffinised in xylene and re-hydrated in a descending ethanol series (100%, 90%, 80%, and 70% ethanol). Heat-induced antigen retrieval was performed in an antigen retrieval solution (Dako) at 105°C for 3 min. Endogenous peroxidase activity was blocked by immersing slides in 0.3% hydrogen peroxide in methanol for 20 min at room temperature (RT). Tissue sections were incubated with protein blocking serum-free reagent (Dako) for 45 min to block nonspecific binding, followed by incubation with primary antibodies overnight at 4°C. Next, the sections were washed with phosphate-buffered saline (PBS) and the slides were sequentially incubated with an appropriate secondary antibody [EnVision + System, HRP labelled polymer anti-rabbit (Dako)] for 30 min at RT. The sections were visualised by developing with 3, 3'-diaminobenzidine as the chromogen (Liquid DAB + Substrate Chromogen System, Dako). Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Canine normal skin, kidney, and liver were employed as positive controls in the current study, as normal keratinocytes, renal tubular

cells, and hepatocytes are known to be positive for PRDX6[99, 100]. Negative controls were performed by substituting PBS for the primary antibodies.

Semi-quantitative evaluation and scoring of PRDX6 immunohistochemical expression

Both the scoring and interpretation of the immunostaining results were determined by two independent, blinded investigators. The percentage of neoplastic cells positive for PRDX6 was determined by analysing 1000 cells (10×400 magnification) obtained from at least five microscopic fields representing the various levels of immunopositivity per each examined slide. The percentage of positive immunostained cells was graded and scored according to Huang *et al.* [101] as follows: 0 = no stained cells; 1+ = 1–25% positive cells; 2+ = 26–50% positive cells; 3+ = 51–75% positive cells; and 4+ = more than 75% positive cells.

Cell Lines and Culture Conditions

The HSA cell lines used (Ud2, Re21, and JuA1 and JuB2) originated from splenic, atrial, and hepatic HSA, respectively [102]. HSA cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM, Wako Pure Chemical Ind., Osaka, Japan) supplemented with 10% foetal bovine serum (FBS, Hyclone, South Logan, UT, USA). Canine aortic endothelial cells (NED), used as normal canine primary endothelial cells, were purchased from Cell Applications Inc. (San Diego, CA, USA) and cultured in canine endothelial cell basal medium with growth supplement (Cell Applications Inc.) according to manufacturer instruction.

Total Protein Extraction and Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE) & Western Blotting Analysis

Cells were detached with Trypsin-EDTA solution (0.05 w/v% Trypsin-0.53 mmol/l EDTA • 4Na solution with Phenol Red, Wako), harvested in cold PBS, pelleted, and washed twice in cold PBS. Total cell protein extracts were prepared by resuspending the pellet in RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA) with a protease inhibitor mixture (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific), which was then briefly sonicated. The protein concentration was quantified in triplicate using a DC protein assay (BioRad, Hercules, CA, USA). For each sample, 20 µg protein was resolved using 4–20% SDS-PAGE (BioRad), and electrotransferred onto a Hybond-ECL membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked overnight at 4°C in bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Membranes were then washed twice with TBS and incubated with primary anti-PRDX6 antibody (rabbit polyclonal, 1:2500 dilution; ab59543; Abcam) in TBS-T for 1 h. After 3 washes in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:5000, Amersham Biosciences) for 1 h. Blots were washed 4 times with TBS-T, followed by 2 washes in TBS and the antigen-antibody complexes were visualized using Western Lightning chemiluminescence (Amersham ECL prime western Blot Detection reagents, Amersham Biosciences) and were scanned by a C-Digit Blot Scanner (LI-COR, Lincoln, NE, USA).

RNA Extraction and Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) for PRDX6 Expression

Total RNA was extracted from subconfluent cells grown in DMEM containing 10% FBS using miRCURY™ RNA Isolation Kits - Cell & Plant (Exiqon, Vedbaek, Denmark) according to manufacturer protocol. RNA concentration and purity were assessed by UV spectrophotometry (NanoDrop lite, Thermo Fischer Scientific). cDNA was generated using PrimeScript™ RT Master Mix (TaKaRa Bio, Ohtsu, Japan) and semi-quantitative real time PCR was performed with a Real Time System Single (TaKaRa Bio). Primers for canine *PRDX6* (forward 5'-TGG CAC CAG AAT TTG CCA AG-3', reverse 5'-TTG CTC CAG GCA AGA TGG TC-3', and for canine ribosomal protein S18 (forward 5'-ATA GCC TTT GCC ATC ACA GCA ATT A-3', reverse 5'-TTG GTG AGA TCG ATG TCT GCT TTC-3') were designed based on GenBank accession Nos. XM_537190.4 and NM_001048082.1, respectively and were provided by TaKaRa Bio. Semi-quantitative real time PCR was performed in triplicate in 12.5 µl reactions containing SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio), 0.4 µmol primers, and 50 ng cDNA as a template. After initial heat activation at 95°C for 30 s, the cycling conditions were as follows: 40 cycles of 95°C for 5 s and 60°C for 30 s. For confirmation of specific amplification, a single peak in the dissociation curve was detected in each reaction. Relative gene expression was calculated by using the 2- Δ Ct method [103, 104]. Each experiment was repeated three times independently.

PRDX6 Knockdown using Small-interfering RNA (siRNA)

All HSA cell lines utilized for siRNA transfection were seeded in 96-well plates at the concentration of 0.5×10^5 per well on the day before the transfection. siRNAs for canine

PRDX6 (siR-PRDX6, sense: 5'- GAA UGU UAA GAU GAU UGC CCU UUC A -3', antisense: 5'- UGA AAG GGC AAU CAU CUU AAC AUU C-3', 1 or 10 nmol/l; Stealth RNAi, Thermo Fischer Scientific), or 10 nmol/l non-specific scramble control siRNA (control siRNA, sense: 5'-GAA UUA AAG GUU AGU CCU CUG UUC A-3', antisense: 5'-UGA ACA GAG GAC UAA CCU UUA AUU C-3', Stealth RNAi) were used for transfection of cells with Lipofectamine RNAi-MAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For western blotting of PRDX6 in knock-down and control cells, cells were transfected with 1 or 10 nmol/l siR-PRDX6, or 10 nmol/l control siRNA, and total protein from each culture was extracted at 48 h after the transfection.

For the evaluation of apoptosis induction, detection of nuclear fragmentation, and Annexin V and propidium iodide (PI) double staining in knock-down and control cells (below), the cells were transfected with 10 nmol/l siR-PRDX6 or 10 nmol/l control siRNA and each experiment was performed at 48 or 72 h after the transfection. Each experiment was repeated three times independently.

Cell Viability Assays

For the cell viability assay, cells transfected with PRDX6 siRNA were incubated for 48 and 72 h after the transfection in 96-well plates in quintuplicate. Viable cell numbers were determined using the WST-1 assay according to the manufacturer's instructions (Premix WST-1 Cell Proliferation Assay System, TaKaRa Bio). The absorbance was measured at 490 nm with an iMark Microplate Absorbance Reader (Bio-Rad) having a reference filter of 630 nm. The values were calibrated using blank wells. Each experiment was repeated three times

independently.

DAPI Nuclear Staining

Nuclear fragmentation was assessed by DAPI staining. In brief, after transfection with siRNAs, the cells were fixed with 4% buffered paraformaldehyde solution (Wako), and incubated with 5 µg/ml 4',6-diamidino-2-phenylindole (DAPI; *Cellstain*, Dojin Chemical, Kumamoto, Japan) at 37°C for 30 min. After the cells had been washed with cold PBS twice, nuclear morphology was visualized using a fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan). The percentage of apoptotic cells was calculated by counting the number of cells with fragmented nuclei among a total of 500 cells.

Annexin V and Propidium Iodide(PI) Double Staining

Translocation of phosphatidylserine in the plasma membrane was measured using Annexin V Alexa Fluor 488 and PI double staining (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Thermo Fischer Scientific) according to the manufacturer's procedure. The percentage of apoptotic cells was calculated by counting the number of Annex V positive/PI negative cells among a total of 500 cells.

Statistical Analysis

The difference of the positive ratio of PRDX6 immunohistochemical staining among splenic HSAs, non-splenic HSAs, and HAs was analysed using the chi squared test. The quantitative data were analysed by unpaired two-tailed t-tests or one-way analysis of variance

(ANOVA). When the P value in the ANOVA test indicated statistical significance, the differences were assessed using Dunnet's test. A value of $P < 0.05$ was considered significant for all tests. All analyses were performed using Excel Statics 2008 (SSRI, Tokyo, Japan)

Results

Immunohistochemical Expression of PRDX6 Protein in Spontaneous HSAs and HAs

Immunohistochemical results showed that PRDX6 was localized in the cytoplasm and nucleus of spontaneous HSA and HA cells with varying staining intensity. Positive staining for PRDX6 was detected in the cytoplasm of newly formed vascular endothelial cells in granulation tissues, as well as in fibroblasts and macrophages (Fig. 6) although the quiescent normal splenic and cutaneous endothelial cells were negative. Neoplastic endothelial cells were positive for PRDX6 and exhibited nuclear and granular cytoplasmic staining (Fig. 7A-C), with scores ranging from (1+ to 4+). The results of PRDX6 labelling for HSAs and cutaneous HAs are summarised in Table 1. PRDX6 expression was observed in all splenic and non-splenic HSAs (100%). Immunoreactivity was detected not only in neoplastic endothelial cells but also in the surrounding tumour stromal cells such as fibroblasts and macrophages of both the HSA and HA tissues (Fig. 7D). However, negative immunostaining associated with the anti-PRDX6 antibody was found in the endothelial cells of all cutaneous HAs (Fig. 7D).

Statistically, no significant differences in PRDX6 expression were detected between splenic ($59.8 \pm 15.3\%$) and non-splenic ($57.8 \pm 19.8\%$) HSAs. Conversely, the proportion of HSAs that were positive for PRDX6 was significantly higher than that of the cutaneous HA samples ($P < 0.001$). Furthermore, considering that the 3+, and 4+ positive-scored HSAs are considered strongly positive whereas the 1+ and 2+ positive-scored HSAs are low to

moderately positive, marked nuclear localization of PRDX6 was detected in splenic and non-splenic HSAs (PI: $42.6 \pm 13.8\%$ and $39.7 \pm 15.9\%$, respectively).

Expression of PRDX6 Protein and mRNA in HSA Cell Lines

The expression of PRDX6 at the protein level was characterized in HSA cell lines and in canine NED. Western blot analysis revealed high expression of PRDX6 corresponding to 27 kD in all HSA cell lines albeit with some variety in the expression. Overall, however, the HSA cell lines were found to have high levels of PRDX6 expression compared with the NED samples (Fig. 8).

Quantification of the differential expression of PRDX6 between HSA cell lines and canine NED by real time-qRT-PCR showed that the relative expression measurements of the *PRDX6* gene exhibited skewed distribution and the results for HSA cell lines were significantly higher compared to those for canine NED cells ($P < 0.01$). The levels of *PRDX6* mRNA expression detected by qRT-PCR was entirely consistent with the data from the western blot (Fig. 8).

Effects of PRDX6 Knockdown in HSA Cells

The results of the mRNA expression levels of *PRDX6* in the siR-PRDX6-transfected or control siRNA-transfected groups at 48 h after transfection measured by real-time qRT-PCR analysis are shown in Fig. 9A. In all HSA cell lines, the *PRDX6* mRNA expression level in the siR-PRDX6-transfected group dramatically decreased ($<10\%$). Furthermore, the relative amount of PRDX6 protein at 48 h after siR-PRDX6-transfection obviously decreased and the

knockdown-effect continued for 72 h after transfection. The regulatory effects of PRDX6-siRNA on PRDX6 protein expression were determined by Western blot at different time intervals after transfection. The results showed that PRDX6-siRNA transfected cells expressed significantly less PRDX6 protein than CONT-siRNA treated ones Fig. 9B. Densitometric analyses also confirmed that PRDX6 expression in post-transfected cells was effectively inhibited by PRDX6-siRNA at protein levels after 48 hours; the inhibition was dramatically increased at 72 hours. The inhibitory effect of PRDX6-siRNA was shown to be specific because siRNA oligos did not cause a nonspecific downregulation of gene expression as demonstrated by the β -actin control.

The cell viabilities of HSA cell lines after siR-PRDX6 transfection are summarised in Fig. 10. In all HSA cell lines at 48 or 72 h after transfection, the cell viabilities of the siR-PRDX6-transfected groups were significantly lower than those of the control siRNA-transfected groups.

DAPI nuclear staining showed that nuclear fragmentation in the siR-PRDX6 transfected groups for all HSA cell lines was significantly increased compared to that of the control siRNA-transfected group at 48 and 72 h after transfection (Fig. 11, and 12). Annexin V positive/ PI double staining showed that Annexin V positive/ PI negative cells, which were regarded as early phase-apoptotic cells, in the siR-PRDX6 transfected groups of all HSA cell lines were significantly increased compared to those of the control siRNA-transfected group at 48 and 72 h after transfection (Fig.13, and 14).

Discussion

Human AS and canine HSA are lethal neoplasms characterized by the aggressive growth of malignant endothelial cells, widespread metastases, and poor response to chemotherapy [64, 65]. Human AS can arise anywhere in the body but most commonly presents as a cutaneous disease [105]. Although less frequent, the disease can also arise within various organs. In dogs, non-splenic HSAs occur less commonly than splenic HSAs, but are still frequently detected and can affect a wide variety of organs. In contrast, cutaneous HAs are more common than visceral HAs, with the latter being particularly uncommon in dogs [12]. Therefore, splenic HSAs accounted for the majority of the samples analysed in the current study.

Cancer cells possess many properties that are characteristic of oxidative stress including increased levels of highly effective ROS such as hydrogen peroxide and superoxide. Elevated ROS levels have been implicated in both cancer initiation, through the induction of DNA damage, as well as cancer promotion, likely through aberrant redox signalling [106]. Whereas high ROS levels are cytotoxic to normal cells, cancer cells are able to tolerate increased oxidative stress and evade cell death. This ability may involve an increased activity of cellular antioxidants, limiting ROS to nonlethal levels, and/or the inactivation of apoptotic pathways. Regardless of the precise mechanism, it is clear that the oxidant/antioxidant balance in cancer cells is shifted to allow increased proliferation and cell survival [107].

In the present study, we investigated the expression of the antioxidant protein PRDX6 in canine HSAs. In these tissues, PRDX6 was highly expressed in nucleus and in the cytoplasm; the proportion of strong PRDX6 immunostaining was 72.2%, with 27.8% of cells showing

moderate and low staining, whereas the normal endothelial cells and HAs showed negative PRDX6 expression. These results are in accordance with those reported by Kinnula *et al.* [108], which found that PRDX6 was overexpressed in both the nucleus and cytoplasm in malignant mesothelioma. In pilocytic astrocytomas, PRDX6 expression was strong in 45% of cases, moderate in 37%, and weak in 15% [109]. The difference in the percentage of strong, moderate and weak positive between these studies is likely due to the differences in cell types. There were no significant differences in the proportions of PRDX6-positive cases between the splenic and non-splenic HSAs in our study. Thus, we propose that PRDX6 immunoreactivity is not dependent on the primary organ of HSAs. Furthermore, PRDX6 mRNA and protein were overexpressed in HSA cell lines compared to NED despite some variation in the expression between the different HSAs cell lines was observed. These results are consistent with some other previous studies that showed upregulation of PRDX6 in malignant tumors. For example, Karihtala *et al.* [108] found that PRDXs, including PRDX6, were overexpressed in breast cancer. Quan *et al.* [110] also demonstrated that enhanced PRDX6 expression was strongly associated with human bladder cancer development. In addition, overexpression of PRDX6 was found in human malignant mesothelioma of the lung and in human cervical squamous cell carcinoma [108, 111].

One possible explanation for the increased PRDX6 levels in neoplastic tissues might be that the PRDX family of peroxidases provides critical defences against oxidative stress through scavenging hydrogen peroxide and thus protects cells from oxidative damage. Therefore, the abundance of PRDX is normally associated with the attenuation of oxidative stress and an increased rate of cell survival under various stress conditions. Owing to an

essential secondary messenger function of hydrogen peroxide, PRDXs are also considered as receptors for cellular hydrogen peroxide and thus play multiple roles in many physiological as well as pathological processes [112].

In particular, in the presence of hydrogen peroxide or other oxidative stress, activated nuclear factor-kappaB (NF- κ B) upregulates death effector genes such as p53 leading to hydrogen peroxide-evoked apoptosis. Through its function as a regulatory factor for the transcription factor NF- κ B, PRDX 6 may prevent hydrogen peroxide-induced NF- κ B activation by reducing hydrogen peroxide. It is thus probable that PRDX6 is able to inhibit ROS-mediated physiological apoptosis in normal cells, resulting in abnormal proliferation and thereby leading to tumorigenesis. The evasion of apoptosis is one of the hallmarks of cancers that promote tumour formation and progression as well as treatment resistance. The latter is likely observed as most current anticancer therapies including chemotherapy as well as radio- and immunotherapies primarily act by activating cell death pathways including apoptosis in cancer cells. Notably, nuclear localization of PRDX6 was markedly detected in HSAs. Hansen et al. [113] have reported that unlike cytoplasmic PRDX1, which inhibited the oxidant-induced activation of NF- κ B and did not protect against oxidant-induced cell death, nuclear PRDX1 was protective as nuclear PRDX1 regulates NF- κ B/DNA binding through elimination of hydrogen peroxide as a p50 subunit oxidant. Therefore, we suggest that the more predominant the nuclear localization of PRDX6, the more resistance the cell exhibits to oxidative stress.

As PRDX6 was shown in this study to be a potent inhibitor of apoptosis, it therefore likely functions upstream of Bcl-2. Accordingly, our siRNA experiments demonstrated an

increased rate of apoptotic cells in HSA cell lines upon downregulation of PRDX6 that was likely due to a decreased capability of the cells to reduce hydrogen peroxide below a critical level. The detection by two different methods of the induction of apoptosis following RNA interference illustrated significant effects in the HSA cell lines. For example, in the siRNA-treated HSA cell line, we found that PRDX6 inhibition had a significant effect on HSA cells viability as determined by the significant changes in the percentage of annexin V-positive cells following PRDX6 knockdown. Furthermore, the visualization of nuclear fragmentation by DAPI staining revealed that the nuclear fragmentation in the siR-PRDX6 transfected groups of all HSA cell lines was significantly increased compared to that of the control siRNA-transfected group. Consistent with our findings, previous transient transfection of Hepa1-6 cells with PRDX6 siRNA was shown to lead to a marked reduction in its expression and an increase in peroxide-induced cytotoxicity mediated by apoptosis; these results suggested that PRDX6 up-regulation might be a tumour-supportive adaptation in cancerous states [39].

In addition to its proposed role in apoptosis, PRDX6 appears to also be involved in regulating the invasive and metastatic potential of breast cancer by upregulating the expression of Urokinase-type plasminogen activator (uPA) receptor, Ets-1, Matrix metalloproteinase (MMP)-9, RhoC, and Tissue inhibitor of metalloproteinase-2[40]. Furthermore, PRDX6 activates the cellular invasiveness and metastasis of lung cancer cells by stimulating the signalling pathway involving p38 kinase, phosphoinositide 3-kinase, Akt, and uPA [41]. Notably, overexpression of uPA, uPAR, and MMPs has been reported in canine HSAs [74, 114]. We therefore suggest that PRDX6 might be associated with the

invasive and metastatic capacity of HSAs by stimulating the upregulation of uPA, uPA receptor, and MMPs. Moreover, PRDX6 appears to also be involved in the regulation of Hif-1 α . It has been reported that stable transfection of thioredoxin significantly increases hypoxia-inducible factor-1 α (Hif-1 α) protein levels as well as the protein products of hypoxia-responsive genes such as vascular endothelial growth factor under both normoxic and hypoxic conditions [115]. This indicates a link between PRDX6 and Hif-1 α expression that might be of interest, since Hif-1 α activates the transcription of genes involved in crucial steps of carcinogenesis including angiogenesis, cell survival, glucose metabolism, and invasion. In conclusion, in this study, we confirmed the overexpression of PRDX6 in canine HSAs and examined the levels of expression in a variety of HSA and NED. We also found that knockdown of PRDX6 using siRNA reduced tumour cell proliferation and caused cell apoptosis. We revealed that the antioxidant PRDX6 protects HSA cells against apoptosis, suggesting that an important mechanism of chemoresistance might involve ROS scavenging and antioxidant enzyme activity. Because cancer resistance to chemotherapies and radiation represents a considerable obstacle to the effective treatment of numerous malignancies including endothelial cell malignancies, PRDX6 expression levels might be a good predictor of tumour response, especially to oxidative stress-producing therapies. Furthermore, the manipulation of PRDX6 expression or inhibition of its ROS scavenging ability might provide a new paradigm for improved cancer treatment.

Abstract

In this chapter the expression levels of PRDX6 in spontaneous primary canine HSAs and HAs was investigated by immunohistochemical analysis, identifying marked significant expression of this protein in canine HSAs than HAs. Furthermore, both PRDX6 mRNA and protein were over expressed in HSA cell lines compared to normal canine endothelial cells, although some non- significant variation was observed between the different HSA cell lines. Notably, the small interfering RNA-induced downregulation of PRDX6 in HSA cell lines promoted apoptosis in the HSA cell lines. It means PRDX6 suppression increased the cytotoxicity of these cells suggests that PRDX6 might play an important cyto-protective role. PRDX6 expression levels might be a good predictor of tumour response, especially to oxidative stress-producing therapies. Furthermore, the manipulation of PRDX6 expression or inhibition of its ROS scavenging ability might provide a new paradigm for improved cancer treatment.

Table 2: Summary of the immunohistochemical results of canine splenic and non-splenic hemangiosarcomas (HSAs) and cutaneous hemangiomas (HAs).

Score ^s	HSA (n = 54)		HA (n = 29)
	spleen (n = 39)	non-spleen (n = 15)	
0	0	0	29
1	1	2	0
2	10	2	0
3	24	8	0
4	4	3	0
+ cases (%)	100 (n = 39) [#]	100 (n = 15) [#]	0 (n = 0)
Positive index (% ± SD)	59.8 ± 15.3	57.8 ± 19.8	0
Nuclear Positive Index (% ± SD)	42.6 ± 13.8	39.7 ± 15.9	0

^s: 0, <10%; 1+, 10–25%; 2+, 26–50%; 3+, 51–75%; 4+, >75%.

Average of PRDX6 positive index (% ± SD)

[#]Significantly higher than that of HA ($P < 0.05$).

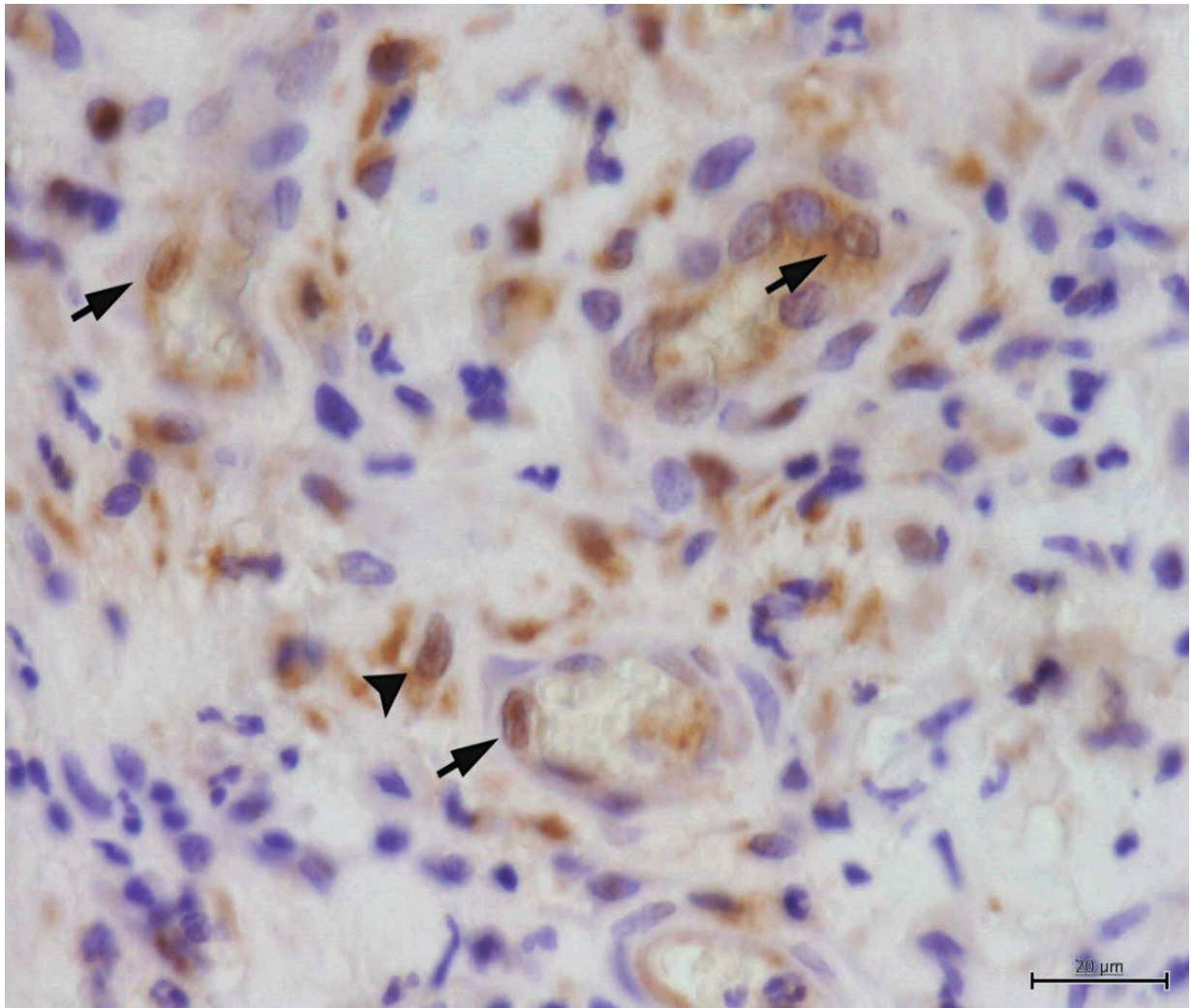


Figure 6. Immunohistochemical detection of PRDX6 in normal canine granulation tissue.

PRDX6 staining was detected in the cytoplasm and nuclei of newly-formed vascular endothelial cells (arrows), fibroblasts, and macrophages (arrowhead). Bar, 20 μ m.

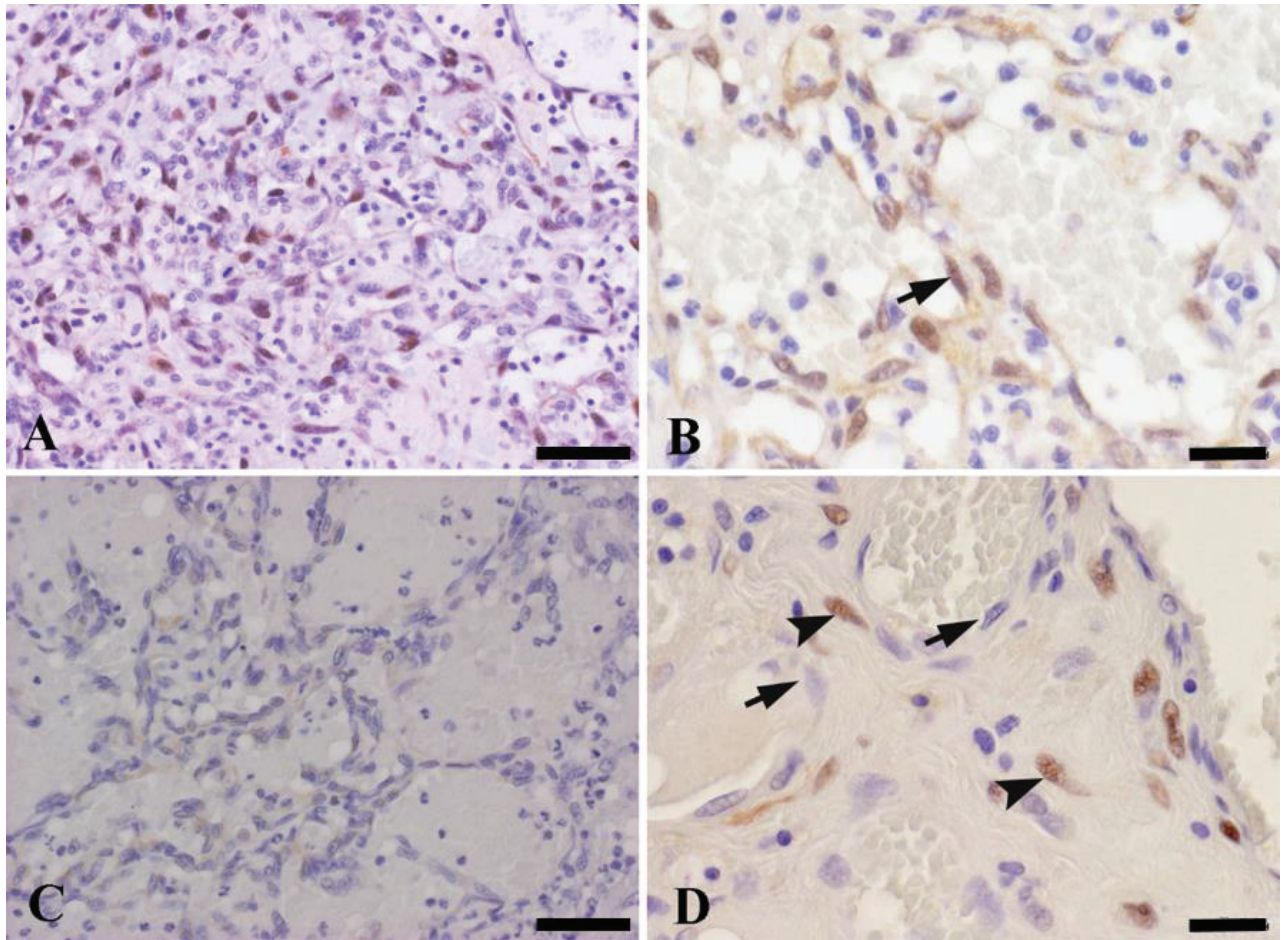


Figure 7. Immunohistochemical detection of PRDX6 in canine HSA and HA tissues. Caninesplenic haemangiosarcoma (HSA) (A, B, and C) and cutaneous haemangioma (HA) (D) tissues were stained using PRDX6-specific antibodies and visualised by light microscopy. (A) Image depicting splenic HSA tissue stained with the PRDX6-specific antibody that was scored as 3+. (B) Image depicting splenic HSA tissue stained with the uPRDX6-specific antibody that was scored as 4+. The neoplastic cells exhibit both nuclear and cytoplasmic-positive staining for PRDX6 (arrow). (C) Image depicting a canine splenic HSA scored as 1+ for PRDX6. (D) Image depicting canine cutaneous HA tissue. A few uPAR-positive fibroblasts (arrowheads) can be observed; however, the neoplastic endothelial cells (arrows) are negative. Bar, A and C, 50 μ m; B and D, 20 μ m.

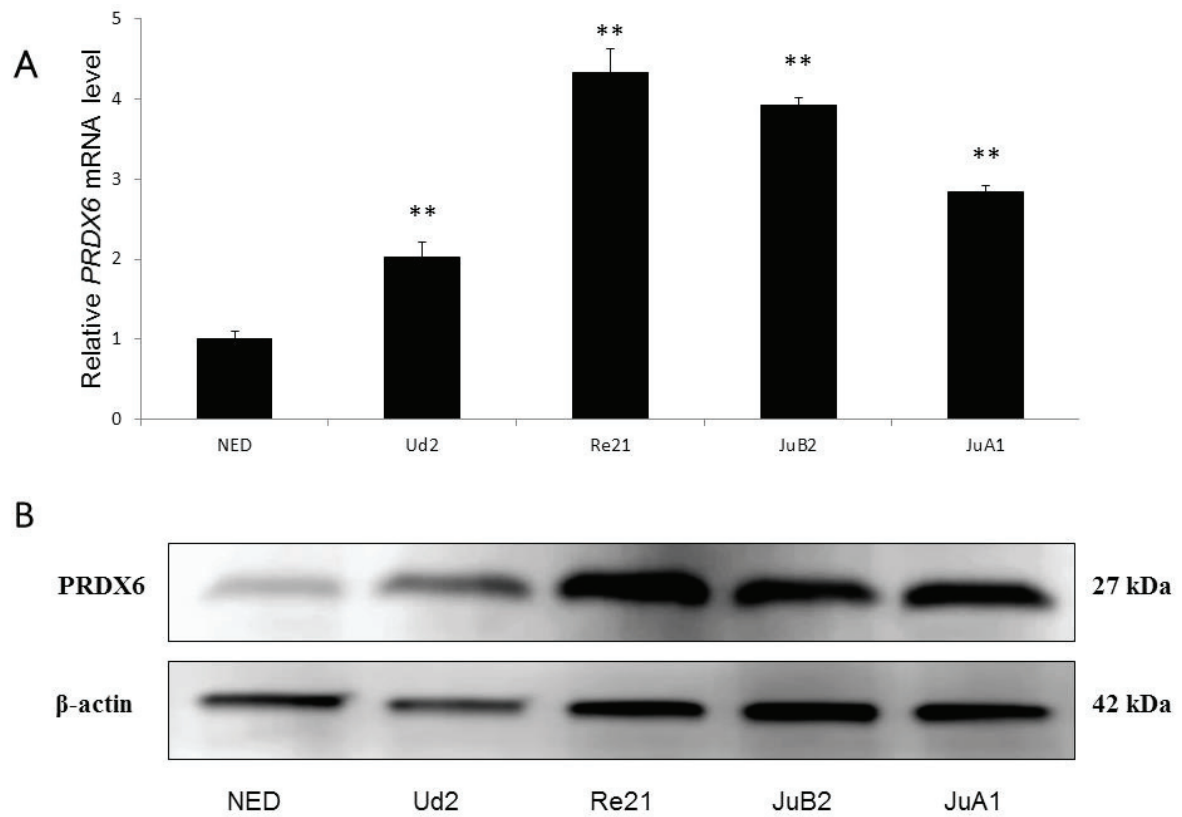


Figure 8. Analysis of *PRDX6* expression in normal endothelial cells (NED) and HSA cell lines by real-time reverse transcription-PCR (A) and western blotting (B). (A) Expression of *PRDX6* mRNA in NED and HSA cell lines (Ud2, Re21, JuB2, and JuA1). (B) Expression of *PRDX6* protein in NED and HSA cell lines. β -actin protein levels were used as controls for sample loading. ** $P < 0.05$ versus NED.

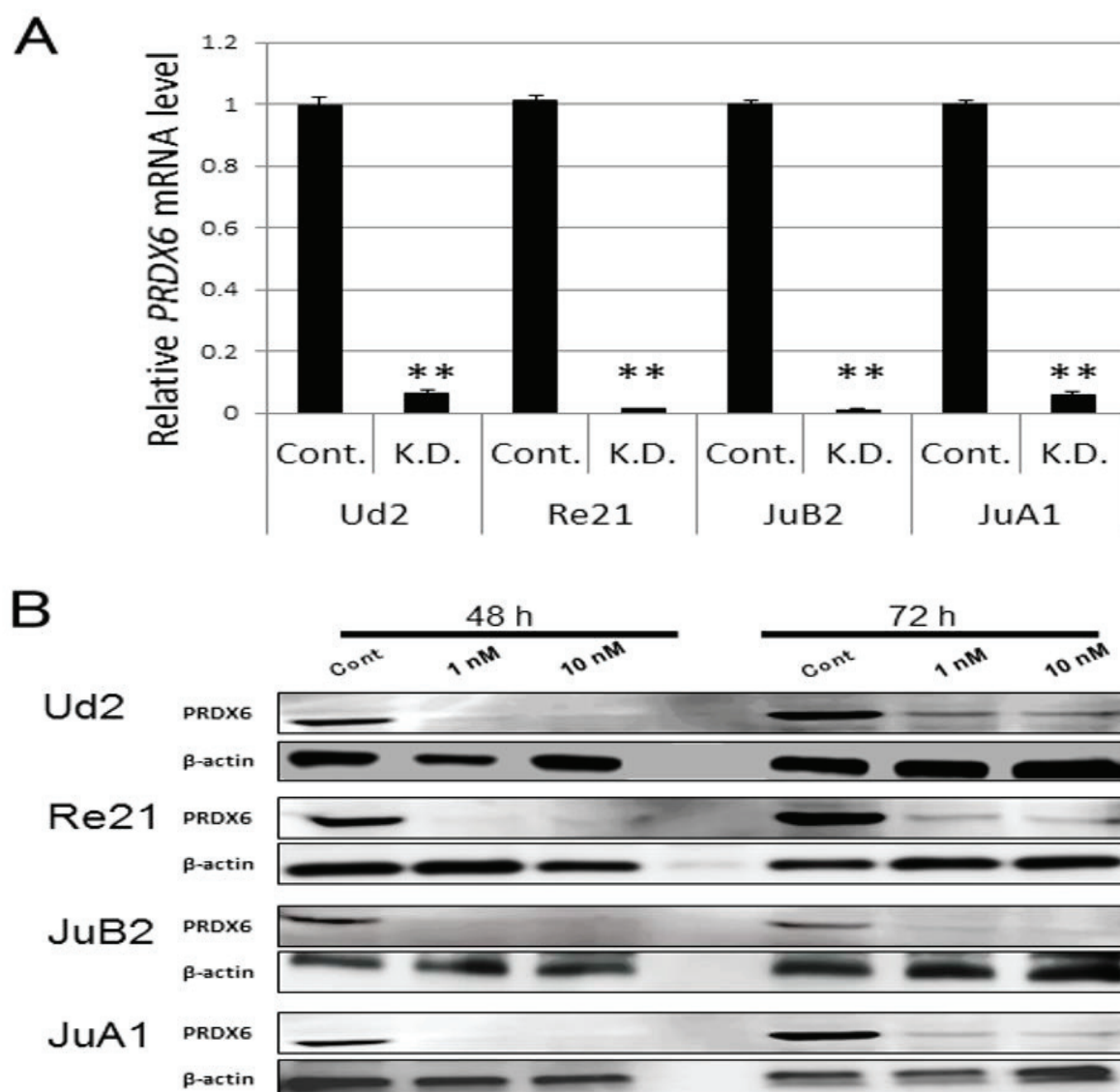


Figure 9. PRDX6 knockdown by siRNA in HSA cell lines (Ud2, Re21, JuB2, and JuA1).

PRDX6 mRNA levels were analysed after transfection with siRNAs. (B) Protein levels of PRDX6 were analysed by western blot 48 h and 72 h after transfection with siRNAs. ** $P < 0.01$ versus the control. Cont. = control, K.D.= knockdown.

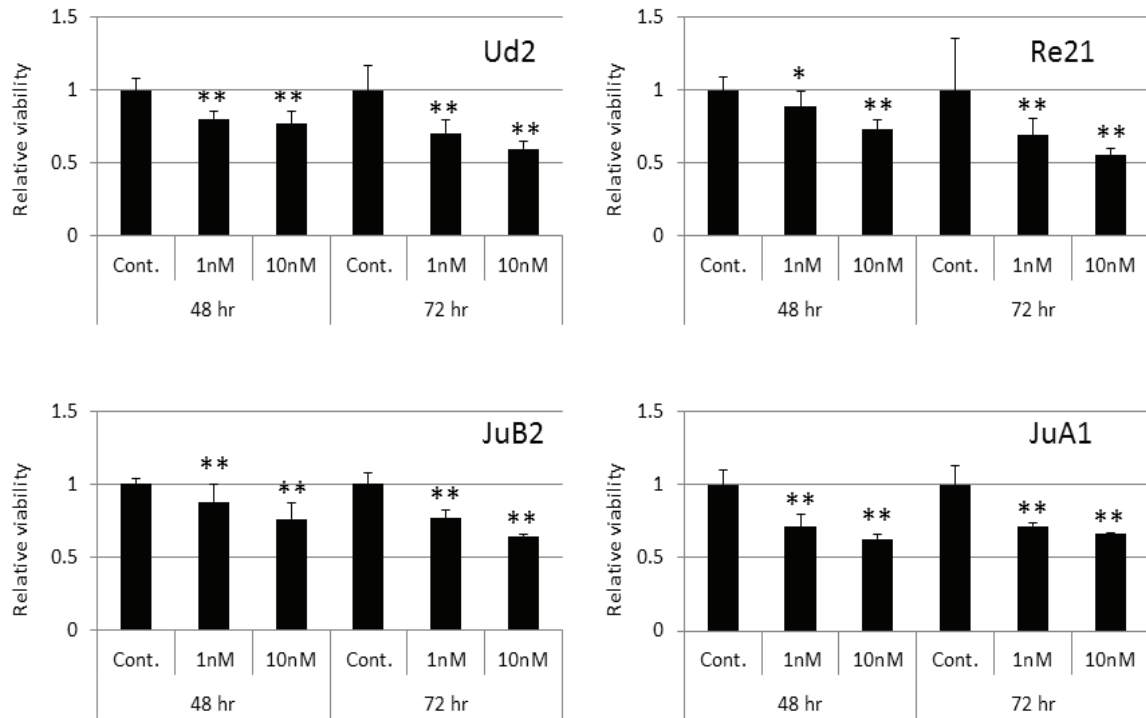


Figure 10. Inhibitory effect on cell proliferation by siRNA in HSA cell lines. The viability of HSA cells transfected with the indicated concentration of siRNA for 48 or 72 h. Marked dose- and time-dependent reductions in cell proliferation can be observed compared to non-treated cells. ** $P<0.01$ and * $P<0.05$ versus the control. Cont. = control, K.D.= knockdown.

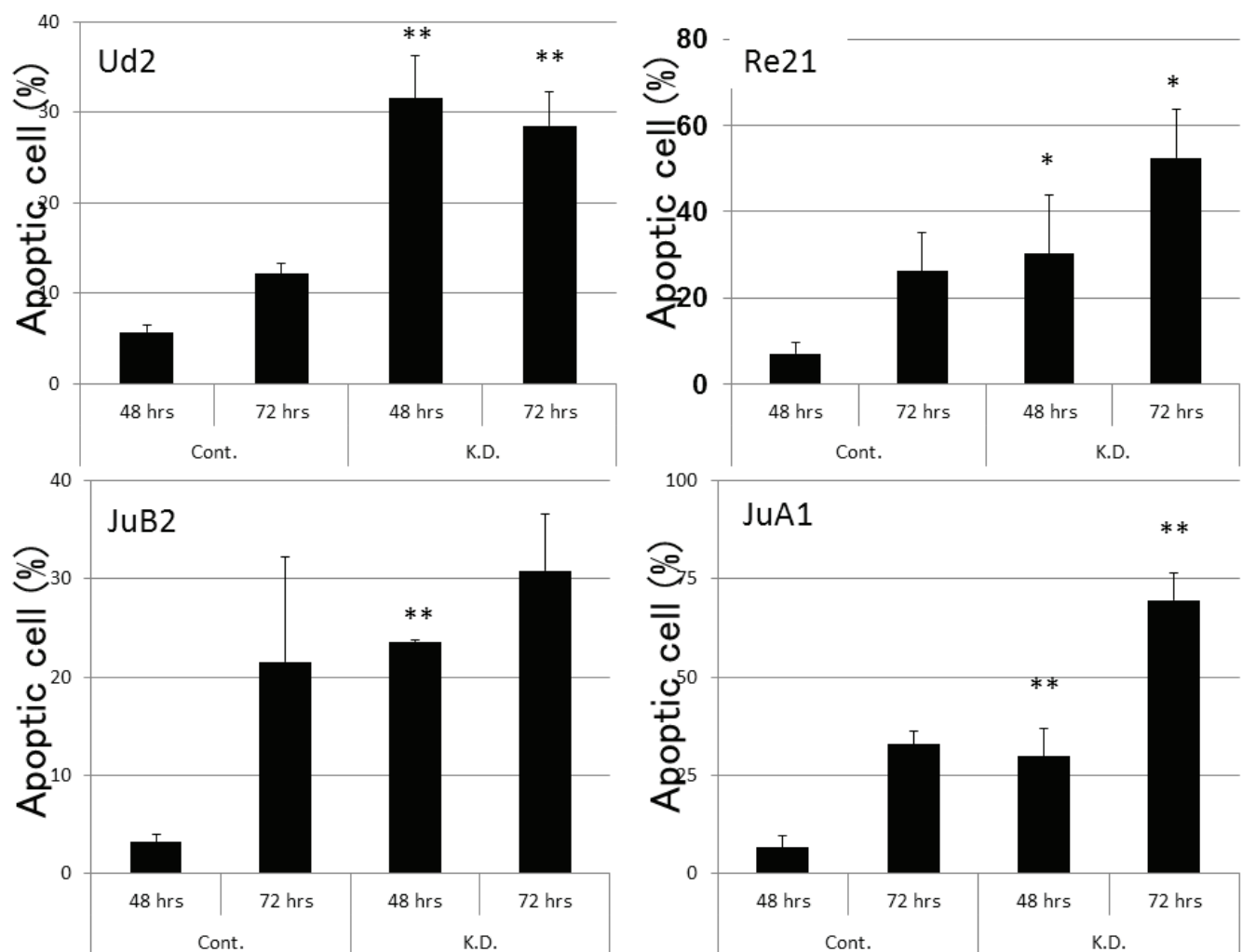


Figure 11. Analysis of apoptosis following down regulation of PRDX6 expression. HSA cells transfected with the PRDX6 targeting siRNAs (K.D., 20 nmol/l) or negative control siRNAs (cont.). Cells were stained with DAPI. (A) Nuclear fragmentation at 48 and 72 h after transfection. ** $P < 0.01$ and * $P < 0.05$ versus the control. Cont. = control, K.D.= knockdown.

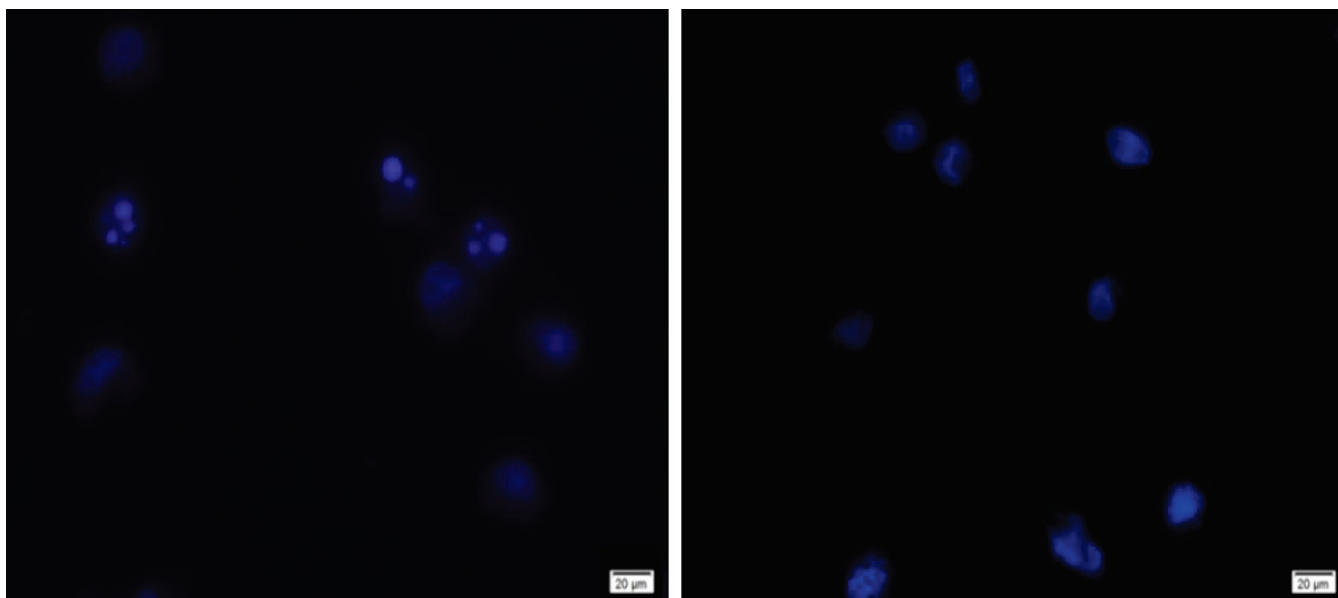


Figure 12. Analysis of apoptosis following down regulation of PRDX6 expression. HSA cells transfected with the PRDX6 targeting siRNAs (K.D., 20 nmol/l) or negative control siRNAs (cont.). Photomicrographs of DAPI staining of HSA cells visualized and photographed under a fluorescence microscope (Olympus, Tokyo, Japan). siR-PRDX6 transfected cells (left) show prominent nuclear fragmentation; non-treated control cells (right) show viable and intact blue-stained nuclei. Bar, 20 μ m.

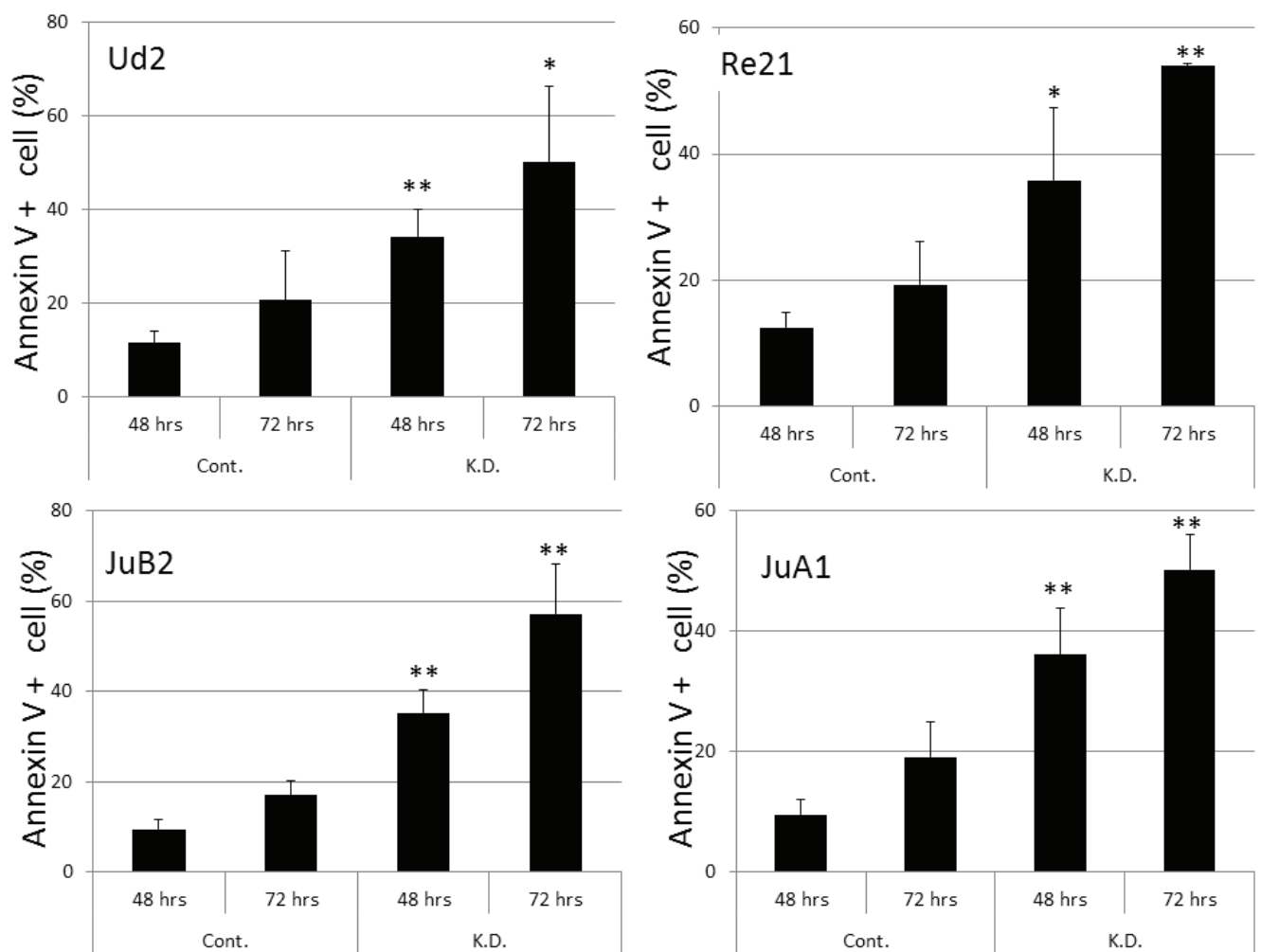


Figure 13: HSA cells transfected with (20 nM) of the sPRDX6 targeting siRNAs (K.D)

Negative Control. Cells were fixed 48, 72 hr post transfection and stained with Annexin-V/PI. Annexin levels in siR-PRDX6 transfected groups of all HSA cells were significantly higher than those of the control siRNA-transfected group at 48 hours and 72 hours after transfection. **Statistically significant with $p < 0.05$

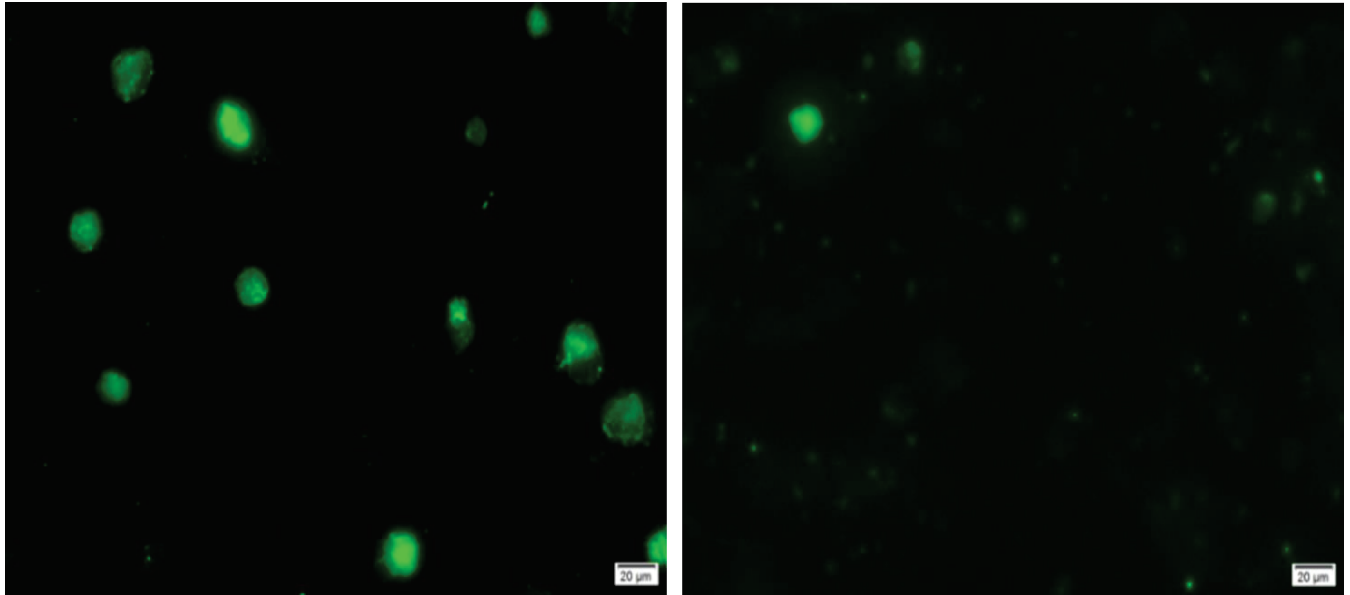


Figure 14. Analysis of apoptosis following down regulation of PRDX6 expression. HSA cells transfected with the PRDX6 targeting siRNAs (K.D., 20 nmol/l) or negative control siRNAs (cont.). Photomicrograph of annexin-V staining of HSA cells visualized and photographed under a fluorescence microscope (Olympus). siR-PRDX6 transfected cells (left) show a higher annexin-V expression level than non-treated control cells. Bar, 20µM.

General Discussion

Comparative oncology is the study of naturally developing cancers in animals as models for human disease. Spontaneous cancers in dogs and cats are an underused group of naturally occurring malignancies that share many features with human cancers such as angiosarcoma, osteosarcoma, prostate and breast cancers, non-Hodgkin's lymphoma, melanoma, soft tissue sarcoma, head and neck carcinoma, and virally induced lymphomas [116, 117]. The evolutionary history of dogs, their position as a family member in many households, and the high level of health care they receive in our world offer tremendous opportunities. Alongside this combined with recently developed genetic resources, makes dogs outstanding models for the studying known genetic pathways, the discovery of genetic and environmental contributions to disease, and translational studies on cancer risk, prevention, and treatments.

Canine HSA is among the most challenging and mysterious diseases encountered in veterinary practice [118]. It is an incurable tumor of ECs. HSA is relatively common in dogs; it accounts for 5-7% of all tumors seen in dogs. Although dogs of any age and breed are susceptible to HSA, it occurs more commonly in dogs beyond middle age (older than 6 years), and in breeds such as Golden Retrievers, German Shepherd Dogs, Portuguese Water Dogs, Bernese Mountain Dogs, Flat Coated Retrievers, Boxers and Skye Terriers, among others. Unlike other cancers, HSA is almost an exclusive disease of dogs [119]. In human, AS occurs only rarely in association with workplace exposure to vinyl chloride and polychlorinated biphenyls (PCBs), such as is found in rubber and tire plants. An even smaller fraction of women who receive high-dose radiation therapy for cancer (usually breast cancer)

can develop AS of the skin [45]. Yet, AS account for much less than 1% of all tumors seen in people. Benign tumors of vascular ECs, called HA, arise in the skin of people and dogs that have extended exposure to sunlight. These tumors are distinct from HSAs and ASs, and they are not life threatening. The common primary sites for canine HSA are the spleen, the right atrium of the heart, and the subcutis, which is the tissue beneath the skin [120]. The pattern of growth for these tumors involves infiltration into normal tissues surrounding the tumor as well as distant spread (metastasis). Since HSA tends to metastasize aggressively to lungs, liver, intestines, and mesentery, distant spread has inevitably occurred once the disease is finally diagnosed. The eventual outcome for patients with this disease often follows the rupture of a large or rapidly growing tumor, which results in acute, severe hemorrhage, collapse, shock, and death.

The scarceness of AS has restricted the field of basic and clinical research on this naturally occurring neoplasm. As a result, attention has turned to naturally occurring canine HSA in companion dogs. Dogs have a much higher incidence of HSA and a more rapid time course of disease progression than AS in humans. Moreover, the canine model also offers some unique, advantageous features that distinguish it from other animal models and that open novel experimental opportunities. First of all, because of selective breeding, genetic variation within canine breeds is very low. Secondly, because each breed is derived from a small group of founders, many of the genes associated with polygenic traits are fixed, so that only a few variable genes determine phenotype. Finally, companion dogs share the same environmental exposures as humans and thus may more accurately reflect the human condition [121].

Clinical HSAs and HAs samples were used during the current studies besides HSAs cell lines. Although advances in genomics during the last decade have opened new tracks for translational research and allowed the direct evaluation of clinical samples, there is still a need for reliable preclinical models to test therapeutic strategies [122]. Cancer-derived cell lines are the most widely used models to study the biology of cancer and to test hypotheses to improve the efficacy of cancer treatment. This opens new avenues for discovery of the basic mechanisms of the origin and progression of cancer, which could be translated to help our understanding of the causes and treatment of neoplasms. Since, endothelial cell lines derived from explants of canine HSA have been established, numerous studies have been done to gain insight into the molecular mechanism of canine HSAs [13, 123-125]. In the current studies, clinical surgical removed canine HSAs and HAs samples, besides, canine HSA cell lines were used.

There is increasing evidence that dysregulation of molecular pathways governing angiogenesis may be important in the biology of HSAs [2]. Stand on the shoulders of this hypothesis, intensive research is needed to get deep understanding the role of angiogenic pathways in the biology of ECs. malignancies, and finding therapies targeting HSA and AS. In the present study, IHC was utilised to confirm that newly formed vascular ECs express both uPA and uPAR in granulation tissue. In contrast, quiescent ECs within normal dermal tissues were negative for these markers. Thus, the uPA and uPAR system are considered that it plays an important role in pathophysiological angiogenesis. Moreover, IHC analysis was performed for detection of uPA and uPAR proteins in clinical HSA and HA samples obtained from dogs. The expression of uPA and uPAR was significantly higher among HSAs than

HAs. In addition, the expression of both ligand and receptor in HSA neoplastic endothelial cells seems to have a phenotype similar to that of active ECs during pathophysiological angiogenesis. Binding of uPA to its receptor promotes uPA activity, which then enhances the activation of plasminogen to plasmin. As a result, the detection of expression of both the ligand and the receptor within individual tumour cells indicates that the cells are likely capable of regulating plasminogen activation via uPA/uPAR production. Substantial evidence exists which implicates the urokinase plasminogen activator system (uPA, and uPAR) in angiogenesis, invasion and metastasis of many solid tumors [126]. Clinical studies have demonstrated an association between high levels of expression of the components of this system in tumors and poor patient prognosis and outcome. Components of the uPA/uPAR system are differentially expressed or activated in motile cells including invading tumor cells and leukocytes, and migrating endothelial cells. In contrast, there is little or no expression on most normal, quiescent cells. Studies performed in vitro have demonstrated the regulation of the expression of uPA and uPAR by growth and differentiation factors as well as by oncogenes [127]. VEGF-initiated angiogenesis requires both the coordinated proteolytic degradation of the ECM, provided by the uPA/uPAR system, and the regulation of cell migration via integrin-matrix interactions [55]. An association between expression of VEGF and VEGFR and enhanced proliferation has been reported in canine HSA [70]. These data suggest that elevated expression of uPA and uPAR may be linked to the activation of the VEGF pathway. There is cooperation between tissue PA, uPA, plasminogen activator inhibitor-1 and VEGF throughout the processes of tumor growth and progression to human malignant gliomas, and invasion and angiogenesis in human gastric cancer [128].

It has been reported that the expression of PRDX-6 contributes to the invasive and metastatic potential of breast cancer cells by stimulating the signaling pathways involving uPA, and uPAR [40, 129]. Based on the aforementioned reports, expression and function of PRDX-6 were investigated in clinical HSAs and HSAs cell lines. we investigated the expression levels of PRDX6 in spontaneous primary canine HSAs by IHC. analysis, identifying marked expression of this protein. Furthermore, both PRDX6 mRNA and protein were overexpressed in HSA cell lines compared to normal canine endothelial cells, although some variation was observed between the different HSA cell lines. PRDX6 was shown in this study to be a potent inhibitor of apoptosis, it, therefore, likely functions upstream of Bcl-2. Accordingly, siRNA experiments demonstrated an increased rate of apoptotic cells in HSA cell lines upon downregulation of PRDX6 that was likely due to a decreased capability of the cells to reduce hydrogen peroxide below a critical level. The detection by two different methods of the induction of apoptosis following RNA interference illustrated significant effects in the HSA cell lines. Consistent with our findings, previous transient transfection of Hepa1-6 cells with PRDX6 siRNA was shown to lead to a marked reduction in its expression and an increase in peroxide-induced cytotoxicity mediated by apoptosis; these results suggested that PRDX6 up-regulation might be a tumor-supportive adaptation in cancerous states [39]. In addition to its proposed role in apoptosis, PRDX6 appears to also be involved in regulating the invasive and metastatic potential of breast cancer by upregulating the expression of uPAR, Ets-1, MMP-9, RhoC, and Tissue inhibitor of metalloproteinase-2[40]. Notably, overexpression of uPA, uPAR, and MMPs has been reported in canine HSAs [74,

114]. We, therefore, suggest that PRDX6 might be associated with the invasive and metastatic capacity of HSAs by stimulating the upregulation of uPA, uPAR.

uPA and uPAR and PRDX-6 expression were detected not only in neoplastic ECs but also in the surrounding tumor stroma. The interaction of these abnormal cells with their microenvironment is essential for tumor development, protection from the body's immune or defence mechanisms, later progression and the development of life-threatening or metastatic disease [130]. Tumor-stroma interaction is mediated by a complex and dynamic crosstalk involving cytokines, chemokines, growth factors, enzymes, microRNAs, and other effector molecules. As our understanding of the role of tumor microenvironment grows, the complexity of the interactions between cancer cells and their surrounding tissues becomes more and more evident.

In dogs, non-splenic HSAs occur less commonly than splenic HSAs, but are still frequently detected and can affect a wide variety of organs. In contrast, cutaneous HAs are more common than visceral HAs. Indeed, the latter are particularly uncommon in dogs [28]. Therefore, splenic HSAs accounted for the majority of the samples analysed in the current studies. However, there were no significant differences in the proportions of uPA, uPAR, and PRDX-6-positive cases between the splenic and non-splenic HSAs. As a result, we propose that immunoreactivity is not dependent on the primary organ of HSAs. Therefore, canine HSA still a good model for human AS, even though the later presents mostly as a cutaneous form (cutaneous AS).

It has been reported that surgical removal and radiotherapy results in long-term survival in many HSAs cases. Up to Date, there is no standard chemotherapy for canine HSA. In the last decade, antiangiogenic therapy has attracted prominent attention as a possible therapy for canine HSA. Given this background, the finding of the present studies indicates that uPA, uPAR, and PRDX-6 genes may be novel targets for the therapy of canine HSA, and *in vivo* models are needed to support further research into the therapy of canine HSA.

Summary

Canine hemangiosarcoma (HSA) is a progressive endothelial cell (ECs) malignant neoplasm of dogs, resembling human angiosarcoma (AS) with poor prognosis. Despite their vascular origin, even the addition of novel anti-angiogenic drugs has shown a minimal to absent response in HSA. Thus, very little therapeutic progress has been made over the past several decades to increase the progression-free survival or overall patient survival of individuals suffering from this sarcoma, and effective therapeutics against this disease are desperately needed.

There is increasing evidence that dysregulation of molecular pathways governing angiogenesis may be important in the biology of HSAs. Stand on the shoulders of this hypothesis, intensive research is needed to get deep understanding the role of angiogenic pathways in the biology of ECs. malignancies, and finding therapies targeting HSA and AS.

In chapter one, Immunohistochemistry was used to assess the expression of urokinase plasminogen activator (uPA) and uPA receptor (uPAR) in canine primary haemangiosarcomas (HSAs), canine cutaneous haemangiomas (HAs) and in control sections of canine cutaneous granulation tissue. Immunohistochemistry was utilised to confirm that newly formed vascular ECs express both urokinase plasminogen activator (uPA) and its receptor uPAR in granulation tissue. In contrast, quiescent ECs within normal dermal tissues were negative for these markers. Thus, the uPA and uPAR system are considered that it plays an important role in pathophysiological angiogenesis. Moreover, IHC analysis was performed for detection of uPA and uPAR proteins in clinical HSA and HA samples obtained

from dogs. The expression of uPA and uPAR was significantly higher among HSAs than HAs. In addition, the expression of both ligand and receptor in HSA neoplastic endothelial cells seems to have a phenotype similar to that of active ECs during pathophysiological angiogenesis. Binding of uPA to its receptor promotes uPA activity, which then enhances the activation of plasminogen to plasmin. As a result, the detection of expression of both the ligand and the receptor within individual tumor cells indicates that the cells are likely capable of regulating plasminogen activation via uPA/uPAR production. The correlation between uPA/uPAR expression and the Ki67 labelling index was estimated in the HSA and HA tissues. Expression of both molecules was significantly higher in HSAs than in cutaneous HAs. The average Ki67 labelling index of the uPA (+)/uPAR (+) HSAs was significantly higher than that of uPA (-)/uPAR (+) HSAs and HA tissues. These results suggest that uPA and uPAR play a significant role in the malignant proliferation of canine HSA, regardless of the primary origin of the tumor.

In chapter 2, we confirmed the overexpression of PRDX6 in canine HSAs and examined the levels of expression in a variety of HSAs and NED. We also found that knockdown of PRDX6 using siRNA reduced tumor cell proliferation and caused cell apoptosis. We revealed that the antioxidant PRDX6 protects HSA cells against apoptosis, suggesting that an important mechanism of chemoresistance might involve ROS scavenging and antioxidant enzyme activity. Because cancer resistance to chemotherapies and radiation represents a considerable obstacle to the effective treatment of numerous malignancies, including endothelial cell malignancies, PRDX6 expression levels might be a good predictor of tumor response, especially to oxidative stress-producing therapies. Furthermore, the

manipulation of PRDX6 expression or inhibition of its ROS scavenging ability might provide a new paradigm for improved cancer treatment.

要約

犬の血管肉腫は、予後不良のヒトの血管肉腫と同様に進行性の内皮細胞性の悪性腫瘍である。血管由来にも関わらず、血管肉腫では、新規抗血管新生薬は無効かわずかな効き目しか示さない。ゆえに、過去数十年間以上、この血管肉腫に罹患した個体の全生存率あるいは無病期間の延長のための治療法開発の進展はほとんどなく、この疾患に対し手の効果的な治療が切望されている。

血管肉腫の生物学において、血管新生に関わる分子経路の異常が重要であることを示す報告が増加している。この仮説から、血管内皮性悪性腫瘍の生物学における血管新生関連経路のより深い理解を得るために、さらなる研究が必要とされている。

第一章では、犬の血管肉腫、血管腫および対照として皮膚の肉芽組織を用い、ウロキナーゼプラスミノゲンアクチベーター（uPA）と uPA 受容体（uPAR）の発現を免疫組織化学的に評価した。肉芽組織において、新生血管の内皮細胞に uPA と uPAR の発現が免疫組織化学的に確認された。対照的に、正常な真皮内の休止した内皮細胞はこれらの分子が陰性を示した。よって、uPA/uPAR システムは、病態生理学的な血管新生において、重要な役割を果たすと考えられた。さらに犬から得られた HSA と HA の臨床材料を用いて uPA と uPAR たんぱく質の検出を免

疫組織化学に行った。uPA および uPAR の発現は、血管腫に比較して血管肉腫の方が有意に高かった。血管肉腫の腫瘍性内皮細胞における uPA および uPAR の発現は病態生理学的な血管新生時の活性化内皮細胞と同様であると考えられた。uPA とその受容体の結合は、プラスミノゲンのプラスミンへの活性化を促進する。腫瘍細胞での両分子の検出は uPA/uPAR 産生を介したプラスミノゲンの活性化を制御しうることが示唆された。血管肉腫と血管腫の組織において uPA および uPAR の発現と Ki-67 陽性率との相関も評価した。uPA (+) /uPAR (+) の血管肉腫の平均 Ki-67 陽性率は、uPA (-) /uPAR (+) の血管肉腫および血管腫の平均 Ki-67 陽性率に比較して高値を示した。これらの結果より、uPA および uPAR が、腫瘍の原発に関わらず、犬血管肉腫の悪性増殖に有意な役割を果たすことが示唆された。

第二章では、犬の血管肉腫および血管腫における peroxiredoxin-6 (PRDX6) の発現を免疫組織学的に検討し、血管腫より血管肉腫において PRDX6 の有意な発現を認めた。さらに、犬の血管肉腫の細胞株において発現の程度には差がみられたが、正常な犬の内皮細胞に比較して、mRNA とタンパク質の過剰発現がみられた。低分子干渉 RNA による PRDX6 の発現抑制によって、血管肉腫細胞にアポトーシスが誘導された。PRDX6 の抑制により、細胞の傷害が起こるという点から、PRDX6 は細胞生存に重要な役割を果たしていることが示唆された。

以上の結果より，uPA，uPAR および PRDX6 は，犬の血管肉腫の新しい治療ターゲットとなりうることが示唆され，さらなる犬の血管肉腫の治療法開発に *in vivo* モデルが必要と考えられた。

Dedication

This thesis is dedicated to my mother and soul of my father. Although they are far away, I always feel their presence encouraging me to achieve my goals in life. To them I dedicate this work. Also to my brothers and sisters who were always a source of constant support and a driving force for me to complete this thesis. To my son Zeyad and my daughters Minna and Jana a constant source of joy and encouragement that was always uplifting and for them faith in me that never failed to inspire me. I also dedicate this work to my father in law professor Adel Gharieb for all his support and help.

great honour to dedicate this to the soul of my professor Salah whose advice have illuminated the pass of my scientific research. I will never forget his words “believe in yourself and do your best to improve yourself and your academic department”.

Finally, I dedicate this work to my dear wife for being patient with me during the difficult times I faced during my research and who have always been inspiration for me.

Acknowledgement

Completion of the Ph.D. has been a part of my commitment to the lifelong learning and the journey of working in the laboratory and then writing this Ph.D. thesis has been challenging, but the rewards are immeasurable. First and Foremost gratitude goes to Allah (God) for providing me with the strength to complete this journey.

The data presented in this thesis has been carried out in Gifu University, Gifu, Japan, during the period of 2012-2016. I am very grateful for the work and study facilities as well as friendly environment provided by Gifu University.

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References

1. Paik, J.-H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z. et al. (2007). FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. *Cell*, 128 (2), 309-323.
2. MacEwen, E.G. (2001). Miscellaneous tumors. In: *Small Animal Clinical Oncology*, SJ Withrow, EG MacEwen, 4th ed., WB Saunders, Philadelphia, pp. 639-671.
3. Bertazzolo, W., Dell'Orco, M., Bonfanti, U., Ghisleni, G., Caniatti, M., Masserdotti, C. et al. (2005). Canine angiosarcoma: cytologic, histologic, and immunohistochemical correlations. *Vet. Clinic. Pathol.* 34 (1), 28-34.
4. Lieberman, L. (1955). Malignant hemangioendothelioma of the canine heart. *J. Am. Vet. Med. Assoc.* 126 (937), 296.
5. Quigley, P., De Saram, W., Dawson, I. and Pryse-Davies, J. (1965). Two cases of haemangiosarcoma of the radius in the dog. *Vet. Rec.* 77 (41), 1207-1209.
6. Geib, L. (1967). Primary angiomatous tumors of the heart and great vessels. A report of two cases in the dog. *The Cornell. Vet.* 57 (2), 292-296.
7. Slaughter, L. and Herman, L. (1969). Hemangiosarcoma in a dog. *J. Am. Vet. Med. Assoc.* 154 (1), 17.
8. Priester, W.A. and McKay, F.W. (1980). The occurrence of tumors in domestic animals. *J. Natl. Cancer Inst. Monographs.* (54), 1-210.

9. Ritt, M., Weiss, D. and Wardrop, K. (2009). Epidemiology of hematopoietic neoplasia. Schalm's Veterinary Hematology, 6th ed.; Weiss, K.J., Eds.; Blackwell Publishing: Ames, IA, USA.
10. Brown, N.O., Patnaik, A.K. and MacEwen, E.G. (1985). Canine hemangiosarcoma: retrospective analysis of 104 cases. J. Am. Vet. Med. Assoc. 186 (1), 56-58.
11. Srebernik, N. and Appleby, E.C. (1991). Breed prevalence and sites of haemangioma and haemangiosarcoma in dogs. Vet. Rec. 129 (18), 408-409.
12. Schultheiss, P.C. (2004). A retrospective study of visceral and nonvisceral hemangiosarcoma and hemangiomas in domestic animals. J. Vet. Diagn. Invest. 16 (6), 522-526.
13. Fosmire, S.P., Dickerson, E.B., Scott, A.M., Bianco, S.R., Pettengill, M.J., Meylemans, H. et al. (2004). Canine malignant hemangiosarcoma as a model of primitive angiogenic endothelium. Lab. Invest. 84 (5), 562-572.
14. Goldschmidt, M.H. and Hendrick, M.J. (2008). Tumors of the Skin and Soft Tissues. In Tumors in Domestic Animals, Iowa State Press, pp. 45-117.
15. von Beust, B.R., Suter, M.M. and Summers, B.A. (1988). Factor VIII-related antigen in canine endothelial neoplasms: an immunohistochemical study. Vet. Pathol. 25 (4), 251-255.
16. Ferrer, L., Fondevila, D., Rabanal, R.M. and Vilafranca, M. (1995). Immunohistochemical detection of CD31 antigen in normal and neoplastic canine endothelial cells. J. Comp. Pathol. 112 (4), 319-326.

17. Italiano, A., Thomas, R., Breen, M., Zhang, L., Crago, A.M., Singer, S. et al. (2012). The miR-17-92 cluster and its target THBS1 are differentially expressed in angiosarcomas dependent on MYC amplification. *Genes Chromosomes Cancer*, 51 (6), 569-578.
18. Fosmire, S.P., Dickerson, E.B., Scott, A.M., Bianco, S.R., Pettengill, M.J., Meylemans, H. et al. (2004). Canine malignant hemangiosarcoma as a model of primitive angiogenic endothelium. *Lab. Invest.* 84 (5), 562-572.
19. Rouhani, P., Fletcher, C.D., Devesa, S.S. and Toro, J.R. (2008). Cutaneous soft tissue sarcoma incidence patterns in the U.S. : an analysis of 12,114 cases. *Cancer*, 113 (3), 616-627.
20. Abraham, J.A., Hornicek, F.J., Kaufman, A.M., Harmon, D.C., Springfield, D.S., Raskin, K.A. et al. (2007). Treatment and outcome of 82 patients with angiosarcoma. *Ann. Surg. Oncol.* 14 (6), 1953-1967.
21. Ogilvie, G.K., Powers, B.E., Mallinckrodt, C.H. and Withrow, S.J. (1996). Surgery and doxorubicin in dogs with hemangiosarcoma. *J. Vet. Intern. Med.* 10 (6), 379-384.
22. Dobson, J.M. (2013). Breed-predispositions to cancer in pedigree dogs. *ISRN Vet. Sci.* 2013; vol. 2013, Article ID 941275, 23 pages, 2013. doi:10.1155/2013/941275
23. Tonomura, N., Elvers, I., Thomas, R., Megquier, K., Turner-Maier, J., Howald, C. et al. (2015). Genome-wide association study identifies shared risk loci common to two malignancies in golden retrievers. *PLoS Genet*, 11 (2), e1004922.

24. Tamburini, B.A., Trapp, S., Phang, T.L., Schappa, J.T., Hunter, L.E. and Modiano, J.F. (2009). Gene expression profiles of sporadic canine hemangiosarcoma are uniquely associated with breed. *PLoS One*, 4 (5), e5549.
25. Modiano, J.F. and Breen, M.(2010). Genetic basis of cancer. In *Cancer management in Small Animal Practice*. Henry, C. Ed. Saunders.Chapter 2, 10-15.
26. Benjamin, S., Hahn, F., Chiffelle, T., Boecker, B., Hobbs, C., Jones, R. et al. (1975). Occurrence of hemangiosarcomas in beagles with internally deposited radionuclides. *Cancer Res.* 35 (7), 1745-1755.
27. Benjamin, S.A., Saunders, W.J., Angleton, G.M. and Lee, A.C. (1991). Radiation carcinogenesis in dogs irradiated during prenatal and postnatal development. *J. Radiat. Res.* 32 (2), 86-103.
28. Wang, Z., Dabrosin, C., Yin, X., Fuster, M.M., Arreola, A., Rathmell, W.K. et al. (2015). Broad targeting of angiogenesis for cancer prevention and therapy. *Semin. Cancer Biol.* 35, S224-S243.
29. Yonemaru, K., Sakai, H., Murakami, M., Yanai, T. and Masegi, T. (2006). Expression of vascular endothelial growth factor, basic fibroblast growth factor, and their receptors (flt-1, flk-1, and flg-1) in canine vascular tumors. *Vet. Pathol.* 43 (6), 971-980.
30. Asa, S.A., Murai, A., Murakami, M., Hoshino, Y., Mori, T., Maruo, K. et al. (2012). Expression of platelet-derived growth factor and its receptors in spontaneous canine hemangiosarcoma and cutaneous hemangioma. *Histol. Histopathol.* 27 (5), 601-607.

31. Murakami, M., Sakai, H., Kodama, A., Yanai, T., Mori, T., Maruo, K. et al. (2009). 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. *Histol. Histopathol.* 24 (4), 437-446.
32. Kodama, A., Sakai, H., Murakami, M., Murai, A., Mori, T., Maruo, K. et al. (2009). Immunohistochemical demonstration of angiogenesis-associated homeobox proteins in canine vascular tumours. *J. Comp. Pathol.* 141 (2-3), 199-203.
33. Kodama, A., Sakai, H., Matsuura, S., Murakami, M., Murai, A., Mori, T. et al. (2009). Establishment of canine hemangiosarcoma xenograft models expressing endothelial growth factors, their receptors, and angiogenesis-associated homeobox genes. *BMC Cancer*, 9, 363.
34. Murai, A., Asa, S.A., Kodama, A., Hirata, A., Yanai, T. and Sakai, H. (2012). Constitutive phosphorylation of the mTORC2/Akt/4E-BP1 pathway in newly derived canine hemangiosarcoma cell lines. *BMC Vet. Res.* 8, 128.
35. Ma, Y.-Y. and Tao, H.-Q. (2012). Role of urokinase plasminogen activator receptor in gastric cancer: a potential therapeutic target. *Cancer Biother. Radiopharm.* 27 (5), 285-290.
36. Chapman, H.A. (1997). Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. *Curr. Opin. Cell Biol.* 9 (5), 714-724.
37. Santibanez, J.F. (2013). Transforming growth factor-Beta and urokinase-type plasminogen activator: dangerous partners in tumorigenesis—implications in skin

cancer. *ISRN Dermatol.* 2013, Article ID 597927, 26 pages.

doi:10.1155/2013/597927

38. Zhang, X.z., Xiao, Z.f., Li, C., Xiao, Z.q., Yang, F., Li, D.j. et al. (2009).
Triosephosphate isomerase and peroxiredoxin 6, two novel serum markers for
human lung squamous cell carcinoma. *Cancer Sci.* 100 (12), 2396-2401.
39. Walsh, B., Pearl, A., Suchy, S., Tartaglio, J., Visco, K. and Phelan, S.A. (2009).
Overexpression of Prdx6 and resistance to peroxide-induced death in Hepa1-6 cells:
Prdx suppression increases apoptosis. *Redox Rep.* 14 (6), 275-284.
40. Chang, X.-Z., Li, D.-Q., Hou, Y.-F., Wu, J., Lu, J.-S., Di, G.-H. et al. (2007).
Identification of the functional role of peroxiredoxin 6 in the progression of breast
cancer. *Breast Cancer Res.* 9 (6), R76.
41. Ho, J.-N., Lee, S.B., Lee, S.-S., Yoon, S.H., Kang, G.Y., Hwang, S.-G. et al. (2010).
Phospholipase A2 activity of peroxiredoxin 6 promotes invasion and metastasis of
lung cancer cells. *Mol. Cancer Ther.* 9 (4), 825-832.
42. Liu, L., Kakiuchi-Kiyota, S., Arnold, L.L., Johansson, S.L., Wert, D. and Cohen,
S.M. (2013). Pathogenesis of human hemangiosarcomas and hemangiomas. *Human
Pathol.* 44 (10), 2302-2311.
43. Coindre, J.M., Terrier, P., Guillou, L., Le Doussal, V., Collin, F., Ranchère, D. et al.
(2001). Predictive value of grade for metastasis development in the main histologic
types of adult soft tissue sarcomas. *Cancer*, 91 (10), 1914-1926.
44. Rouhani, P., Fletcher, C.D., Devesa, S.S. and Toro, J.R. (2008). Cutaneous soft
tissue sarcoma incidence patterns in the US. *Cancer*, 113 (3), 616-627.

45. Young, R.J., Brown, N.J., Reed, M.W., Hughes, D. and Woll, P.J. (2010). Angiosarcoma. *Lancet Oncol* .11 (10), 983-991.
46. Kao, Y.-C., Chow, J.-M., Wang, K.-M., Fang, C.-L., Chu, J.-S. and Chen, C.-L. (2011). Primary pleural angiosarcoma as a mimicker of mesothelioma: a case report. *Diagn. Pathol.* 6, 130.
47. Lamerato-Kozicki, A.R., Helm, K.M., Jubala, C.M., Cutter, G.C. and Modiano, J.F. (2006). Canine hemangiosarcoma originates from hematopoietic precursors with potential for endothelial differentiation. *Exp. Hematol.* 34 (7), 870-878.
48. Gázquez, A., Martín, d.L.M.J., Millán, R.Y., García, A., Masot, A. and Redondo, E. (2012). Widespread epithelioid angiosarcoma with ventricular wall involvement in a dog. *Histol. Histopathol.* 27 (7), 865-872.
49. Clifford, C.A., Mackin, A.J. and Henry, C.J. (2000). Treatment of canine hemangiosarcoma: 2000 and beyond. *J. Vet. Intern. Med.* 14 (5), 479-485.
50. Pearson, G. and Head, K. (1976). Malignant haemangioendothelioma (angiosarcoma) in the dog. *J. Small Anim. Pract.* 17 (11), 737-745.
51. Brown, N., Patnaik, A. and MacEwen, E. (1985). Canine hemangiosarcoma: retrospective analysis of 104 cases. *J. Am. Vet. Med. Assoc.* 186 (1), 56-58.
52. Raghu, H., Lakka, S.S., Gondi, C.S., Mohanam, S., Dinh, D.H., Gujrati, M. et al. (2010). Suppression of uPA and uPAR attenuates angiogenin mediated angiogenesis in endothelial and glioblastoma cell lines. *PLoS One*, 5 (8), e12458.
53. Sasaki, T., Nishi, H., Nagata, C., Nagai, T., Nagao, T., Terauchi, F. et al. (2014). A retrospective study of urokinase-type plasminogen activator receptor (uPAR) as a

- prognostic factor in cancer of the uterine cervix. *Int. J. Clin. Oncol.* 19 (6), 1059-1064.
54. Alexander, R.A., Prager, G.W., Mihaly-Bison, J., Uhrin, P., Sunzenauer, S., Binder, B.R. et al. (2012). VEGF-induced endothelial cell migration requires urokinase receptor (uPAR)-dependent integrin redistribution. *Cardiovasc. Res.* 94 (1), 125-135.
 55. Uhrin, P. and Breuss, J.M. (2013). uPAR: a modulator of VEGF-induced angiogenesis. *Cell Adh. Migr.* 7 (1), 23-26.
 56. Prager, G.W., Breuss, J.M., Steurer, S., Olcaydu, D., Mihaly, J., Brunner, P.M. et al. (2004). Vascular endothelial growth factor receptor-2–induced initial endothelial cell migration depends on the presence of the urokinase Receptor. *Circ. Res.* 94 (12), 1562-1570.
 57. Welti, J., Loges, S., Dimmeler, S. and Carmeliet, P. (2013). Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *J. Clin. Invest.* 123 (8), 3190-3200.
 58. Wakui, S. (1988). Two-and three-dimensional ultrastructural observation of two cell angiogenesis in human granulation tissue. *Virchows Archiv B.* 56 (1), 127-139.
 59. Kämmerer, U., Kapp, M., Gassel, A.M., Richter, T., Tank, C., Dietl, J. et al. (2001). A new rapid immunohistochemical staining technique using the EnVision antibody complex. *J. Histochem. Cytochem.* 49 (5), 623-630.

60. Kennedy, S., Duffy, M., Duggan, C., Barnes, C., Rafferty, R. and Kramer, M. (1998). Semi-quantitation of urokinase plasminogen activator and its receptor in breast carcinomas by immunocytochemistry. *Br. J. Cancer* 77 (10), 1638.
61. Mondino, A. and Blasi, F. (2004). uPA and uPAR in fibrinolysis, immunity and pathology. *Trends Immunol.* 25 (8), 450-455.
62. Sakai, H., Yamane, T., Yanai, T., Shirai, N. and Masegi, T. (2001). Expression of Cyclin Kinase Inhibitor p27 Kip1 in Skin Tumours of Dogs. *J. Comp. Pathol.* 125 (2), 153-158.
63. Santos, A., Lopes, C., Marques, R.M., Amorim, I., Ribeiro, J., Frias, C. et al. (2011). Immunohistochemical analysis of urokinase plasminogen activator and its prognostic value in canine mammary tumours. *Vet. J.* 189 (1), 43-48.
64. Johnson, K.D., Glinskii, O.V., Mossine, V.V., Turk, J.R., Mawhinney, T.P., Anthony, D.C. et al. (2007). Galectin-3 as a potential therapeutic target in tumors arising from malignant endothelia. *Neoplasia*, 9 (8), 662-670.
65. Babarović, E., Zamolo, G., Mustać, E. and Strčić, M. (2011). High grade angiosarcoma arising in fibroadenoma. *Diagn. Pathol.* 6, 125.
66. Lin, X.-Y., Liu, Y., Zhang, Y., Yu, J.-H. and Wang, E.-H. (2012). The co-expression of cytokeratin and p63 in epithelioid angiosarcoma of the parotid gland: a diagnostic pitfall. *Diagn. Pathol.* 7 (1), 118.
67. Pettersson, A., Nagy, J.A., Brown, L.F., Sundberg, C., Morgan, E., Jungles, S. et al. (2000). Heterogeneity of the angiogenic response induced in different normal adult

tissues by vascular permeability factor/vascular endothelial growth factor. *Lab.*

Invest. 80 (1), 99-115.

68. Clifford, C.A., Hughes, D., Beal, M.W., Mackin, A.J., Henry, C.J., Shofer, F.S. et al. (2001). Plasma vascular endothelial growth factor concentrations in healthy dogs and dogs with hemangiosarcoma. *J. Vet. Intern. Med.* 15 (2), 131-135.
69. Yu, Y., Varughese, J., Brown, L.F., Mulliken, J.B. and Bischoff, J. (2001). Increased Tie2 expression, enhanced response to angiopoietin-1, and dysregulated angiopoietin-2 expression in hemangioma-derived endothelial cells. *Am. J. Pathol.* 159 (6), 2271-2280.
70. Yonemaru, K., Sakai, H., Murakami, M., Yanai, T. and Masegi, T. (2006). Expression of vascular endothelial growth factor, basic fibroblast growth factor, and their receptors (flt-1, flk-1, and flg-1) in canine vascular tumors. *Vet. Pathol.* 43 (6), 971-980.
71. Kim, J.-H., Frantz, A.M., Anderson, K.L., Graef, A.J., Scott, M.C., Robinson, S. et al. (2014). Interleukin-8 promotes canine hemangiosarcoma growth by regulating the tumor microenvironment. *Exp. Cell Res.* 323 (1), 155-164.
72. Kodama, A., Sakai, H., Matsuura, S., Murakami, M., Murai, A., Mori, T. et al. (2009). Establishment of canine hemangiosarcoma xenograft models expressing endothelial growth factors, their receptors, and angiogenesis-associated homeobox genes. *BMC cancer*, 9 (1), 363.

73. Murakami, M., Sakai, H., Kodama, A., Mori, T., Maruo, K., Yanai, T. et al. (2008). Expression of the anti-apoptotic factors Bcl-2 and survivin in canine vascular tumours. *J. Comp. Pathol.* 139 (1), 1-7.
74. Murakami, M., Sakai, H., Kodama, A., Mori, T., Yanai, T., Maruo, K. et al. (2009). 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. *Histol. Histopathol.* 139(1),1-7.
75. Duggan, C., Maguire, T., McDermott, E., O'higgins, N., Fennelly, J. and Duffy, M. (1995). Urokinase plasminogen activator and urokinase plasminogen activator receptor in breast cancer. *Int. J. Cancer.* 61 (5), 597-600.
76. Seddighzadeh, M., Steineck, G., Larsson, P., Wijkström, H., Norming, U., Onelöv, E. et al. (2002). Expression of UPA and UPAR is associated with the clinical course of urinary bladder neoplasms. *Int. J. Cancer.* 99 (5), 721-726.
77. Annes, J.P., Munger, J.S. and Rifkin, D.B. (2003). Making sense of latent TGF β activation. *J. Cell. Sci.* 116 (2), 217-224.
78. Andreasen, P., Egelund, R. and Petersen, H. (2000). The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell. Mol. Life Sci.* 57 (1), 25-40.
79. Wang, L., Madigan, M.C., Chen, H., Liu, F., Patterson, K.I., Beretov, J. et al. (2009). Expression of urokinase plasminogen activator and its receptor in advanced epithelial ovarian cancer patients. *Gynecol. Oncol.* 114 (2), 265-272.

80. Sandström, M., Johansson, M., Sandström, J., Bergenheim, A.T. and Henriksson, R. (1999). Expression of the proteolytic factors, tPA and uPA, PAI-1 and VEGF during malignant glioma progression. *Int. J. Dev. Neurosci.* 17 (5), 473-481.
81. Kirchheimer, J.C., Wojta, J., Christ, G. and Binder, B.R. (1989). Functional inhibition of endogenously produced urokinase decreases cell proliferation in a human melanoma cell line. *Proc. Natl. Acad. Sci. U.S.A.* 86 (14), 5424-5428.
82. Cantero, D., Friess, H., Deflorin, J., Zimmermann, A., Bründler, M., Riesle, E. et al. (1997). Enhanced expression of urokinase plasminogen activator and its receptor in pancreatic carcinoma. *Br. J. Cancer.* 75 (3), 388.
83. Asuthkar, S., Gondi, C.S., Nalla, A.K., Velpula, K.K., Gorantla, B. and Rao, J.S. (2012). Urokinase-type plasminogen activator receptor (uPAR)-mediated regulation of WNT/ β -catenin signaling is enhanced in irradiated medulloblastoma cells. *J. Biol. Chem.* 287 (24), 20576-20589.
84. Dumler, I., Stepanova, V., Jerke, U., Mayboroda, O., Vogel, F., Bouvet, P. et al. (1999). Urokinase-induced mitogenesis is mediated by casein kinase 2 and nucleolin. *Curr. Biol.* 9 (24), 1468-1476.
85. Ulisse, S., Baldini, E., Sorrenti, S. and D'Armiento, M. (2009). The urokinase plasminogen activator system: a target for anti-cancer therapy. *Curr. Cancer Drug Targets.* 9 (1), 32-71.
86. Fukumoto, S., Saida, K., Sakai, H., Ueno, H., Iwano, H. and Uchide, T. (2016) Therapeutic potential of endothelin inhibitors in canine hemangiosarcoma. *Life Sci.* doi: 10.1016/j.lfs.2016.01.047

87. Lan, J., Huang, B., Liu, R., Ju, X., Zhou, Y., Jiang, J. et al. (2015). Expression of cancer stem cell markers and their correlation with pathogenesis in vascular tumors. *Int. J. Clin. Exp. Pathol.* 8 (10), 12621-12633.
88. Fukumoto, S., Miyasho, T., Hanazono, K., Saida, K., Kadosawa, T., Iwano, H. et al. (2015). Big endothelin-1 as a tumour marker for canine haemangiosarcoma. *Vet. J.* 204 (3), 269-274.
89. Hanahan, D. and Weinberg, Robert A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, 144 (5), 646-674.
90. Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 35 (4), 495-516.
91. Johnstone, R.W., Ruefli, A.A. and Lowe, S.W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. *Cell*, 108 (2), 153-164.
92. Fulda, S. (2010). Evasion of apoptosis as a cellular stress response in cancer. *Int. J. Biochem. Cell Biol.* 2010. doi:10.1155/2010/370835
93. Woo, H.A., Chae, H.Z., Hwang, S.C., Yang, K.-S., Kang, S.W., Kim, K. et al. (2003). Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science (New York, N.Y.)*, 300 (5619), 653-656.
94. Aguilar-Melero, P., Prieto-Álamo, M.-J., Jurado, J., Holmgren, A. and Pueyo, C. (2013). Proteomics in HepG2 hepatocarcinoma cells with stably silenced expression of PRDX1. *J. Proteomics* 79, 161-171.
95. Hall, A., Karplus, P.A. and Poole, L.B. (2009). Typical 2-Cys peroxiredoxins—structures, mechanisms and functions. *FEBS journal*, 276 (9), 2469-2477.

96. Fisher, A.B. (2011). Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A2 activities. *Antioxid. Redox Signal.* 15 (3), 831-844.
97. Xu, X., Lu, D., Zhuang, R., Wei, X., Xie, H., Wang, C. et al. (2015). The phospholipase A2 activity of peroxiredoxin 6 promotes cancer cell death induced by tumor necrosis factor alpha in hepatocellular carcinoma. *Mol. Carcinog.* doi: 10.1002/mc.22371.
98. Yun, H.-M., Park, K.-R., Park, M.H., Kim, D.H., Jo, M.R., Kim, J.Y. et al. (2015). PRDX6 promotes tumor development via the JAK2/STAT3 pathway in a urethane-induced lung tumor model. *Free Radic. Biol. Med.* 80, 136-144.
99. Fujii, T., Fujii, J. and Taniguchi, N. (2001). Augmented expression of peroxiredoxin VI in rat lung and kidney after birth implies an antioxidative role. *Eur. J. Biochem.* 268 (2), 218-225.
100. Kümin, A., Schäfer, M., Epp, N., Bugnon, P., Born-Berclaz, C., Oxenius, A. et al. (2007). Peroxiredoxin 6 is required for blood vessel integrity in wounded skin. *J. Cell Biol.* 179 (4), 747-760.
101. Huang, C.F., Sun, Z.J., Zhao, Y.F., Chen, X.M., Jia, J. and Zhang, W.F. (2011). Increased expression of peroxiredoxin 6 and cyclophilin A in squamous cell carcinoma of the tongue. *Oral Dis.* 17 (3), 328-334.
102. Murai, A., Asa, S.A., Kodama, A., Hirata, A., Yanai, T. and Sakai, H. (2012). Constitutive phosphorylation of the mTORC2/Akt/4E-BP1 pathway in newly derived canine hemangiosarcoma cell lines. *BMC Vet. Res.* 8 (1), 128.

103. Heishima, K., Mori, T., Sakai, H., Sugito, N., Murakami, M., Yamada, N. et al. (2015). MicroRNA-214 Promotes Apoptosis in Canine Hemangiosarcoma by Targeting the COP1-p53 Axis. *PloS one*, 10 (9), e0137361.
104. Yonemaru, K., Sakai, H., Murakami, M., Kodama, A., Mori, T., Yanai, T. et al. (2007). The significance of p53 and retinoblastoma pathways in canine hemangiosarcoma. *J. Vet. Med. Sci.* 69 (3), 271-278.
105. Mark, R.J., Poen, J.C., Tran, L.M., Fu, Y.S. and Juillard, G.F. (1996). Angiosarcoma. *Cancer*, 77, 2400-2406.
106. Waris, G. and Ahsan, H. (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* 5 (1), 14.
107. Goncalves, K., Sullivan, K. and Phelan, S. (2012). Differential expression and function of peroxiredoxin 1 and peroxiredoxin 6 in cancerous MCF-7 and noncancerous MCF-10A breast epithelial cells. *Cancer Res.* 30 (1), 38-47.
108. Kinnula, V., Lehtonen, S., Sormunen, R., Kaarteenaho-Wiik, R., Kang, S., Rhee, S. et al. (2002). Overexpression of peroxiredoxins I, II, III, V, and VI in malignant mesothelioma. *J. Pathol.* 196 (3), 316-323.
109. Zhou, J., Liu, Q., Wang, J., Guo, X. and Song, L. (2012). Expressions of peroxiredoxin 1, peroxiredoxin 6 and GFAP in human brain astrocytoma and their clinical significance. *J. South. Med. Univ.* 32 (9), 1255-1259.
110. Quan, C., Cha, E.-J., Lee, H.-L., Han, K.H., Lee, K.M. and Kim, W.-J. (2006). Enhanced expression of peroxiredoxin I and VI correlates with development, recurrence and progression of human bladder cancer. *J. Urol.* 175 (4), 1512-1516.

111. Li, C., Wu, J., He, M., Zhou, Q., Chen, X., Chen, K. et al. Peroxiredoxin 6 highly expressed in human cervical squamous cell carcinoma. *JSAB*. 2014;2(6):32-35.
112. Rhee, S.G. and Woo, H.A. (2011) Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H₂O₂, and protein chaperones. *Antioxid. Redox Signal*.15 (3), 781-794.
113. Hansen, J.M., Moriarty-Craige, S. and Jones, D.P. (2007). Nuclear and cytoplasmic peroxiredoxin-1 differentially regulate NF- κ B activities. *Free Radic. Biol. Med.* 43 (2), 282-288.
114. Anwar, S., Yanai, T. and Sakai, H. (2015). Immunohistochemical Detection of Urokinase Plasminogen Activator and Urokinase Plasminogen Activator Receptor in Canine Vascular Endothelial Tumours. *J. Comp. Pathol.* 153 (4), 278-282.
115. Welsh, S.J., Bellamy, W.T., Briehl, M.M. and Powis, G. (2002). The Redox Protein Thioredoxin-1 (Trx-1) Increases Hypoxia-inducible Factor 1 α Protein Expression Trx-1 Overexpression Results in Increased Vascular Endothelial Growth Factor Production and Enhanced Tumor Angiogenesis. *Cancer Res.* 62 (17), 5089-5095.
116. Vail, D.M. and MacEwen, E.G. (2000) Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest.* 18 (8), 781-792.
117. Cekanova, M. and Rathore, K. (2014). Animal models and therapeutic molecular targets of cancer: utility and limitations. *Des. Dev. Ther.* 8, 1911-1922.
118. Jakab, C., Halász, J., Kiss, A., Schaff, Z., Rusvai, M., Gálfi, P. et al. (2009). Claudin-5 protein is a new differential marker for histopathological differential diagnosis of canine hemangiosarcoma. *Histol. Histopathol.*24(7), 801-13.

119. Kim, J.-H., Graef, A.J., Dickerson, E.B. and Modiano, J.F. (2015). Pathobiology of hemangiosarcoma in dogs: Research advances and future perspectives. *Vet. Sci.* 2 (4), 388-405.
120. Yoon, H.-Y., Kang, H.-M. and Lee, M.-Y. (2014). Primary cranial mediastinal hemangiosarcoma in a young dog. *Ir. Vet. J.* 67 (1), 1.
121. Van Gramberg, J.L., de Veer, M.J., O'Hehir, R.E., Meeusen, E.N. and Bischof, R.J. (2013). Use of animal models to investigate major allergens associated with food allergy. *J. Allergy*, 2013.
122. Gillet, J.-P., Varma, S. and Gottesman, M.M. (2013). The clinical relevance of cancer cell lines. *J. Natl. Cancer Inst.* 105(7):452-8.
123. Rodriguez, A.M., Graef, A.J., LeVine, D.N., Cohen, I.R., Modiano, J.F. and Kim, J.H. (2015). Association of Sphingosine-1-phosphate (S1P)/S1P Receptor-1 Pathway with Cell Proliferation and Survival in Canine Hemangiosarcoma. *J. Vet. Intern. Med.* 29 (4), 1088-1097.
124. Wirth, K.A., Kow, K., Salute, M.E., Bacon, N.J. and Milner, R.J. (2014). In vitro effects of Yunnan Baiyao on canine hemangiosarcoma cell lines. *Vet. Comp. Oncol.* DOI: 10.1111/vco.12100.
125. Kim, J.H., Frantz, A.M., Anderson, K.L., Graef, A.J., Scott, M.C., Robinson, S. et al. (2014). Interleukin-8 promotes canine hemangiosarcoma growth by regulating the tumor microenvironment. *Exp. Cell Res.* 323 (1), 155-164.

126. Mazar, A.P., Henkin, J. and Goldfarb, R.H. (1999). The urokinase plasminogen activator system in cancer: implications for tumor angiogenesis and metastasis. *Angiogenesis*, 3 (1), 15-32.
127. Pepper, M.S. (2001). Role of the matrix metalloproteinase and plasminogen activator–plasmin systems in angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* 21 (7), 1104-1117.
128. Zhang, L., Zhao, Z.-S., Ru, G.-Q. and Ma, J. (2006). Correlative studies on uPA mRNA and uPAR mRNA expression with vascular endothelial growth factor, microvessel density, progression and survival time of patients with gastric cancer. *World J. Gastroenterol.* 12 (25), 3970.
129. Lee, S.B., Ho, J.-N., Yoon, S.H., Kang, G.Y., Hwang, S.-G. and Um, H.-D. (2009). Peroxiredoxin 6 promotes lung cancer cell invasion by inducing urokinase-type plasminogen activator via p38 kinase, phosphoinositide 3-kinase, and Akt. *Molecules and cells*, 28 (6), 583-588.
130. Goubran, H.A., Kotb, R.R., Stakiw, J., Emara, M.E. and Burnouf, T. (2014). Regulation of tumor growth and metastasis: the role of tumor microenvironment. *Cancer growth metastasis*, 7, 9-18.