The study on the expression of angiogenesis-related factors and the angiogenesis in canine mammary tumors

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(イヌの乳腺腫瘍における血管新生関連因子の発現と
血管新生の関連に関する研究)

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General introduction

Angiogenesis is extremely important for the development and metastasis of solid tumors because tumor-associated vessels can supply oxygen and nutrition to tumor cells (Folkman, 1990). Angiogenesis is modulated by many factors because angiogenesis is a complex process that involves endothelial cell migration in response to chemotactic factors, endothelial cell prolifelation, capillary budding, establishment of capillary loops, and neovascular remodeling(Carmeliet, 2003; Jain, 2003; Yancopoulos et al., 2000). These angiogenesis factors have been investigated as tumor prognostic factors. Among them, vascular endothelial growth factor (VEGF) plays a central role in angiogenesis and mediates vascular permeability by binding to its receptors, flt-1 and flk-1 (Leung et al., 1989). Up-regulated VEGF in many human solid tumors correlate with a poor prognosis. Recently combination therapy using anti-VEGF drug received accelerated approval from the US Food and Drug Administration (FDA) for use in the first-line treatment of patients with metastatic breast cancer.

However, expression of and correlation among angiogenic factors including VEGF and its receptors in canine tumors are still poorly understood, although several studies have demonstrated significant correlation among angiogenic factors, microvessel density, and tumor grade in mammary gland tumors, squamous cell carcinoma, meningioma, melanoma and prostate tumors in dogs(Chevalier et al., 2002; Maiolino et al., 2000; Platt et al., 2006; Rawlings et al., 2003; Restucci et al., 2002). This study was aimed to investigate the expression of angiogenic factors, mainly VEGF, and the correlation between these factors and vascular marker, including endothelial markers and intratumoral microvessel density in canine tumors. For this purpose, the author examined canine mammary gland tumors because of the reason as follows. 1) human breast cancer has been well investigated for angiogenesis and was beginning to be treated with anti-VEGF drug. 2) mammary gland tumors are the most common neoplasms in female dogs and have both benign and malignant counterparts. 3) it is comparable between tumor tissues and non-neoplastic tissues in the same cases because non-neoplastic mammary tissues were resected together in most cases. Thus, in chapter 1, the author examined the expression and distribution of VEGF and its receptor, flt-1 and flk-1, in the normal canine tissues. In chapter 2, the author evaluated two endothelial markers in canine mammary tumors. One is a pan-endothelial marker, vWF, and the

other is recently used as a marker of "activated" endothelial cells, endoglin. In chapter 3, the author investigated the expression of VEGF and its receptor in canine mammary tumors to evaluate the correlation among these factors and the potential as markers of tumor aggressiveness. The author also analyzes the correlation of VEGF and its regulator, HIF-1α and COX-2, in canine mammary tumors.

Chapter 1

Expression and distribution of vascular endothelial growth factor and its receptor, flt-1 and flk-1, in the normal canine tissues

Abstract

Angiogenesis is essential for tumor progression and is regulated by several angiogenic factors such as vascular endothelial growth factor (VEGF). Recently, VEGF-targeting therapy was received from FDA, but side effects of the therapy have been reported. Therefore, it is important to clarify the distribution and function of VEGF and its receptors in normal physiology. Thus, the author investigated the expression and distribution of VEGF and its receptor, flt-1 and flk-1 in 13 normal canine tissues using immunohistochemistry, RT-PCR and real-time RT-PCR. Immunohistochemical staining showed that both VEGF and flt-1 were expressed in many tissues and their mRNAs were detected in all organs examined by RT-PCR. Levels of VEGF164 and flt-1 mRNA expression were high in tissues containing many intensely immunopositive cells. The expression levels of VEGF164, flt-1, and flk-1 mRNA tended to be similar. These results indicated that VEGF, flt-1, and flk-1 are closely associated in canine, as in human tissues, and quantifying their mRNAs might be helpful in evaluating angiogenesis.

Introduction

Recently anti-VEGF therapy has demonstrated clinical benefit in metastatic colorectal cancer, non-small-cell lung cancer, and breast carcinomas(Ferrara et al., 2004). However, the side effects of anti-VEGF therapy, including proteinuria, hypertension, and thrombus, have been reported(Gordon and Cunningham, 2005). This suggested that VEGF and its receptors have crucial roles not only in tumor angiogenesis but in non-neoplastic tissues. Therefore, it is important to clarify the distribution and function of VEGF and its receptors in normal tissues.

At least four isoforms of VEGF (VEGF 120, 164, 182, 188) have been identified in the dog and they are produced by alternative splicing during transcription(Jingjing et al., 2000). Although each isoforms has specific biological activities, VEGF164 has been considered to be the most predominant form in tumor associated angiogenesis(Dvorak et al., 1995; Keyt et al., 1996; Soker et al., 1997).

The expression and distribution of the mRNAs and proteins for these angiogenic factors in normal canine tissues have not been well understood. Therefore, the present study examines VEGF, flt-1, and flk-1 expression in normal canine tissues using immunohistochemistry, RT-PCR, and real-time RT-PCR.

Materials and methods

Samples

Six normal beagle dog tissue samples including lung, renal cortex, heart, adrenal, liver, skin, thyroid, intestine, bladder, mesenteric lymph node, pancreas, and spleen were kindly provided by Dr. Nakagaki (Nippon veterinary and life science university, Tokyo, Japan). Four mammary gland tissue samples from four normal beagle dogs were kindly provided by Dr. Fujii (Azabu university, Kanagawa, Japan). These samples were confirmed as normal using hematoxylin-eosin (HE) staining.

Immunohistochemistry of VEGF and flt-1

Immunohistochemical staining was performed using a universal immunoenzyme polymer method. Four-micron thick sections were cut from paraffin blocks and mounted on silane-coated glass slides and dried in a 37 °C oven. Slides were deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Endogenous peroxidase was blocked with hydrogen peroxide 0.3% in absolute methanol for 20 min. The primary antibodies, polyclonal rabbit anti-human VEGF (A-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 in 100 and polyclonal rabbit anti-human flt-1 (C-17, Santa Cruz Biotechnology) diluted 1 in 50 were applied overnight at 4 °C. After rinsing in PBS (three times for 5 min each), in both staining, peroxidase-labeled goat anti-rabbit IgG (Histofine® Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan) was used as the second antibody for 20 min at room temperature. After rinsing in PBS (three times for 5 min each), immunoreactions was visualized with DAB and hematoxylin was used as counterstain. Negative controls included normal rabbit IgG (Chemicon, Temecula, CA, USA) instead of primary antibody at the same concentration.

Qualitative and quantitative analysis of gene expression

Total RNA was isolated from the normal canine tissues with a TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) follows the manufacturer's instructions. Genomic DNA was digested by incubation with 1 U/μg DNase I (Promega, Madison, WI, USA) for 30 minutes at 37°C. The quantity of RNA was determined using absorption at 260 nm on a spectrophotometer. The first-strand complementary DNA (cDNA) was

reverse transcribed from 2 μg total RNA using the SuperScript First-Strand Synthesis System III for RT-PCR (Invitrogen Life Technologies) primed by an random primer in 20 μl final volume. After reverse transcription, the cDNA was diluted 1:10 with sterile distilled water (DW) and used as a template for the RT-PCR and real-time RT-PCR analysis.

Oligonucleotide primers (Table 1) were designed to amplify cDNA fragments of canine all isoforms of VEGF, flt-1, flk-1, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The PCR reaction with Takara Taq (Takara Shuzo, Ohtsu, Japan) proceeded as follows: denaturation at 94°C for 5min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were separated on 2% agarose gels and stained with ethidium bromide.

Real-time PCR proceeded in duplicate on ABI PRISM 7700 Sequence Analyzer (Applied Biosystems, Foster City, CA, USA) using the primer sets for VEGF164, flt-1, and flk-1 and TaqMan Universal PCR Mastermix® (Applied Biosystems). Expression levels of each mRNA were standardized against 18S ribosomal RNA(Applied Biosystems) in corresponding samples and then the amount of expression relative to that in the liver was calculated. Values are expressed as means ± SE of six dogs.

Results

Immunohistochemistry

Table 2 shows the immunohistochemical findings obtained from normal canine tissues using both anti-VEGF and -flt-1 antibodies. The staining profiles did not significantly differ according to vessel size or between arteries and veins. Anti-VEGF antibody weakly reacted with some, whereas anti-flt-1 antibody moderately reacted with many vascular smooth muscle cells. However, neither antibody reacted with endothelial cells. Bronchiolar and alveolar epithelial cells, as well as bronchiolar smooth muscles were intensely stained with both antibodies (Figure 1A). In the kidney, glomeruli were negative, whereas proximal, distal and collecting tubules were weakly stained for both antibodies. The myocardium was moderately and diffusely stained with both antibodies. Anti-flt-1 antibody moderately stained the secretory cells of the adrenal cortex and medulla, whereas anti-VEGF antibody moderately stained only cortical cells. In the liver, hepatocytes and the bile duct reacted with both antibodies, whereas Kupffer cells were immunopositive only against anti-VEGF antibody. The epidermis, sebaceous and apocrine glands, as well as erector pili muscles in the skin were moderately to strongly positive for both VEGF and flt-1. Anti-VEGF and anti-flt-1 antibody strongly reacted with upper and hair follicles, respectively. No thyroid structures other than vessels were positive for both antibodies. Anti-VEGF antibody weakly stained the goblet cells and muscularis of the intestine, whereas anti-flt-1 antibody was weakly to moderately reactive in the muscularis, the nerve plexus, and intensely stained in the microvilli. The transitional epithelium of the bladder moderately reacted with both antibodies and intensely with anti-flt-1 antibody in umbrella cells (Figure 1B). Anti-flt-1 antibody reacted with some lymphocytes in T-cell regions of lymph nodes, whereas anti-VEGF antibody weakly reacted with lymphocytes in both T-cell and B-cell regions. No pancreatic structures were VEGF-positive except for the vessels, whereas intercalated ducts and islets weakly reacted with flt-1 antibody. In the spleen, only vascular smooth muscle cells reacted with both antibodies. The mammary epithelial cells were negative to weakly positive for VEGF and moderately positive for flt-1. The mammary myoepithelial cells were negative for both VEGF and flt-1.

Qualitative analysis of gene expression

Bands corresponding to VEGF120, VEGF164, and VEGF182 or VEGF188 appeared after RT-PCR for VEGF in all normal canine tissues examined. The VEGF182 and VEGF188 products migrated as a single band on the gel because the sizes of these cDNAs were similar. More VEGF164 isoform was expressed than any other isoform. All normal tissues in all dogs examined expressed flt-1 and flk-1 mRNA (Figure 2).

Quantitative analysis of gene expression

The degree of expression of VEGF164 was correlated with that of flt-1 and flk-1 mRNA, the high level expression of both VEGF164, flt-1 and flk-1 mRNA was seen in the lung, renal cortex, heart, and adrenal though expression levels were low in pancreas, lymph node, and spleen (Figure 3).

Discussion

The present study found that VEGF and flt-1 were expressed in all normal canine tissues at the protein level and VEGF, flt-1, and flk-1 in mRNA level. These results are consistent with findings in human, mouse, rat, and guinea pig tissues(Berse et al., 1992; Claffey et al., 1992; Monacci et al., 1993). Consistent with the relatively high level of VEGF expression in the lung and heart, the capillaries of these organs are considered to be more permeable than those of many other tissues. The high level of VEGF expression in the renal cortex could be because renal glomeruli have an exceptionally permeable filtration system(Berse et al., 1992). The reasons for the negative staining of the renal gromeruli for both VEGF and flt-1 may include that the limit of detection and major VEGF receptor in the glomeruli is not flt-1 but flk-1(Masuda et al., 2001). The findings that the level of VEGF expression in the spleen was low despite the abundance of vessels indicate that only a few cells express VEGF under normal conditions. Neutralizing VEGF in the lung results in alveolar endothelial cell apoptosis(Kasahara et al., 2000), significant capillary regression in pancreatic islets, thyroid, adrenal cortex, pituitary, choroid plexus, small-intestinal villi, and epididymal adipose tissue(Kamba et al., 2006). These results indicate that VEGF functions in vascular preservation, a notion supported by the results of the present study. The results that vascular smooth muscle cells stained with both VEGF and flt-1 antibodies were consistent with those reported by Berse *et al. (Berse et al., 1992)*. Those suggested that VEGF plays a role in maintaining not only the vascular endothelium but also vascular smooth muscle cells by autocrine and paracrine manners. The expression levels of VEGF164, flt-1, and flk-1 mRNA among tissues tended to correlate in the present study, indicating that they are closely associated in canine tissues. flk-1 has more tyrosine kinase activity than flt-1. This suggested flk-1 is the main receptor that mediates the angiogenic effect of VEGF and flt-1 adjust the signaling (Fong et al., 1995) (Shalaby et al., 1995) (Hiratsuka et al., 1998). But some reports have demonstrated that increased flt-1 correlates with that of VEGF and tumor grade(Pallares et al., 2006). Yonemaru *et al. (Yonemaru et al., 2006)* recently reported that VEGF and its receptors might be associated with the malignant proliferation of canine hemangiosarcoma. In addition, flt-1 plays

an important role in the pulmonary metastasis of tumors(Hiratsuka et al., 2002). Therefore tumor characteristics might be better understood by studying flt-1 expression. The present study showed that intensely VEGF or flt-1 immunopositive tissues also expressed high levels of their mRNAs, indicating that quantifying these mRNAs would help to evaluate angiogenesis in canine tissues. Approaches that target VEGF might become a therapeutic strategy against canine tumors, and advances in understanding the role of VEGF in normal physiology will provide insight into the basis of side-effects attributed to the administration of VEGF inhibitors.

Table 1

Sequence of primers and probes for RT-PCR or real-time RT-PCR

Gene	Sequence	Amplicon size
VEGF (all isoforms) F	5'-TTCTGTATCAGTCTTTCCTGGTGAG-3'	405, 534, 606 bp
VEGF (all isoforms) R	5'-CGAAGTGGTGAAGTTCATGGATG-3'	
VEGF164 F	5'-CCCACTGAGGAGTTCAACATCAC-3'	143 bp
VEGF164 R	5'-CAGGGATTTTCTTGCCTTGCT-3'	
VEGF164 probe	5'-TGCGGATCAAACCTCATCAAGGCC-3'	
flt-1 F	5'-GATGCACAGTGAAATACCCGAAA-3'	147 bp
flt-1 R	5'-CAGGTTATTCGCTTCCCATCA-3'	
flt-1 probe	5'-AGATCGTCATCCCCTGCCGGGT-3'	
flk-1 F	5'-CGTGATATCTCTGGTTGTGAATGTC-3'	149 bp
flk-1 R	5'-GCCAGTACCAGCGGATGTG-3'	
flk-1 probe	5'-TGGCACCACGCAGTCGCTGAC-3'	
GAPDH F	5'-GGGGCCATCCACAGTCTTCT-3'	229 bp
GAPDH R	5'-GCCAAAAGGGTCATCATCTC-3'	

Table 2A. Results of immunohistochemistry for VEGF in normal canine tissues.

	Dog no.					
	1	2	3	4	5	6
g						
pe I alveolar cells	++	++	+	+	++	++
pe II alveolar cells	+++	+++	+++	+++	+++	+++
onchial epithelium	+++	++	++	++	++	+++
onchiolar smooth muscle	++	++	++	++	++	++
ney						
omerulus	–	–	–	–	–	–
oximal tubule	+	+	+	+	++	+
stal tubule	+	+	+	+	++	+
llecting tubule	++	+	++	+	++	+
rt	++	++	++	++	+++	++
enal gland						
omerulosa	++	+	++	+	++	++
sciculate	+	+	+	+	++	+
ticularis	++	+	+	+	+	++
edulla	–	+	–	–	–	–
er						
patocytes	+	+	+	+	++	+
le ducts	+	+	+	+	++	+
pffer cell	++	+	++	+	++	+
n						
idermis/hair	++[a]	++[a]	++[a]	++[a]	++[a]	++[a]
baceous glands	++	++	++	+	++	++
ocrine glands	+++	++	+++	++	+++	+++
rector muscle	++	++	++	++	+++	++
roid	–	–	–	NA	–	–
stine						
icrovilli	–	–	–	–	–	–
terocyte	+	–	+	+	+	–
uscularis	+	+	++	+	++	+
rve plexus	–	–	–	–	+	–
dder						
ithelium	++	++	++	++	++	++
uscularis	+	+	+	+	++	+
nph node						
cell region	+	+	+	–	+	+
cell region	+	–	+	–	+	+
creas						
ini	–	–	–	–	–	–
cts	–	–	–	–	+	–
ets	–	–	–	+	–	–
een	–	–	–	–	–	–

– = negative; + = weak; ++ = intermediate; +++ = strong reaction; NA = not available

a) upper follicles: strongly positive

Table 2B. Results of immunohistochemistry for flt-1 in normal canine tissues.

	Dog no.					
	1	2	3	4	5	6
Lung						
Type I alveolar cells	++	++	++	++	++	++
Type II alveolar cells	+++	+++	+++	+++	+++	+++
Bronchial epithelium	++	++	++	++	++	++
Bronchiolar smooth muscle	++	+++	++	+++	++	++
Kidney						
Glomerulus	–	–	–	–	–	–
Proximal tubule	+	++	+	+	+	+
Distal tubule	++	+	++	+	++	+
Collecting tubule	++	+	+	+	++	++
Heart	++	++	++	+	++	++
Adrenal gland						
Glomerulosa	+	+	+	+	++	+
Fasciculate	++	++	++	++	++	++
Reticularis	++	++	+	++	+	++
Medulla	+	+	+	+	+	+
Liver						
Hepatocytes	++	++	++	++	++	+
Bile ducts	++	++	+	++	+	+
Kupffer cell	–	–	–	–	–	–
Skin						
Epidermis/hair	++[a]	+ - ++[a]	++[a]	++[a]	+[a]	++[a]
Sebaceous glands	++	++	++	+	++	++
Apocrine glands	++	++	++	++	++	++
Arrector muscle	++	++	++	++	++	++
Thyroid	–	– - +	–	NA	–	–
Intestine						
Microvilli	++	+++	++	++	+++	+++
Enterocyte	–	–	–	–	–	–
Muscularis	+	+	++	+	+	+
Nerve plexus	++	++	++	+	++	++
Bladder						
Epithelium	++[b]	++[b]	++[b]	++[b]	++[b]	++[b]
Muscularis	++	++	++	++	++	++
Lymph node						
T-cell region	++	++	+	+	+	+
B-cell region	–	–	–	–	–	–
Pancreas						
Acini	–	–	–	–	–	–
Ducts	+	+	+	+	–	+
Islets	+	+	–	+	+	+
Spleen	–	–	–	–	–	–

– = negative; + = weak; ++ = intermediate; +++ = strong reaction; NA = not available

a) lower follicles: strongly positive

b) umbrella cells: strongly positive

Table 2C. Results of immunohistochemistry for VEGF in normal canine tissues.

	Dog no.			
	7	8	9	10
Mammary epithelial cell	+	+	− - +	−
Mammary myoepithelial cell	−	− - +	−	−

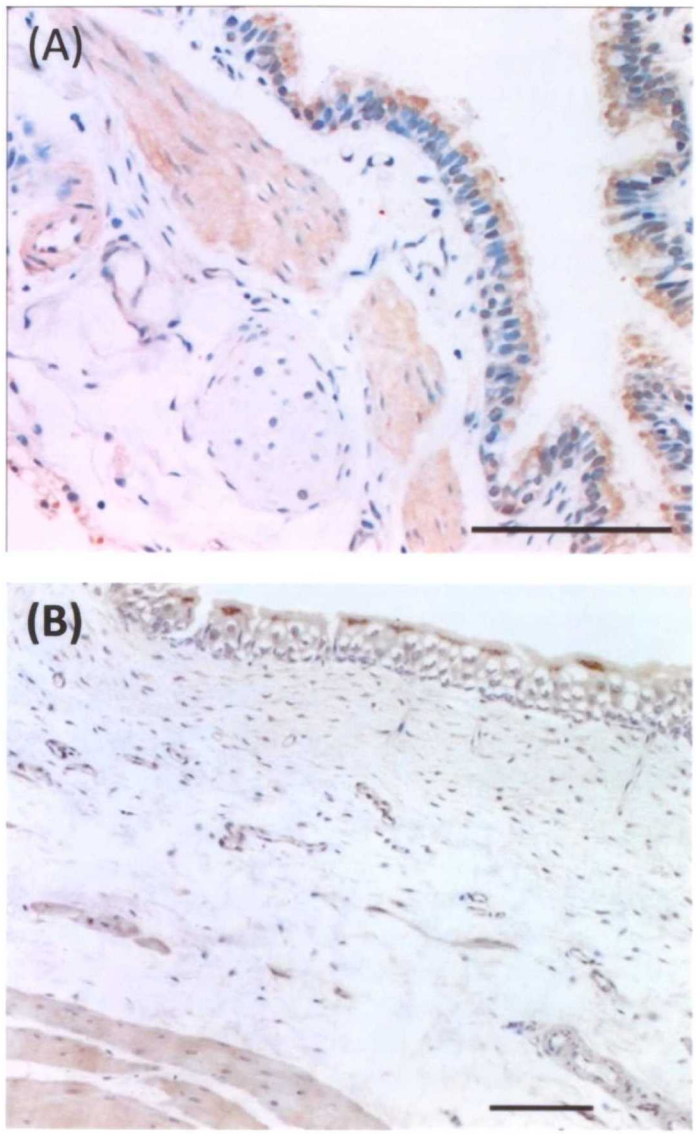
− = negative; + = weak; ++ = intermediate; +++ = strong reaction

Table 2D. Results of immunohistochemistry for flt-1 in normal canine tissues.

	Dog no.			
	7	8	9	10
Mammary gland	++	++	+	++
Mammary myoepithelial cell	−	−	−	−

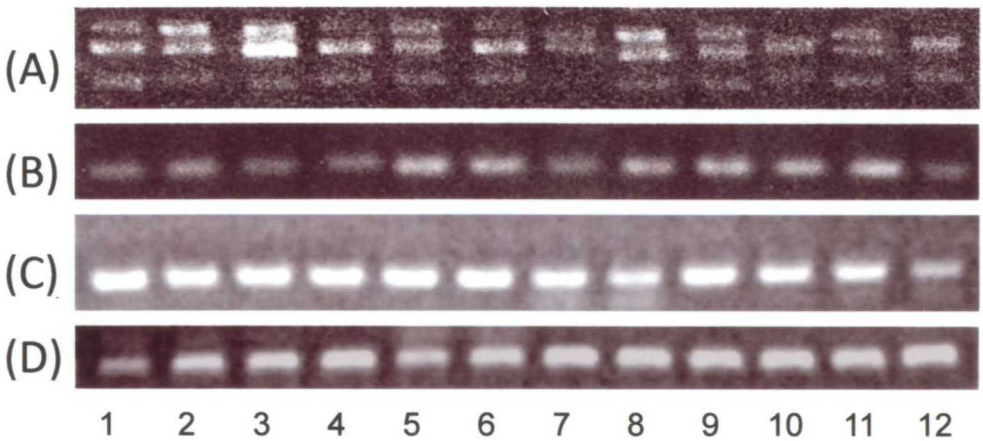
− = negative; + = weak; ++ = intermediate; +++ = strong reaction

Figure 1



(A) Dog 1. Immunohistochemical staining for VEGF in lung. Bronchiolar epithelial cells, bronchiolar smooth muscle layer and vascular smooth muscle in the lungs are immunopositive for VEGF. Bar = 100μm. (B) Dog 1. Immunohistochemical staining for flt-1 in urinary bladder. The transitional epithelium, umbrella cells, and vascular smooth muscle cells are immunopositive for flt-1. Bar = 100μm.

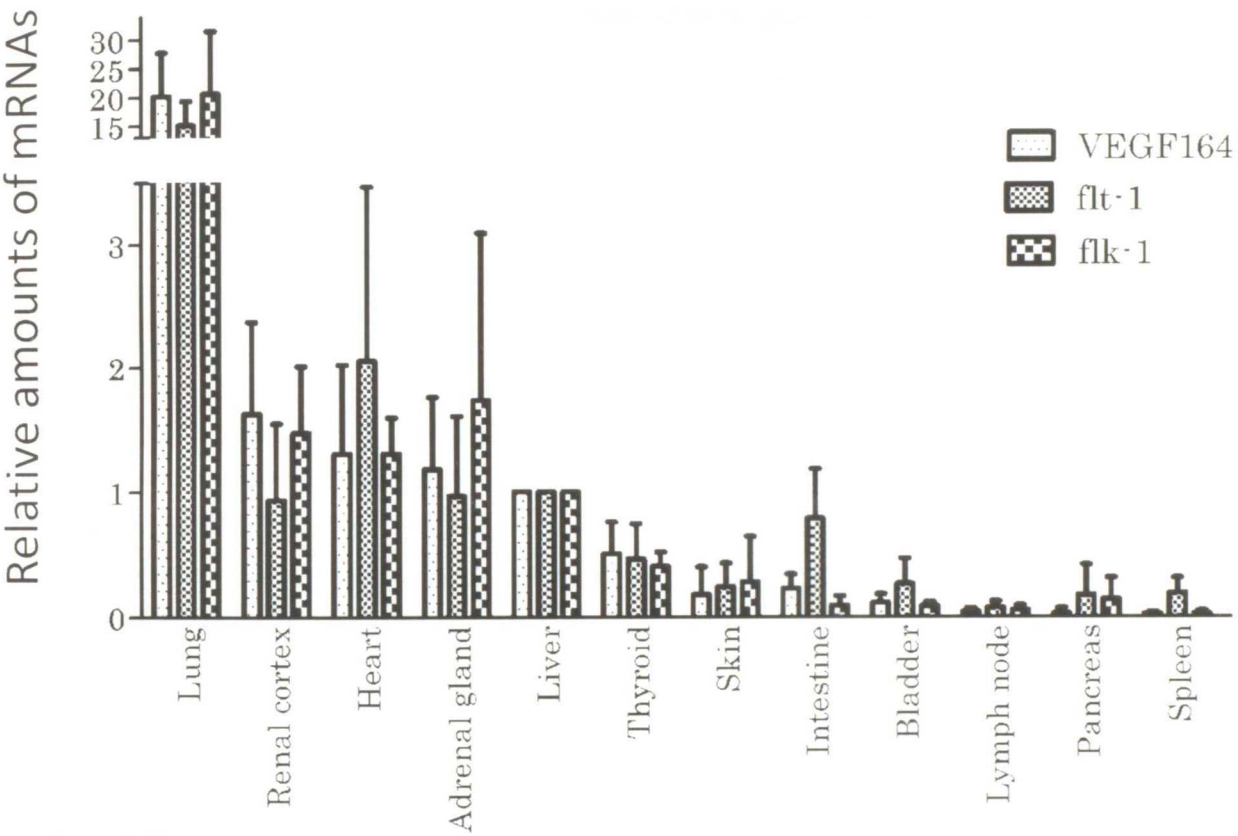
Figure 2



RT-PCR findings of dog 6. Tissues are numbered as follows: 1, lung; 2,renal cortex; 3, heart; 4, adrenals; 5, liver; 6, skin; 7, thyroid; 8, intestinal mucosa[a)]; 9, bladder[a)]; 10, lymph node; 11, pancreas; 12, spleen. (A) RT-PCR for VEGF shows VEGF120 (405 bp), VEGF164 (534 bp), and VEGF188 (606 bp). Among VEGF isoforms, VEGF164 was most intensely stained. (B) RT-PCR for flt-1. All tissues examined expressed flt-1 mRNA. (C) RT-PCR for flk-1. All tissues examined expressed flk-1 mRNA. (D) RT-PCR for GAPDH to verified RNA integrity.

[a)]Intestinal mucosa and bladder specimen obtained by scratching luminal surface.

Figure 3



Expression levels of mRNAs encoding VEGF164, flt-1, and flk-1 in normal canine tissues. The mRNA expression was evaluated by real-time PCR. The data were normalized by 18S ribosomal RNA level in each sample. Levels of each mRNA in tissues are expressed relative to those in liver. The data are shown as means ± SE of six dogs. Intestinal mucosa and bladder specimen obtained by scratching luminal surface.

Chapter 2

Evaluation of endothelial markers in dogs with benign mammary gland tumor

Abstract

Tumor angiogenesis is essential for tumor growth. Although intratumoral microvessel density correlates with prognosis in many tumors, some study showed no correlation between these factors. Recently, it has been demonstrated that endoglin is highly expressed on activated endothelial cells and is suggested as a more useful angiogenesis marker than pan-endothelial markers such as vWF and CD31. In the present study, the author evaluated the expression of vWF mRNA, endoglin mRNA, and proliferation activity in vascular endothelial cells in canine normal, adjacent non-neoplastic, and benign mammary gland tumors. The author also investigated the correlation between the expression of VEGF164 mRNA and the expression levels of vWF or endoglin mRNAs. Expression levels of endoglin and VEGF164 mRNAs were quantified by real-time RT-PCR. Sections of formalin-fixed, paraffin-embedded tissues were analyzed using immunofluorescense double staining for PCNA and vWF. The percentage of endoglin mRNA expression was significantly higher in tumor tissues than adjacent non-neoplastic tissues. The proliferation activity of endothelial cells and the percentage of anti-VEGF positive microvessels were increased from normal to adjacent and from adjacent to tumor tissues. Both the percentage of PCNA-positive endothelial cells and VEGF-positive microvessel were significantly higher in endoglin mRNA positive tissues than those in endoglin mRNA negative tissues. Although the expression levels of endoglin mRNA were positively correlated with both the expression levels of VEGF164 mRNA and vWF mRNA, the correlation between endoglin and VEGF164 mRNAs shows stronger correlation than the correlation between endoglin and vWF mRNAs. Thus, the present study indicates that VEGF may play an important role in the positive regulation of vascular endothelial cell activity and endoglin mRNA may be a better angiogenic marker than vWF mRNA.

Introduction

From the results of chapter 1 that suggest VEGF is regulated at transcriptional level in canine tissues, it is thought that the amount of VEGF mRNA reflects the degree of tumor angiogenesis. To evaluate the degree of angiogenesis, intratumoral microvessel density (IMVD) and the amounts of endothelial marker gene expression are used and many clinical studies have demonstrated the correlation between IMVD or the amount of endothelial marker expression and tumor growth. Although most of these studies showed a positive correlation between IMVD or endothelial marker and prognosis (de Jong et al., 2000) (Weidner et al., 1992), some studies have fail to find these correlation (Siitonen et al., 1995). A possible reason for this discrepancy between these results may be the target of endothelial marker used in the evaluation of angiogenesis. In most reports, antibodies against pan-endothelial cells, such as anti-von Willebrand factor (vWF) and anti-CD31 antibodies, have been used as endothelial markers. These antibodies can react with not only newly formed blood vessels but also normal blood vessels just trapped in the tumor tissues(Miller et al., 1999). The mammary tissues intended in the present study contains abundant vessels in normal conditions and angiogenic evaluation with pan-endothelial marker included these normal blood vessels. Thus, pan-endothelial cell antibodies may not be the ideal markers to evaluate tumor-associated angiogenesis in canine mammary gland tumors.

Proliferation activities of vascular endothelial cells in human breast carcinoma was significantly higher than those in adjacent non-neoplastic tissues (Vartanian and Weidner, 1994). It has been demonstrated that endoglin (CD105) is highly expressed on activated endothelial cells, but weakly or not expressed on normal vessels(Kumar et al., 1996; Wang et al., 1994). Therefore it is suggested endoglin is more useful as an angiogenesis marker than pan-endothelial cell markers. Some tumor-associated markers are known as a post-transcriptional regulator but some angiogenic marker mRNA expressions, including vWF and endoglin, directly correlate with the degree of angiogenesis (Zanetta et al., 2000) (Bellone et al., 2007). Although several studies have demonstrated significant correlation among angiogenic factors and endothelial markers in human tumors, little studies were reported for endoglin expression and proliferation activity of vascular

endothelial cells in canine tumors (Fosmire et al., 2004). Therefore, it is unclear the utility of endoglin as angiogenesis marker in canine mammary tumors.

In this study, the author evaluated the correlation among the expression of vWF mRNA, endoglin mRNA, and proliferation activity in vascular endothelial cells in canine normal, adjacent non-neoplastic, and mammary gland tumors. The author also investigated the correlation among the gene expression of VEGF164, the rate of VEGF-positive microvessel, and the expression levels of vWF and endoglin mRNAs. For these analysis, the author investigated benign mammary gland tumors but not malignant mammary gland tumors because malignant tumor cells tend to infiltrate adjacent structures which may influence the results of adjacent tissues.

Materials and methods

Samples

Samples were classified into 3 groups: (1) 4 mammary gland in 4 healthy female beagle dogs (normal mammary gland), the same as those used in chapter 1, (2) 10 normal mammary glands from 10 tumor bearing dogs (adjacent to tumor) (Dog 5-8, 10, 12-16), these samples were confirmed as normal using HE staining, (3) 13 benign mammary gland tumors (complex adenoma) from 13 dogs, the same dogs used in adjacent tissues. The profiles of the samples are presented in Table 1. All the samples were obtained by surgery at Azabu University Veterinary Teaching Hospital (Kanagawa, Japan) and private clinics. These samples were fixed with 10% formalin or frozen at –80°C prior to analysis.

Immunohistochemistry

Formalin-fixed, deparaffinized tissue sections were immunohistochemically stained using universal immunoenzyme polymer method. The primary antibody were polyclonal rabbit anti-VEGF antibody (A-20; Santa Cruz Biotechnology) diluted 1 : 100. Peroxidase-labeled goat anti-rabbit IgG (Histofine® Simple Stain MAX-PO kit; Nichirei) was used as the second antibody and the immunoreactions was visualized with DAB. Negative controls included normal rabbit IgG (Chemicon, Temecula, CA, USA) instead of primary antibody at the same concentration.

Double immunostaining for proliferating cell nuclear antigen (PCNA) and vWF was also accomplished using fluorescence antibody method. Briefly, deparaffinized sections were incubated with polyclonal rabbit anti-vWF antibody (Dako, Glostrup, Denmark), prediluted, for 2 h. After the samples were rinsed with PBS, they were incubated with FITC-conjugated anti-rabbit IgG antibody (Chemicon), diluted 1 : 500, for 30 min. After incubation, samples were rinsed with PBS and then incubated with monoclonal mouse anti-PCNA antibody (Dako), diluted 1 : 100, for 2 h. After the samples were rinsed with PBS, they were incubated with rhodamine-conjugated anti-mouse IgG antibody (Chemicon), diluted 1 : 200, for 30 min. The samples were then rinsed and mounted with Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA). All reactions were conducted at room temperature. The images were captured using

fluorescence microscopy system.

Gene expression analysis

Total RNA was isolated from the sample stored in a frozen condition with a TRIzol Reagent (Invitrogen) follows the manufacturer's instructions. Genomic DNA was digested by incubation with 1 U/μg DNase I (Promega) for 30 minutes at 37°C. The quantity of RNA was determined using absorption at 260 nm on a spectrophotometer. The first-strand cDNA was reverse transcribed from 2 μg total RNA using the SuperScript First-Strand Synthesis System III for RT-PCR (Invitrogen) primed by an random primer in 20 μl final volume. After reverse transcription, the cDNA was diluted 1:10 with sterile DW and used as a template for the RT-PCR analysis of endoglin and vWF and real-time RT-PCR analysis of endoglin, vWF, and VEGF164. The PCR reaction with Takara Taq (Takara Shuzo) proceeded as follows: denaturation at 94°C for 5min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were separated on 2% agarose gels and stained with ethidium bromide. The specific TaqMan probe (Applied Biosystems) and primer pairs described in Table 2 were designed using Primer Express software (Applied Biosystems) based on their sequence. For analysis of ribosomal 18S RNA, TaqMan ribosomal 18S RNA (Applied Biosystems) were used in this study. All PCR amplifications were carried out in duplicate for each sample, and the mean values of mRNA expression were calculated as the ratio to those of ribosomal 18S RNA. Values are expressed as means ± SE.

Assessment of immunostaining

The percentage of PCNA-positive vascular endothelial cells defined as the ratio of positively stained cells (red) that also had concomitant positive cytoplasmic staining (green) with vWF to the total cell count. For this analysis, more than 200 individual endothelial cell nuclei were counted in each case. The percentage of VEGF-positive microvessels defined as the ratio of VEGF-positive microvessels which have VEGF-positive endothelial cells to total microvessel count. For this analysis, more than 100 microvessels were counted in each case. Values are expressed as means ± SE.

Statistical analysis

Fisher's exact test was used to compare the frequency of endoglin mRNA expression, PCNA-positive endothelial cells, and VEGF-positive microvessels among normal, adjacent, and tumor tissues. The Pearson correlation coefficients was used to assess the relationship among the expression levels of VEGF164, vWF, and endoglin mRNAs and PCNA-positive vascular endothelial cells. A p-value of < 0.05 was considered to be statistically significant difference.

Results

Immunohistochemistry

Proliferation activity of intratumoral vascular endothelial cells were counted using with anti-PCNA antibody and compared to those of normal and adjacent non-neoplastic mammary tissues (Figure 1A). The percentage of positively labeled endothelial cells with anti-PCNA antibody increased from normal (0%) to adjacent tissues (2.6%) and from adjacent to tumor tissues (27.8%). The percentage of anti-VEGF positive microvessels was seen in 0% of normal, 21.2% of adjacent, 60.7% of tumor tissues. Both percentage of PCNA-positive endothelial cells and VEGF-positive microvessels significantly increased from normal to adjacent and from adjacent to tumor tissues (Table 3).

endoglin mRNA expression

The percentage of vWF and endoglin mRNAs expression were assessed and compared among normal, adjacent non-neoplastic, and tumor mammary tissues. Although vWF mRNA expression was detected in all samples examined, endoglin mRNA expression was detected in 1 of 4 (25.0%) normal, 4 of 10 (40.0%) adjacent non-neoplastic, and 11 of 13 (84.6%) tumor tissues (Table 3) (Figure 1B). The percentage of endoglin mRNA expression was significantly higher in tumor tissues than adjacent non-neoplastic tissues.

Correlation between the expression of endoglin mRNA and the percentage of VEGF-positive microvessels or PCNA-positive vascular endothelial cells

The percentage of PCNA-positive vascular endothelial cells was 5.4 ± 3.0 in endoglin mRNA negative and 19.9 ± 4.2 in endoglin mRNA positive tissues, respectively (Figure 2A). The percentage of VEGF-positive microvessels was 13.2 ± 3.9 in endoglin mRNA negative and 51.9 ± 6.5 in endoglin mRNA positive tissues, respectively (Figure 2B). Both the percentage of PCNA-positive endothelial cells and VEGF-positive microvessels were significantly higher in endoglin mRNA positive tissues than those in endoglin mRNA negative tissues ($p<0.05$).

Compare the correlation between the endothelial marker and proliferating

marker

Two samples (1 adjacent tissue and 1 tumor tissue) were not detectable by real-time RT-PCR. Expression levels of endoglin mRNA were positively correlated with the percentage of PCNA-positive endothelial cells (Spearman's $r = 0.699$, $p < 0.05$), although vWF mRNA did not show significant correlation (Spearman's $r = 0.625$ and $p > 0.05$) (Figure 3). Although the expression levels of endoglin mRNA were positively correlated with the both expression levels of VEGF164 mRNA (Spearman's $r = 0.783$, $p < 0.05$), and vWF mRNA (Spearman's $r = 0.643$ and $p < 0.05$) (Figure 4), the correlation between endoglin and VEGF164 mRNAs shows stronger correlation than the correlation between endoglin and vWF mRNAs.

Discussion

Angiogenesis plays an essential role in tumor progression providing the nutrients and growth factors for growth. Many experimental models and many studies in human tumors have shown that tumor-associated angiogenesis involves endothelial cell proliferation (Vartanian and Weidner, 1994) (Vartanian and Weidner, 1995) (Hobson and Denekamp, 1984). In human cancers, studies of endothelial cell proliferation using breast cancer have been reported (Edel et al., 2000) (Vartanian and Weidner, 1994). The expression of endoglin, appears exclusively associated with the endothelial cells in the newly formed blood vessels and in the immature tumor vessels, have been investigated as a vascular endothelial marker and showed positive correlation with prognosis (Minhajat et al., 2006) (Fonsatti et al., 2001). The present study investigated the gene expression of endoglin and correlation between endoglin and proliferation activity and VEGF expression on microvessels in benign mammary gland tumor of dogs.

The present study has shown that the percentage of endoglin mRNA expression was significantly higher in tumor tissues compared to non-neoplastic adjacent tissues although vWF mRNA expression was detected in all tissues examined. This results suggest endoglin mRNA is weakly or not at all expressed on normal vessels and the expression of endoglin is regulated at transcriptional level in canine mammary tumors, which is congruent with human cancers(Bellone et al., 2007; Miller et al., 1999).

In this study, the author found significantly elevated both PCNA-positive vascular endothelial cells and VEGF-positive microvessels in tumor tissues compared to non-neoplastic mammary gland tissues. This finding indicates that, in canine mammary gland tumor associated angiogenesis, proliferation activity of vascular endothelial cells are up-regulated in tumor tissues and endothelial cells in tumor tissues may be activated by VEGF. The present study has also shown that the positive correlation between endoglin mRNA expression and PCNA-positive vascular endothelial cells. This result is accordance with those reported for human breast cancer that endoglin is a sensitive marker for microvessel proliferation (Bodey et al., 1998). The present study has also shown that the positive correlation between endoglin mRNA expression and both the

percentage of VEGF-positive and PCNA-positive endothelial cells. These results suggest endoglin mRNA may be a useful angiogenic marker in canine mammary tumors because PCNA-positive and VEGF-positive endothelial cells are thought to be endothelial cells of newly formed blood vessels. The expression level of endoglin mRNA showed stronger correlation with VEGF164 mRNA expression compared with the correlation between the expression levels of vWF and VEGF164 mRNAs. These results were consistent with the findings that VEGF is one of the strongest endothelial mitogens (Kushlinskii and Gershtein, 2002) and endoglin expresses on activated endothelial cells. These results also suggest the expression levels of VEGF164 mRNA seem to be directly associated with VEGF functions.

Finally, the present study indicates that endoglin mRNA may be a useful angiogenic marker than vWF mRNA in canine mammary tumors.

Table 1

Details of 17 dogs and their diagnosis and the size of tumor

Dog no.	Sex	Age (years)	Breed	diagnosis	tumor size (cm)
1	female	ND	beagle	Normal	–
2	female	ND	beagle	Normal	–
3	female	ND	beagle	Normal	–
4	female	ND	beagle	Normal	–
5	spayed	14	mongrel	Complex adenoma	4 x 3 x 2
6	female	9	miniature dachshund	Complex adenoma	3 x 2 x 1
7	female	11	golden retriever	Complex adenoma	1 x 1 x 1
8	female	10	Kishu	Complex adenoma	1 x 1 x 1.5
9	female	3	American cocker spaniel	Complex adenoma	1.2 x 1.2 x 0.8
10	female	14	pomeranian	Complex adenoma	ND
11	female	8	welsh corgi	Complex adenoma	ND
12	female	8	shih tzu	Complex adenoma	3 x 2 x 2
13	female	1	shih tzu	Complex adenoma	5 x 3 x 2
14	female	6	papillon	Complex adenoma	1 x 1 x 1
15	female	10	miniature dachshund	Complex adenoma	ND
16	female	ND	shiba	Complex adenoma	5 x 3 x 2
17	spayed	8	shiba	Complex adenoma	ND

ND = no data

Table 2

Sequence of primers and probes for PCR and real-time RT-PCR

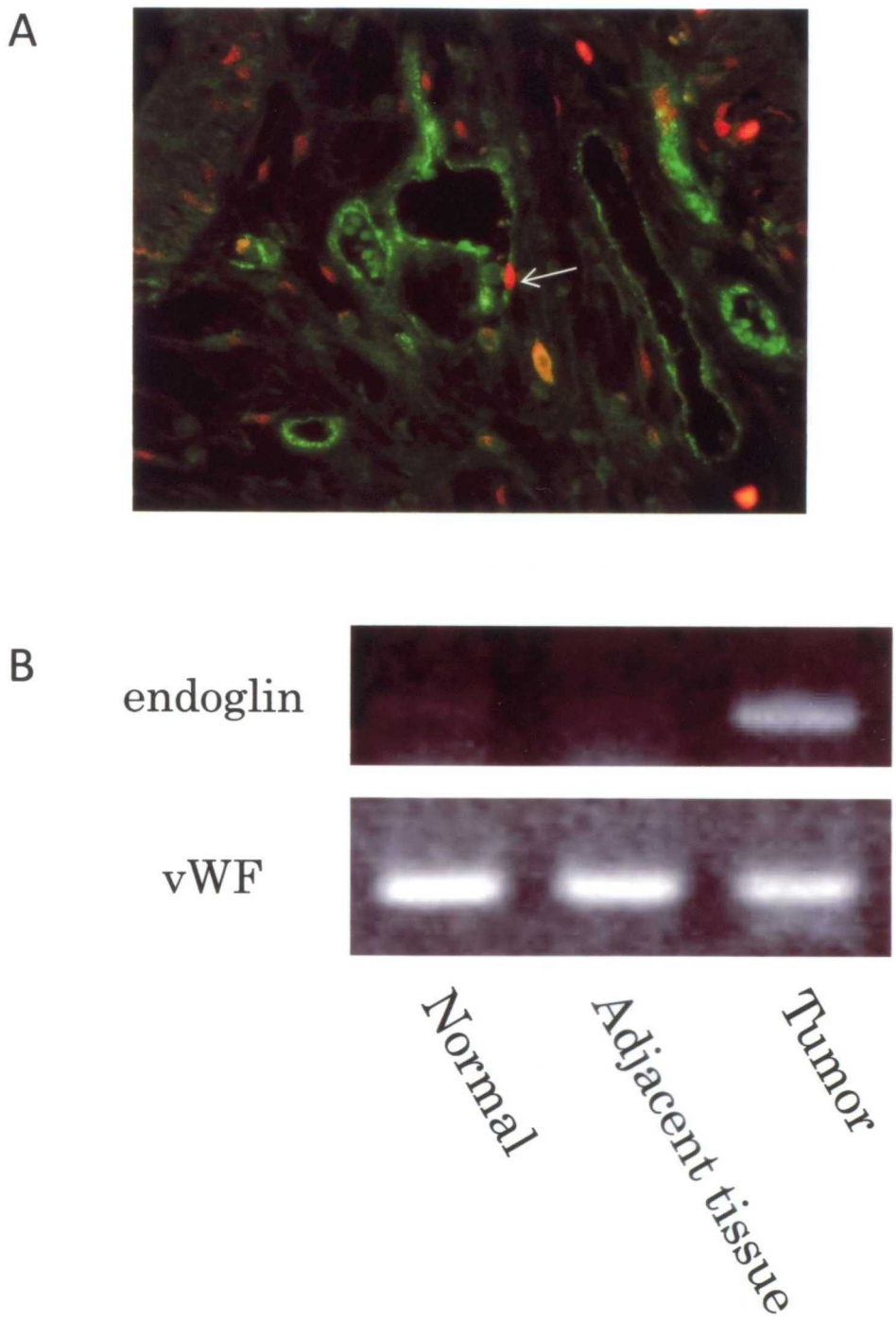
Gene	Sequence	Amplicon size
VEGF164 F	5'-CCCACTGAGGAGTTCAACATCAC-3'	143 bp
VEGF164 R	5'-CAGGGATTTTCTTGCCTTGCT-3'	
VEGF164 probe	5'-TGCGGATCAAACCTCATCAAGGCC-3'	
vWF F	5'-AAAGCGGTGGTTATCCTAGTCACA-3'	112 bp
vWF R	5'-ACCGATCCCCGATTCCAA-3'	
vWF probe	5'-AGGCCGCCAGATCCAACCGAGT-3'	
endoglin F	5'-CCGGAAACCCACAGAACTATCT-3'	84 bp
endoglin R	5'-CACAGCGGGCAGGACAAG-3'	
endoglin probe	5'-CAGCCCTGGCCTGCCTGACAA-3'	

Table 3

	No. of Samples	Percentage of endoglin mRNA expression	Positive rate	
			PCNA	VEGF
Normal	4	25.0%	0%	0%
Adjacent	10	40.0%	2.6 ± 0.65%*	21.2 ± 3.37%*
Tumor	13	84.6%†	27.8 ± 2.77%*†	60.7 ± 4.19%*†

Percentage of endoglin mRNA expression, PCNA-positive endothelial cells, and VEGF-positive microvessels. The data are shown as mean ± S.E. * $p<0.05$ vs normal, † $p<0.05$ vs adjacent tissues.

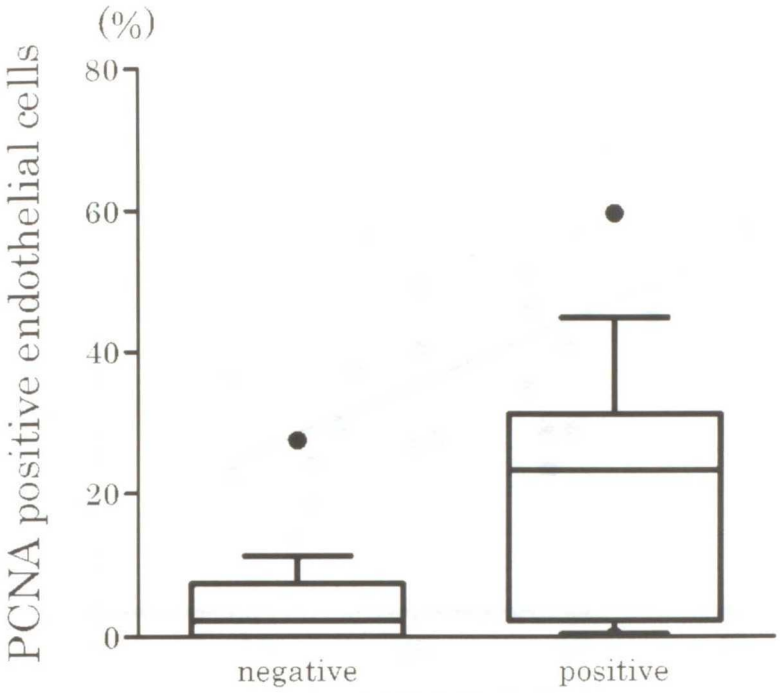
Figure 1



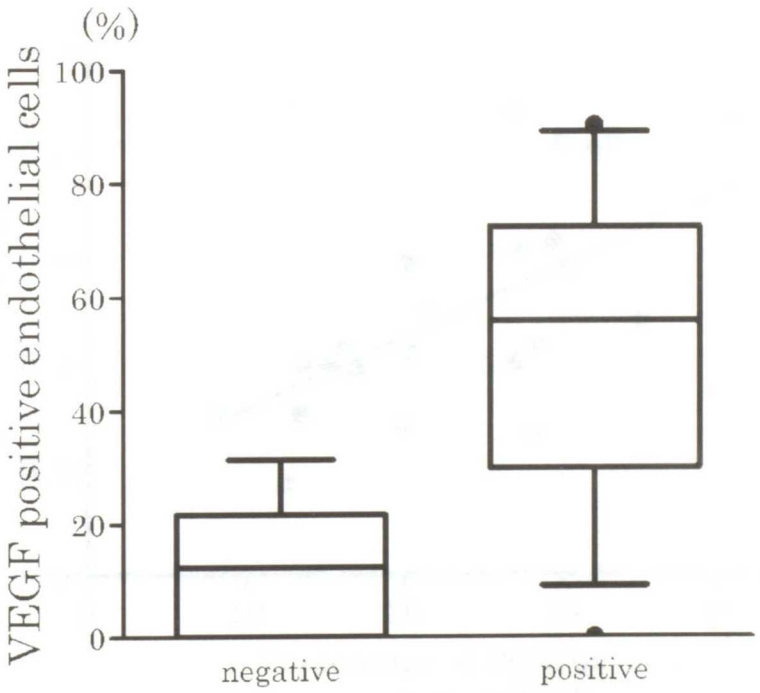
A) Dog 12. Immunofluorescense double staining of mammary tumor tissue for PCNA and vWF. Combined images of PCNA-immunostaining (red) and vWF-immunostaining (green). The arrow shows PCNA-positive endothelial cells. B)RT-PCR. Expression of endoglin and vWF mRNAs in normal, adjacent, and tumor tissues.

Figure 2

A



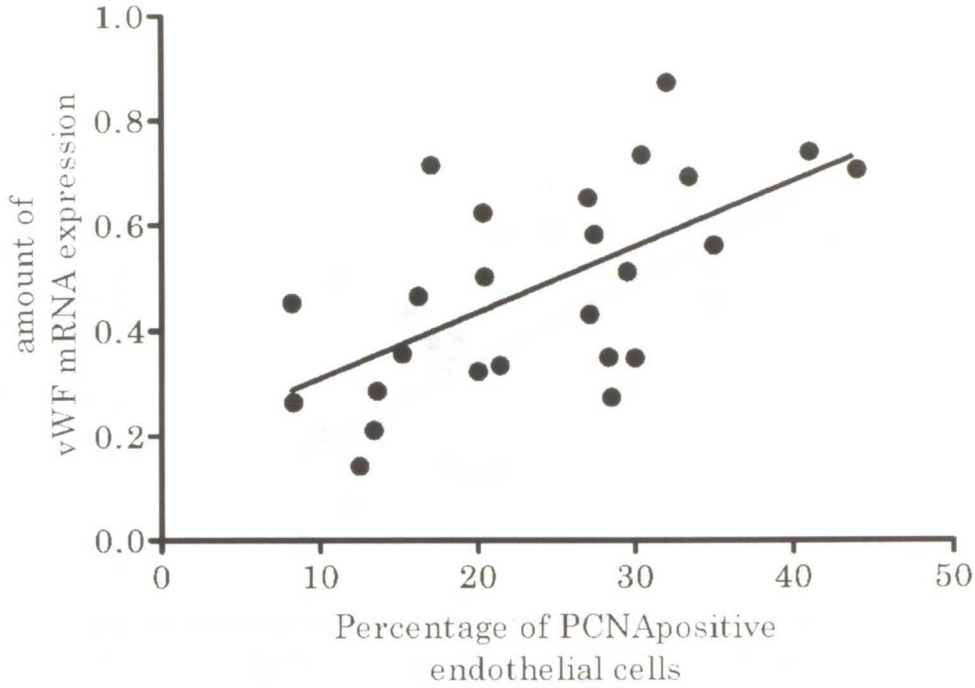
B



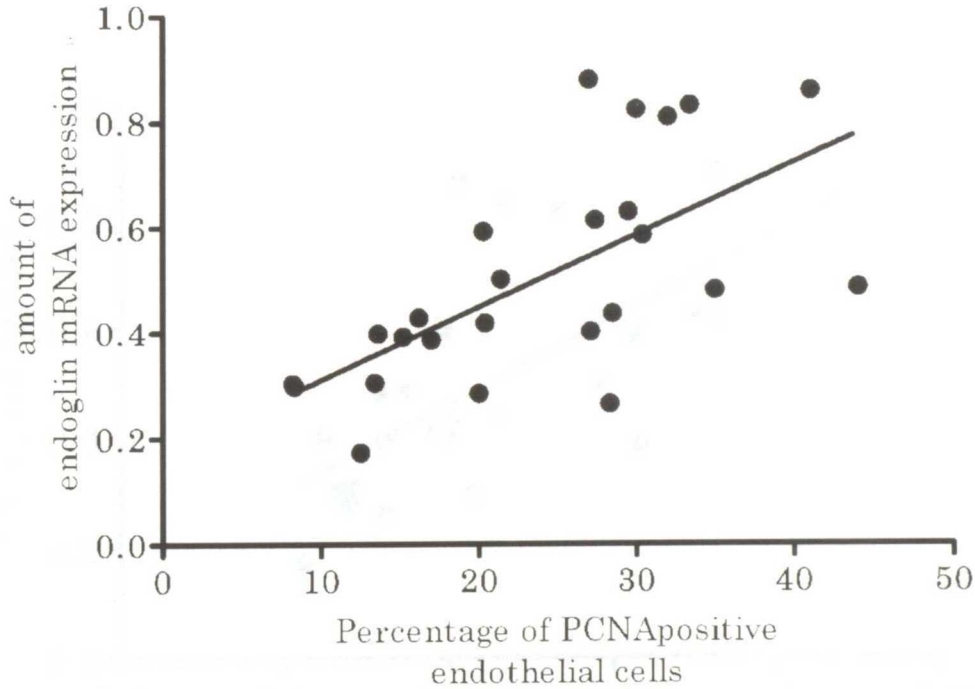
Box plot representing the median value, 25% and 75%, 10% and 90% and observations < 10% or > 90%. A) Correlation between the expression of endoglin mRNA and the rate of PCNA-positive endothelial cells. endoglin mRNA-positive tissues showed high rates of PCNA positivity ($p<0.05$). B) Correlation between the expression of endoglin mRNA and the rate of VEGF-positive microvessels. endoglin mRNA-positive tissues showed high rates of VEGF positivity ($p<0.05$). These data were obtained from endoglin mRNA-positive tissues ($n = 11$) and endoglin mRNA-negative tissues ($n = 14$).

Figure 3

A

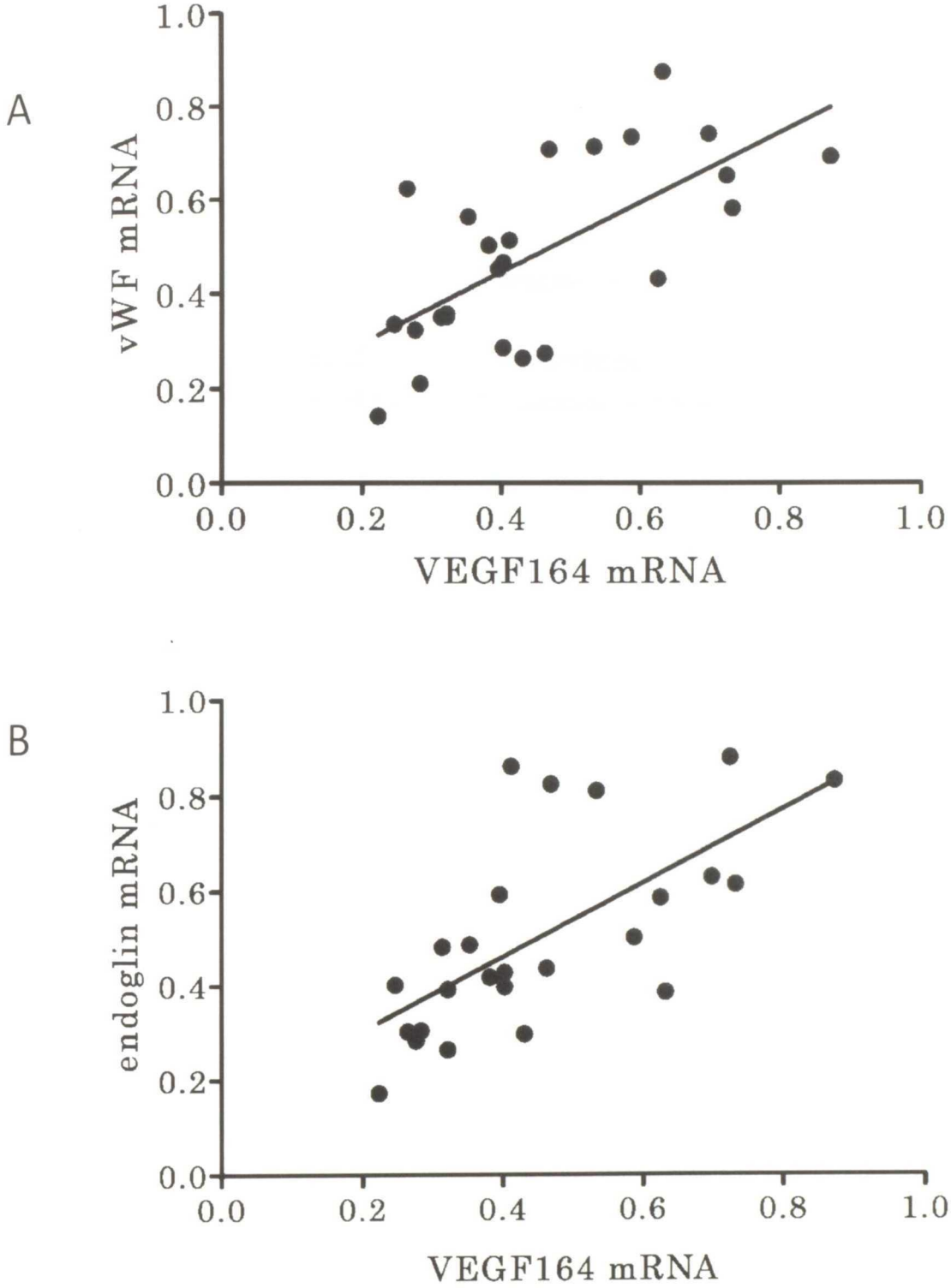


B



A) Correlation between the expression levels of vWF mRNA and percentage of PCNA positive endothelial cells. (Pearson's r=0.625, p=0.096)
B) Correlation between the expression levels of endoglin mRNA and percentage of PCNA positive endothelial cells. (Pearson's r=0.699, p=0.043)

Figure 4



A) Correlation between the expression levels of vWF and VEGF164 mRNAs. (Pearson's r=0.643, p=0.048)

B) Correlation between the expression levels of endoglin and VEGF164 mRNAs (Pearson's r=0.783, p=0.017)

Chapter 3

Expression of vascular endothelial growth factor and its related factors in canine mammary tumors

Abstract

The growth of solid tumors is dependent on angiogenesis. The early investigator demonstrated that VEGF and flk-1 correlated with intratumoral microvessel density and were greater in malignant than in benign mammary gland tumors of dog, however, little is known about expression of flt-1 and expressions of VEGF, flt-1, and flk-1 mRNAs in canine mammary tumors. Solid tumor tissues have been used for quantitative analysis of angiogenic factor gene expression, but these tissues are composed of two distinct compartments, the neoplastic cells themselves and tumor stroma. The recently developed techniques of laser microdissection (LMD) and quantitative RT-PCR are powerful tools that allow the measurement of mRNA expression levels in tumor cells and stroma separately. Although several factors regulate VEGF expression, the main stimulation factor for VEGF expression is hypoxia. Intratumoral hypoxia is a potent inducer of tumor angiogenesis. Hypoxia-inducible factor 1 (HIF-1) is one of the most important factors induced by hypoxia. Overexpression of HIF-1α is reported in many types of cancers in humans and HIF-1α considered to be a transcriptional activator of VEGF in cancer cell lines. However, only few studies report about the expression of HIF-1α in canine tumors. Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandins, which contributes to the regulation of angiogenesis. COX-2 is highly expressed in many canine tumors, including mammary tumors, however little is known about the correlation between COX-2 and tumor-associated angiogenesis in canine mammary tumors. In the present study, the author investigated the expression levels of VEGF164, flt-1, and flk-1 mRNA and angiogenesis marker, microvessel density and expression levels of endoglin mRNA, in benign and malignant canine mammary tumors to evaluate the potential as a malignancy markers. The author also investigated the relationship among HIF-1α, COX-2, and VEGF in canine mammary tumors. In addition, the author examined the mRNA expression levels in neoplastic cells and stromal cells separately using laser microdissection method. The percentage of VEGF positive cells increase from adjacent to benign, and from benign to malignant tumor tissues however those of flt-1 were not increased significantly. The amount of VEGF164 and flk-1 mRNA in tumor tissues increased from adjacent tumor tissues although

flt-1 has not shown significant difference among adjacent, benign, and malignant tissues. The amounts of VEGF164 mRNA in tumor epithelial cells of benign and malignant tumors were significantly higher than mammary epithelial cells of adjacent mammary tissues although there is no significant difference between benign and malignant tumor tissues. The present study showed significant correlation between the expression levels of HIF-1α and VEGF164 or COX-2 mRNA although there is no correlation between VEGF164 and COX-2 mRNA expressions. Microvessel density and expression levels of endoglin mRNA were greater in benign and malignant tumors than in adjacent tissues although there is no significant difference between benign and malignant tumors. These results indicates that VEGF in canine mammary tumors is mainly expressed in tumor epithelial cells but not in stromal cells and suggests VEGF expressed in tumor epithelial cells binds flk-1. Although it is not shown the correlation between tumor aggressiveness and the gene expression levels of VEGF164, flt-1, flk-1, COX-2, and HIF-1α, the expression of VEGF164, flk-1, and COX-2 mRNAs are increased in canine mammary tumors. These results indicate these angiogenesis related factors plays an important role in canine mammary tumors. The author also suggests the COX-2/HIF-1α/VEGF pathway possibly playing an important role in canine mammary tumors.

Introduction

It is well known that solid tumors, including mammary tumors, require the growth of new blood vessels into them in order to supply oxygen and nutrients(Folkman, 1990). VEGF is one of the most important factors that stimulate angiogenesis and is considered as a prognostic factor and target of drug therapy(Gasparini et al., 1997; Grunstein et al., 1999; Klement et al., 2000; Soh et al., 2000). Therefore, further detailed analysis of the mechanism of angiogenesis in tumor tissues is very important. In canine mammary tumors, VEGF and its receptor flk-1 have also been detected and discussed as factors of tumor aggressiveness (Restucci et al., 2002) (Restucci et al., 2004). However, little is known about expression of flt-1 and expression of VEGF, flt-1, and flk-1 mRNAs.

Solid tumor tissues have been used for quantitative analysis of angiogenic factor gene expression, but these tissues are composed of two distinct compartments, the neoplastic cells themselves and tumor stromal cells including inflammatory cells, fibroblast, and endothelial cells. From the result of chapter 1 that many type of cells, including stromal cells, express VEGF in normal tissues, it is thought that VEGF is expressed not only tumor cells but also stromal cells in tumor tissues. Therefore, it is unclear that up-regulation of VEGF gene expression in tumor tissue is attributed to tumor cells and/or stromal cells. The recently developed techniques of laser microdissection (LMD) and quantitative RT-PCR are powerful tools that allow the measurement of mRNA expression levels in tumor cells and stroma separately.

Although several factors regulate VEGF expression, the main stimulation factor for VEGF expression is hypoxia(Wenger and Gassmann, 1997). Hypoxia-inducible factor 1 (HIF-1) is one of the most important factors induced by hypoxia (Wang et al., 1995). HIF-1 is a heterodimeric protein that consists of two subunits, HIF-1α and HIF-1β. While HIF-1β is constitutively expressed, HIF-1α is targeted to proteosome degradation under normal oxygen conditions and is rapidly induced under hypoxic conditions. Overexpression of HIF-1α is reported in many types of cancers in humans (Ioachim et al., 2006) (Maxwell et al., 1997) and HIF-1α considered to be a transcriptional activator of VEGF in cancer cell lines (Jones et al., 2001). However, only few studies report about the expression of HIF-1α in

canine tumors.

Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandins and two isoforms of COX have been characterized. While COX-1 is expressed constitutively in many tissues and many cell types, COX-2 is usually absent in normal cells but can be induced by several factors, such as growth factors, cytokines, and tumor promoters. Some studies have suggested that COX-2 and prostaglandin (PG) E_2, one of major (PG) products, is a potent inducer of angiogenic factor, including VEGF, in tumors (Form and Auerbach, 1983) (Wang et al., 2005). It is reported that COX-2 is highly expressed in many canine tumors, including mammary tumors (Kirkpatrick et al., 2002) (Queiroga et al., 2007), but little is known about the correlation between COX-2 and tumor-associated angiogenesis in canine tumors.

The aims of this chapter were to clarify the expressions and the distributions of VEGF and VEGF-related factors and the relationship between VEGF and its regulator, HIF-1α and COX-2, and to evaluate the relationship between tumor aggressiveness and expression of angiogenesis factors in caine mammary tumors. For these objectives, the author investigated the expression levels of VEGF164, flt-1, flk-1, HIF-1α, COX-2, and endoglin mRNAs, immunohistochemical expressions of VEGF, flt-1, and COX-2, and IMVD in normal, adjacent, benign and malignant canine mammary tumors to evaluate the potential as markers of tumor aggressiveness. In addition, the author examined the expression levels of neoplastic cells and stromal cells separately using LMD and quantitative RT-PCR methods.

Materials and methods

Samples

Samples were classified into 4 groups: (1) 4 mammary glands in 4 healthy female beagle dogs (normal mammary gland), the same as those used in chapter 1 and 2, (2) 18 normal mammary glands from 18 tumor-bearing dogs (adjacent to tumor) (Dog 5-8, 10, 12-16, 18-20, 22, 24, 25, 27, 28), these samples were confirmed as normal using HE staining and a part of these samples were the same as those used in chapter 2, (3) 13 benign mammary gland tumors diagnosed as complex adenoma from 13 dogs, the same as those used in chapter 2, (4) 11 malignant mammary gland tumors diagnosed as tubulopapillary carcinoma from 11 dogs. Mammary tumors were diagnosed according to the WHO classification system of canine mammary tumors (Misdorp, 1999). The profiles of the samples are shown in Table 1. All the samples were obtained by surgery at Azabu University Veterinary Teaching Hospital (Kanagawa, Japan) and private clinics. These samples were fixed with 10% formalin or frozen at -80°C prior to analysis.

Immunohistochemistry

All samples were fixed in 10% formalin and embedded in paraffin. Sections (4 μm in thick) were deparaffinized in xylene and hydrated in graded alcohols. For VEGF and flt-1, no pretreatment was used. For COX-2, before the immunohistochemical procedure, tissue samples in 0.01M citrate buffer (pH 6.0) were heated for 10 min in a microwave oven. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in absolute methanol for 20 minutes. The primary antibodies were polyclonal rabbit anti-VEGF antibody (A-20; Santa Cruz Biotechnology), diluted 1 : 100, polyclonal rabbit anti-flt-1 antibody (C-17; Santa Cruz Biotechnology), diluted 1 : 50, and polyclonal rabbit anti-COX-2 antiserum (Cayman Chemical Company, Ann Arbor, MI, USA), diluted 1 : 300. Tissue sections were incubated with primary antibody overnight at 4°C. After washing, section were incubated with Histofine Simple Stain MAX-PO (Nichirei) for 20 min at room temperature. To demonstrate the immunolabeling, DAB was used as a chromogen, and hematoxylin was used as a counterstain. Negative controls included normal rabbit IgG instead of primary antibody at the same concentration.

Total RNA extraction

Total RNA was isolated from the normal canine tissues with a TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) follows the manufacturer's instructions. Genomic DNA was digested by incubation with 1 U/μg DNase I (Promega, Madison, WI, USA) for 30 minutes at 37°C. The quantity of RNA was determined using absorption at 260 nm on a spectrophotometer. The first-strand complementary DNA (cDNA) was reverse transcribed from 2 μg total RNA using the SuperScript First-Strand Synthesis System III for RT-PCR (Invitrogen) primed by an random primer in 20 μl final volume. After reverse transcription, the cDNA was diluted 1:10 with sterile distilled water (DW) and used as a template for the real-time PCR analysis.

Laser microdissection

Frozen tissue samples embedded in Tissue-Tek OCT compound (SAKURA Finetechnical, Tokyo, Japan) were cut into 8 μm sections in a cryostat. These frozen sections mounted on glass slides covered with PEN foil (Leica Microsystems, Wetzlar, Germany) were fixed using air drying for 1 min, with 70% ethanol for 15 sec, and then washed with ultrapure water for 15 sec. The sections were stained with 0.05% toluidine blue solution, pH 4.1, (Wako Pure Chemical Industries, Osaka, Japan) for 20 sec , and washed twice with ultrapure water for 15 sec each. The stained sections were air dried and tumor epithelial cells and stromal cells were collected into two separate tube caps filled with an appropriate volume of buffer RLT (RNeasy Plus Micro Kit) (Qiagen, Valentia, Calif., U.S.A.) with the Laser Microdissection System (Leica Microsystems) (Figure 1). Fig. 4–1 shows TB–stained sections before and after LMD. The total RNAs were extracted using RNeasy Plus Micro Kit according to the manufacturer's instructions. cDNAs for quantitative PCR were synthesized from 12 μl of the total RNA solution using a Sensiscript RT kit (Qiagen), 1μM random primer (Invitrogen), and 10U of RNasin ribonuclease inhibitor (Promega) in a 20μl final volume.

Quantitative PCR

The specific TaqMan probe (Applied Biosystems) and primer pairs described in table 2. For analysis of ribosomal 18S RNA, TaqMan ribosomal

18S RNA (Applied Biosystems) were used in this study. All PCR amplifications were carried out in duplicate for each sample, and the mean values of mRNA expression were calculated as the ratio to those of ribosomal 18S RNA.

Intratumoral microvessel density

To assess the IMVD, the immunolabelled sections were scanned at low magnification (x100) to identify the areas of the tissues with the highest number of immunolabelled microvessels, so called "hot spot" (Weidner et al., 1991). For microvessel scoring, images of the hotspot areas were captured using picture analysis system (Mac Scope, MITANI Corp.) and 10 fields at high magnification (x400) were selected and counted the density.

Scoring of immunohistochemistry

The percentages of VEGF-, flt-1-, and COX-2-positive neoplastic epithelial cells were defined as the ratio of positively stained cells to the total cell count at high magnification (x400). At least 1000 neoplastic cells were counted.

Statistical analysis

Fisher's exact test was used to compare the percentages of VEGF-, flt-1-, and COX-2-positive normal mammary epithelial cells or neoplastic epithelial cells among normal, adjacent, benign tumor, and malignant tumor tissues and to compare the expression levels of VEGF164 between normal or neoplastic mammary epithelial cells and stroma. The Pearson correlation coefficients were used to assess the relationship among the expression levels of VEGF164, COX-2, and HIF-1α mRNAs. A p-value of < 0.05 was considered to be statistically significant difference.

Results

Immunohistochemistry

Immunohistochemical stainings for VEGF, flt-1, and COX-2 were performed on normal, adjacent, and benign and malignant mammary tumor tissues. In tumor tissues, VEGF was localized in tumor epithelial cells, vascular endothelial cells, vascular smooth muscle cells, and macrophages and showed diffuse pattern in cytoplasm. In normal and non-neoplastic adjacent tissues, VEGF was detected in some vascular smooth muscle cells and macrophages and little in normal mammary epithelial cells. Some tumor specimens showed the strong staining in neoplastic cells that abutting necrotic area (Figure 1). flt-1 was detected not only tumor epithelial cells but also normal mammary epithelial cells diffusely and often restricted in cellular pole. flt-1 was also detected in vascular endothelial cells and vascular smooth muscle cells in all sample tissues (Figure 2). COX-2 was detected in the cytoplasm with diffuse to coarsely granular patterns of tumor epithelial cells, vascular endothelial cells, vascular smooth muscle cells, and macrophages in tumor tissues. In normal and adjacent tissues, COX-2 was detected in macrophages and some normal mammary epithelial cells (Figure 3).

The results of VEGF, flt-1, and COX-2 staining are shown in Figure 4, Figure 5, and Figure 6, respectively. The percentage of VEGF-positive cells significantly increased from normal and adjacent to benign, and from benign to malignant tumor tissues however those of flt-1 was not increased significantly. The percentage of COX-2-positive cells in benign and malignant tissues significantly increased from normal and adjacent tissues, although there is no significant correlation between benign and malignant tumor tissues. In all results of immunostaining, there are no significant correlation between normal and adjacent tissues.

Quantification analysis of gene expression

Eleven samples (4 adjacent tissue and 3 benign and 4 malignant tumor tissue) were not detectable by real-time RT-PCR. Expression levels of VEGF164, flt-1, flk-1, HIF-1α and COX-2 mRNAs in normal, adjacent, benign tumor, and malignant tumor tissues were determined using

quantitative RT-PCR method. However the amount of VEGF164 and flk-1 mRNAs in benign and malignant tumor tissues increase from normal and adjacent tissues, expression level of flt-1 mRNA show no significant difference among normal, adjacent, benign tumor, and malignant tumors. In contrast, the expression level of flt-1 has not shown significant difference among adjacent, benign, and malignant tissues (Figure 7). Although the expression level of HIF-1α mRNA increased from normal and adjacent tissues to benign and malignant tumor tissues (Figure 8), there is no significant correlation among these tissues. The level of COX-2 mRNA expression significantly increased from adjacent to tumor tissues. In all the gene expression examined, there is no correlation between benign and malignant tumor tissues (Figure 9).

LMD

The author determined the amount of VEGF164 mRNA expression in the normal mammary epithelial cells and stroma isolated from non-neoplastic adjacent tissues and tumor epithelial cells and stroma from benign tumor tissues using LMD method (Figure 10). The amounts of VEGF164 mRNA in tumor epithelial cells of benign tumors were significantly higher than normal mammary epithelial cells of adjacent mammary tissues although there is no significant difference between those in stroma from adjacent and tumor tissues (Figure 11).

Intratumoral microvessel density and endothelial marker

Microvessel density was greater in benign and malignant tumors than in normal and adjacent mammary tissues although there is no significant difference between benign and malignant tumor tissues (Figure 12, 13). Similarly, the expression levels of endoglin mRNA was greater in benign and malignant tumors than in normal and adjacent tissues although there is no significant difference between benign and malignant tumors (Figure 14).

Correlation among the expression of HIF-1α, COX-2, and VEGF164 mRNA

A statistically positive correlation was observed between VEGF164 and HIF-1α mRNAs (Spearman's r = 0.5494, p = 0.0036) (Figure 15A). Furthermore, between HIF-1α and COX-2 mRNAs have significant difference (Spearman's r = 0.7091, p = 0.0268) (Figure 15B). However, there was no significant correlation between VEGF164 and COX-2 mRNAs (Spearman's r = 0.6429, p = 0.1389) (Figure 15C).

Discussion

In this chapter, the author examined the expression of VEGF and its related factors, flt-1, flk-1, HIF-1α, and COX-2 in normal, adjacent non-neoplastic, and benign and malignant mammary tissues of dogs. The results of the present study show that the amount of VEGF164 mRNA increased in benign and malignant tumor tissues as well as immunohistochemical staining. These findings are in agreement with the result in chapter 1 suggested that VEGF expression is up-regulates at transcriptional level. Strong staining against VEGF in neoplastic cells were seen in abutting necrotic area. This may be considered that abutting necrotic area is likely to be hypoxic and the hypoxia induces both increasing transcription and decreasing degradation of VEGF mRNA (Levy et al., 1995) (Ikeda et al., 1995). Many studies are reported about expression of VEGF mRNA but mostly these used a mixture of neoplastic cells and stromal cells. These samples may yield confusing data. In the present study, the author use LMD method to separate neoplastic epithelial cells from stroma in tumor tissues. In chapter 2, the author shows the results of immunohistochemical analysis for VEGF on endothelial cells that the percentages of VEGF-positive microvessel are increased in tumor tissues. In this chapter, the expression level of VEGF164 mRNA in tumor epithelial cells but not stromal cells significantly increased than that in adjacent tissues. These results suggest tumor epithelial cells up-regulate mRNA synthesis of VEGF164 and secret into vascular endothelial cells in a paracrine manner. The results that obtained with flt-1 and flk-1 mRNAs showed expression level of flk-1 mRNA was significantly higher in benign and malignant tumor tissues than normal and adjacent tissues but not of flt-1. These results are concurrent with the report that flk-1 is the main receptor that mediates the angiogenic effect of VEGF in tumor tisseus (Yoshiji et al., 1999) (Takahashi et al., 1995). The present study showed no significant correlation between the expression of HIF-1α mRNA and sample types, including adjacent, benign, and malignant tumor tissues. On the other hand, expression level of COX-2 mRNA was significantly increased in benign and malignant tumor tissues than normal and adjacent tissues. These results suggested that the gene expression levels of VEGF164, flk-1, and COX-2 are increased in tumor tissues but those should not be regarded as a marker of tumor aggressiveness.

Both the expression levels of endoglin mRNA and IMVD were significantly increase in benign and malignant tumor tissues although no statistically significant differences of those appeared between benign and malignant canine mammary tumor tissues. These results suggested that angiogenesis occurs in canine mammary tumor but there is no difference at the degrees of angiogenesis between benign and malignant tumors. These results may be explained that canine mammary gland tumors are known that has a lot of variation in their histology and biologic behavior within the same diagnosis (Misdorp, 1999).

Hypoxia is considered that one of the most potent inducer of VEGF expression (Wenger and Gassmann, 1997). In fact, the results presented in this chapter demonstrated a strong VEGF staining abutting necrotic area. VEGF expression is regulated at transcriptional level by HIF-1α in response to hypoxia and growth factor (Forsythe et al., 1996). Although the expression levels of HIF-1α mRNA did not show significant difference between non-neoplastic tissues and tumor tissues, significant correlation was found between the expression levels of HIF-1α mRNA and VEGF164 mRNA. This findings are agreement with the report that HIF-1α is a transcriptional activator of VEGF (Jones et al., 2001). In the present study, the author also found a significant correlation between HIF-1α mRNA and COX-2 mRNA. PGE_2, prostaglandin product of COX-2, mediates the hypoxic induction of VEGF in human cancers (Lukiw et al., 2003) (Hemmerlein et al., 2004) (Zhi et al., 2005) and it is reported HIF-1α mediates COX-2 and VEGF pathway (Huang et al., 2005). Some studies support the COX-2/HIF-1α/VEGF pathway possibly playing an important role in tumor angiogenesis. A study in prostate cancer cells found that NSAIDs reduce HIF-1 protein levels but the inhibitory effect might be COX-2 independent. Studies on a lung cancer cell line investigated the role of HIF-1α and the results showed that an HIF-1α transcriptional inhibitor suppressed VEGF expression induced by COX-2 (Rapisarda et al., 2002) (Jung et al., 2003). The results in this chapter support the COX-2/HIF-1α/VEGF pathway in canine mammary gland tumor related angiogenesis.

In summary, the present study indicates VEGF in canine mammary tumors is mainly expressed in tumor epithelial cells but not in stromal cells and suggests VEGF expressed in tumor epithelial cells binds flk-1, main receptor that mediates the angiogenic effect of VEGF. Although it is not

shown the correlation between tumor aggressiveness and the gene expression levels of VEGF164, flt-1, flk-1, COX-2, and HIF-1α, the expression of VEGF164, flk-1, and COX-2 mRNAs are increased in canine mammary tumors. These results indicate these angiogenesis related factors plays an important role in canine mammary tumors. The author also suggests the COX-2/HIF-1α/VEGF pathway possibly playing an important role in canine mammary tumors.

Table 1 Details of 17 dogs and their diagnosis and the size of tumor

Dog no.	Sex	Age (years)	Breed	diagnosis	tumor size (cm)
1	female	ND	beagle	Normal	–
2	female	ND	beagle	Normal	–
3	female	ND	beagle	Normal	–
4	female	ND	beagle	Normal	–
5	spayed	14	mongrel	Complex adenoma	4 x 3 x 2
6	female	9	miniature dachshund	Complex adenoma	3 x 2 x 1
7	female	11	golden retriever	Complex adenoma	1 x 1 x 1
8	female	10	Kishu	Complex adenoma	1 x 1 x 1.5
9	female	3	American cocker spaniel	Complex adenoma	1.2 x 1.2 x 0.8
10	female	14	pomeranian	Complex adenoma	ND
11	female	8	welsh corgi	Complex adenoma	ND
12	female	8	shih tzu	Complex adenoma	3 x 2 x 2
13	female	1.	shih tzu	Complex adenoma	5 x 3 x 2
14	female	6	papillon	Complex adenoma	1 x 1 x 1
15	female	10	miniature dachshund	Complex adenoma	ND
16	female	ND	shiba	Complex adenoma	5 x 3 x 2
17	spayed	8	shiba	Complex adenoma	ND
18	female	8	papillon	Tubulopapillary carcinoma	4 x 4 x 2
19	female	9	miniature dachshund	Tubulopapillary carcinoma	1.5 x 1 x 1
20	female	10	mongrel	Tubulopapillary carcinoma	2 x 1.2 x 1
21	spayed	ND	mongrel	Tubulopapillary carcinoma	ND
22	female	8	golden retriever	Tubulopapillary carcinoma	5 x 4 x 4
23	female	10	pekingese	Tubulopapillary carcinoma	3 x 2 x 1
24	female	9	welsh corgi	Tubulopapillary carcinoma	1.5 x 1.2 x 1
25	spayed	10	yorkshire terrier	Tubulopapillary carcinoma	2 x 1 x 1
26	female	14	pomeranian	Tubulopapillary carcinoma	2 x 2 x 1
27	female	8	shih tzu	Tubulopapillary carcinoma	1.5 x 1.5 x 1.0
28	female	10	miniature dachshund	Tubulopapillary carcinoma	3 x 3 x 2
29	female	ND	shiba	Tubulopapillary carcinoma	ND
30	female	10	mongrel	Tubulopapillary carcinoma	5 x 4 x 4

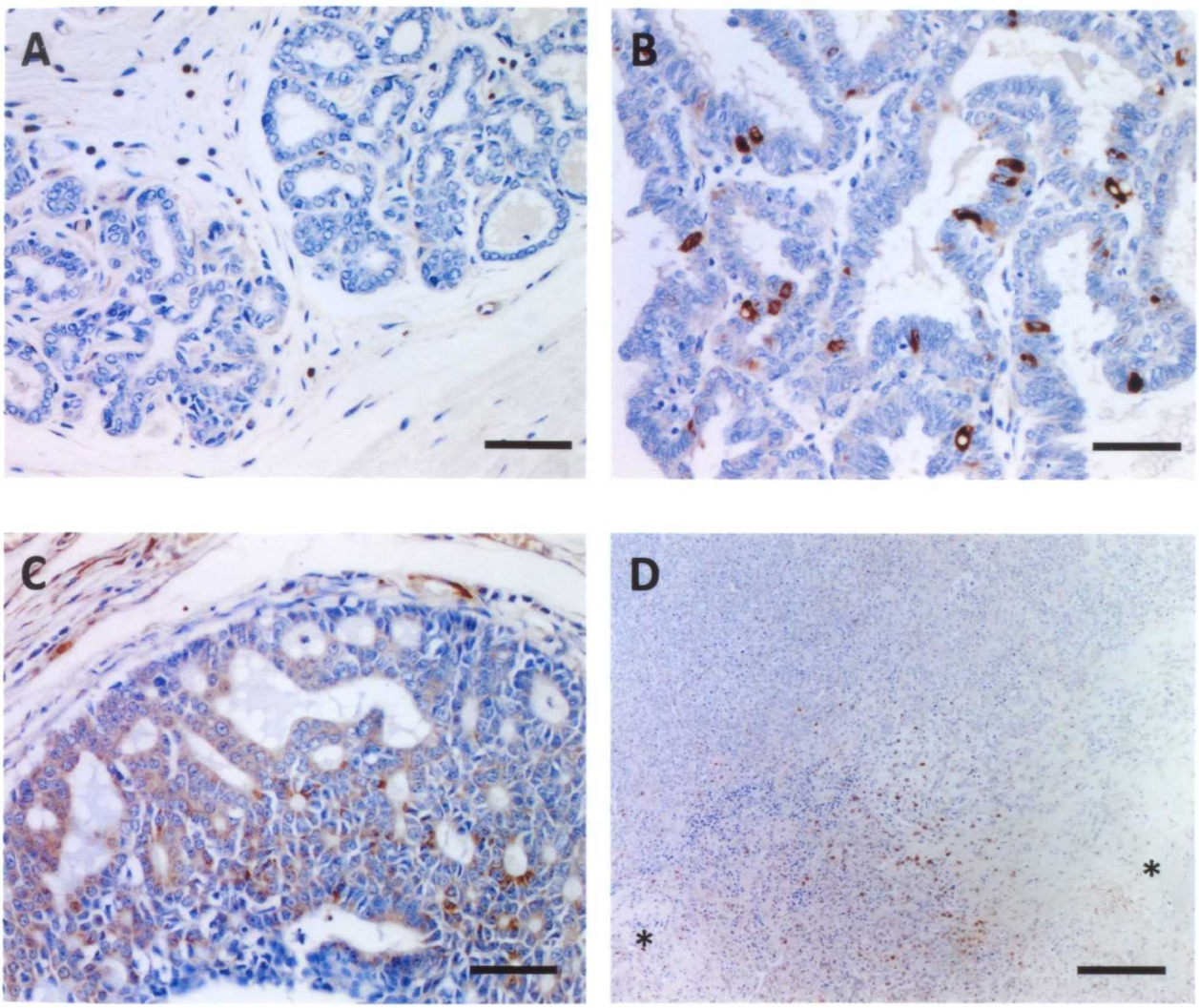
ND = no data

Table 2

Sequence of primers and probes for PCR and real-time RT-PCR

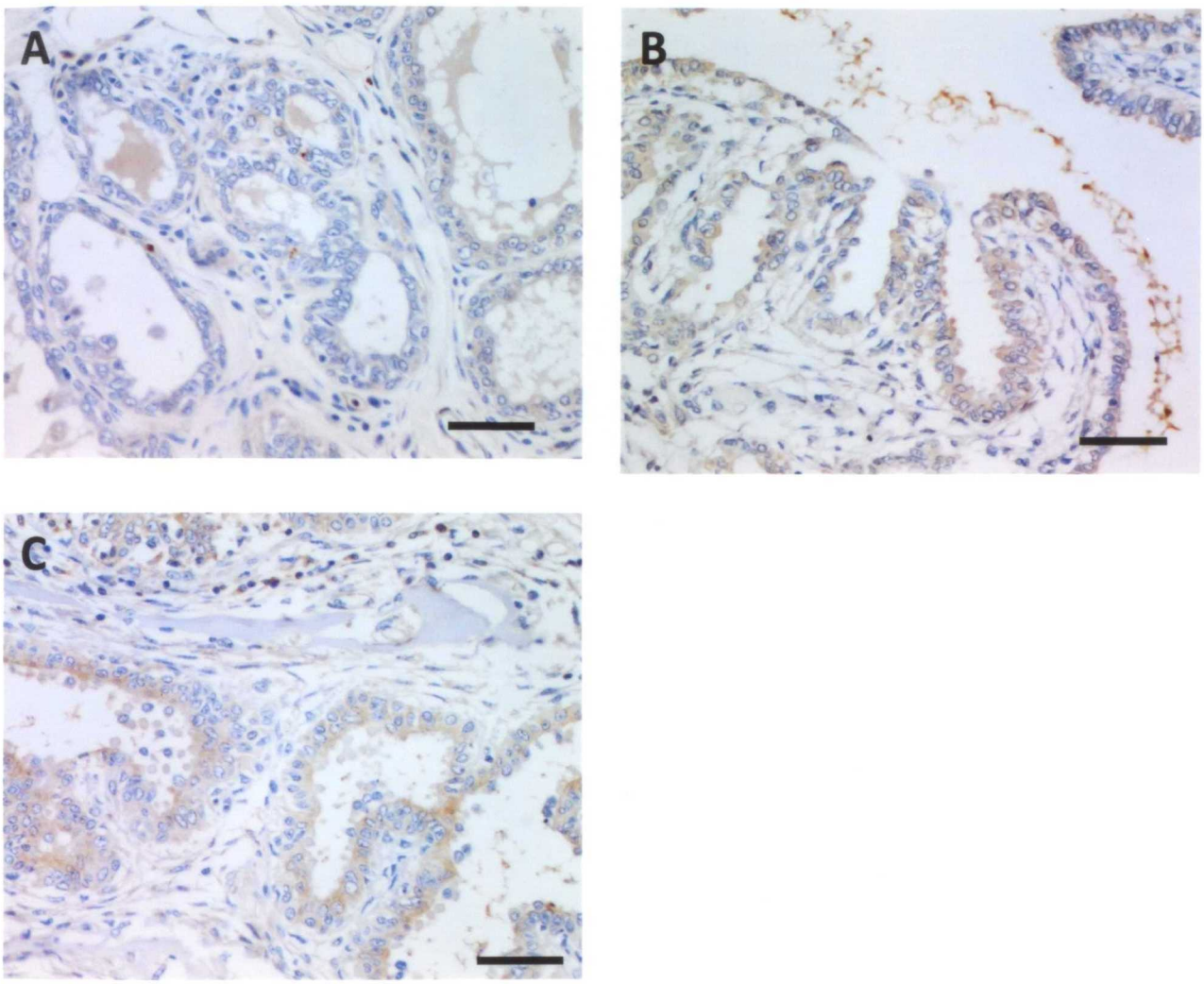
Gene	Sequence	Amplicon size
VEGF164 F	5'-CCCACTGAGGAGTTCAACATCAC-3'	143 bp
VEGF164 R	5'-CAGGGATTTTCTTGCCTTGCT-3'	
VEGF164 probe	5'-TGCGGATCAAACCTCATCAAGGCC-3'	
WF F	5'-AAAGCGGTGGTTATCCTAGTCACA-3'	112 bp
WF R	5'-ACCGATCCCCGATTCCAA-3'	
WF probe	5'-AGGCCGCCAGATCCAACCGAGT-3'	
ndoglin F	5'-CCGGAAACCCACAGAACTATCT-3'	84 bp
ndoglin R	5'-CACAGCGGGCAGGACAAG-3'	
ndoglin probe	5'-CAGCCCTGGCCTGCCTGACAA-3'	
HIF-1α F	5'-TGCTGACCCGGCACTCA-3'	108 bp
HIF-1α R	5'-GGACTAGCTGGCTGATCTTGAATC-3'	
HIF-1α probe	5'-CAGAGTCACTGGAACTTTCTTTTACTATGCCCCA-3'	
COX-2 F	5'-CGTCCGCGCAGCAAA-3'	142 bp
COX-2 R	5'-TGTCAGAAATTCCGGTGTTGAG-3'	
COX-2 probe	5'-ACCCGAACAGGATTCTACGGCGAAAA-3'	

Figure 1



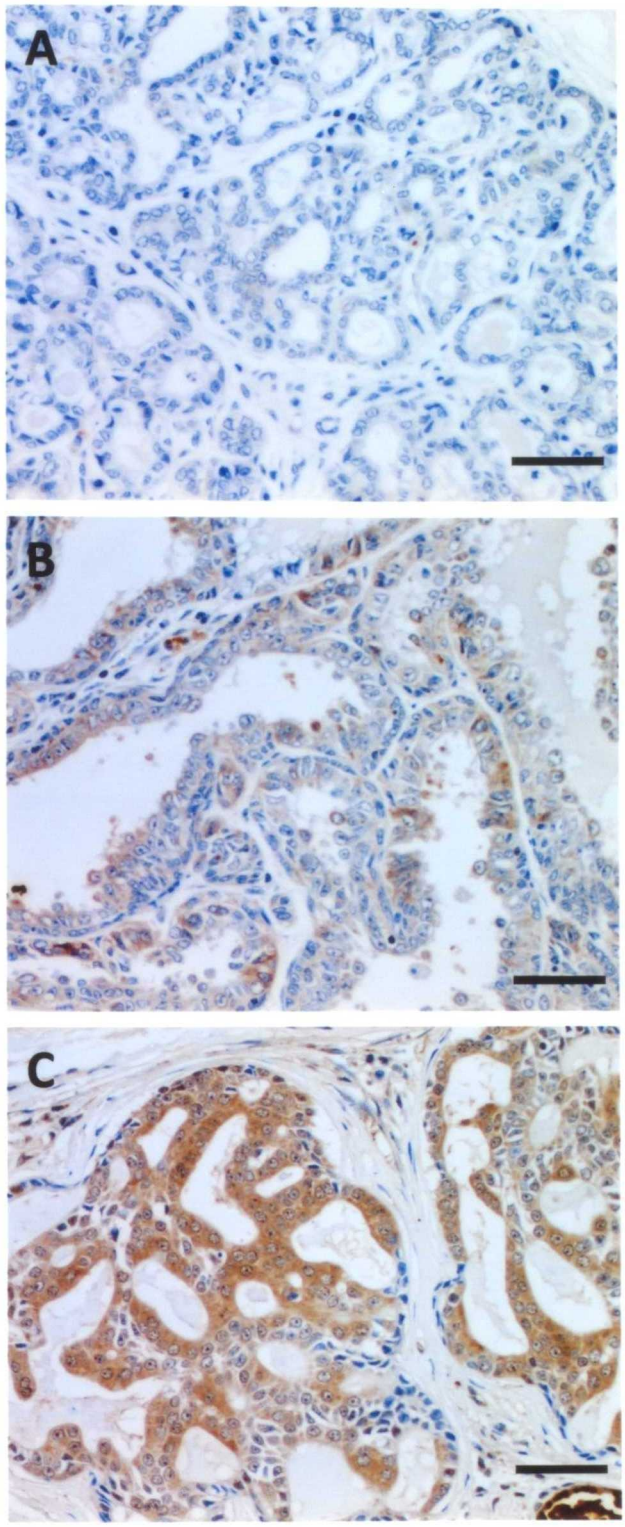
Immunohistochemical staining for VEGF. (A) Dog 13. Non-neoplastic adjacent tissue. (B) Dog 13. Benign tumor. (C) Dog 20. Malignant tumor. (D) Dog 21. Neoplastic cells abutting necrotic area showed strong staining. Asterisk shows necrotic area. Bar = 50 μm (A-C) or 200 μm (D).

Figure 2



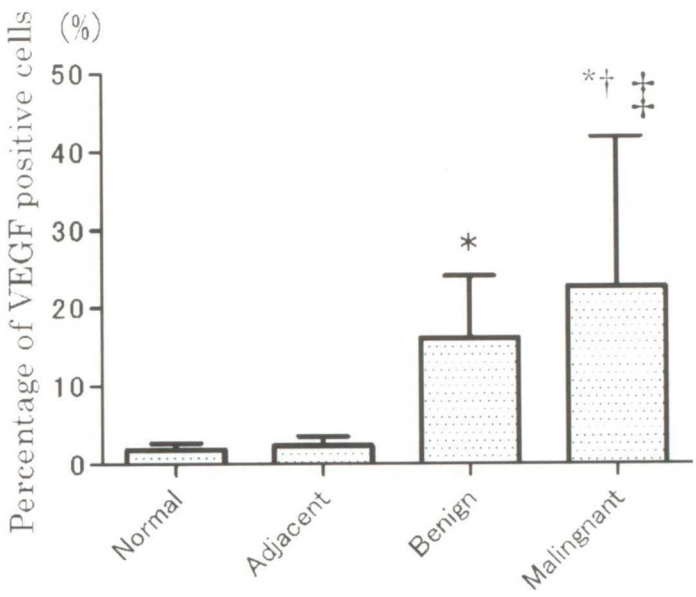
Immunohistochemical staining for flt-1. (A) Dog 13. Non-neoplastic adjacent tissue. (B) Dog 13. Benign tumor tissue. (C) Dog 20. Malignant tumor tissue. Bar = 50 μm.

Figure 3



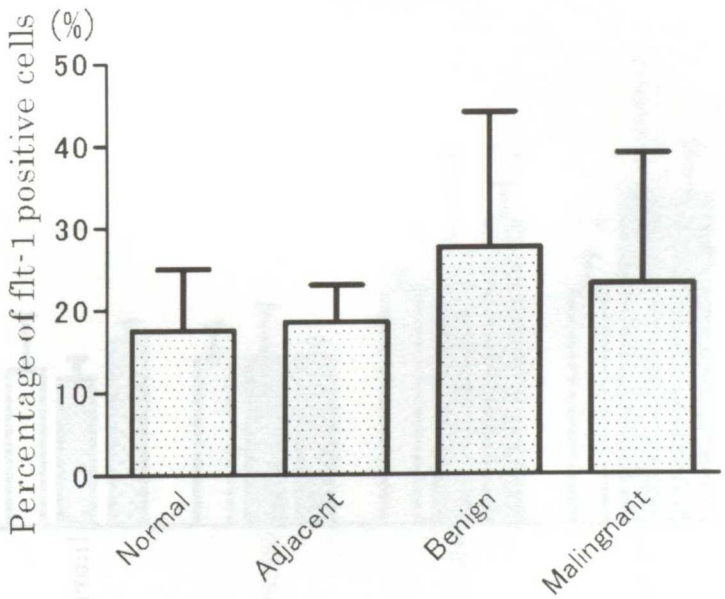
Immunohistochemical staining for COX-2. (A) Dog 13. Non-neoplastic adjacent tissue. (B) Dog 13. Benign tumor tissue. (C) Dog 20. Malignant tumor tissue. Bar = 50 μm.

Figure 4



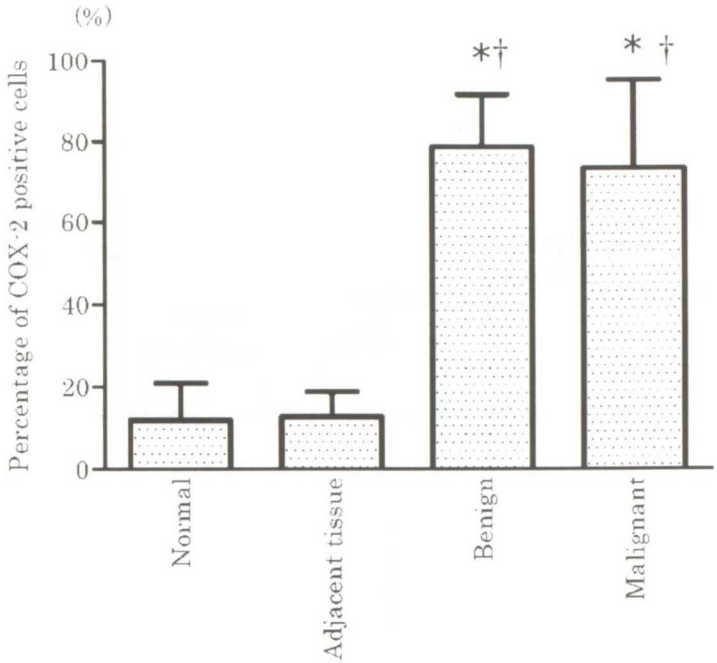
Semi-quantitative analysis of immunohistochemical staining for VEGF. * $p < 0.05$ vs normal, † $p < 0.05$ vs adjacent, and ‡ $p < 0.05$ vs benign tissues.

Figure 5



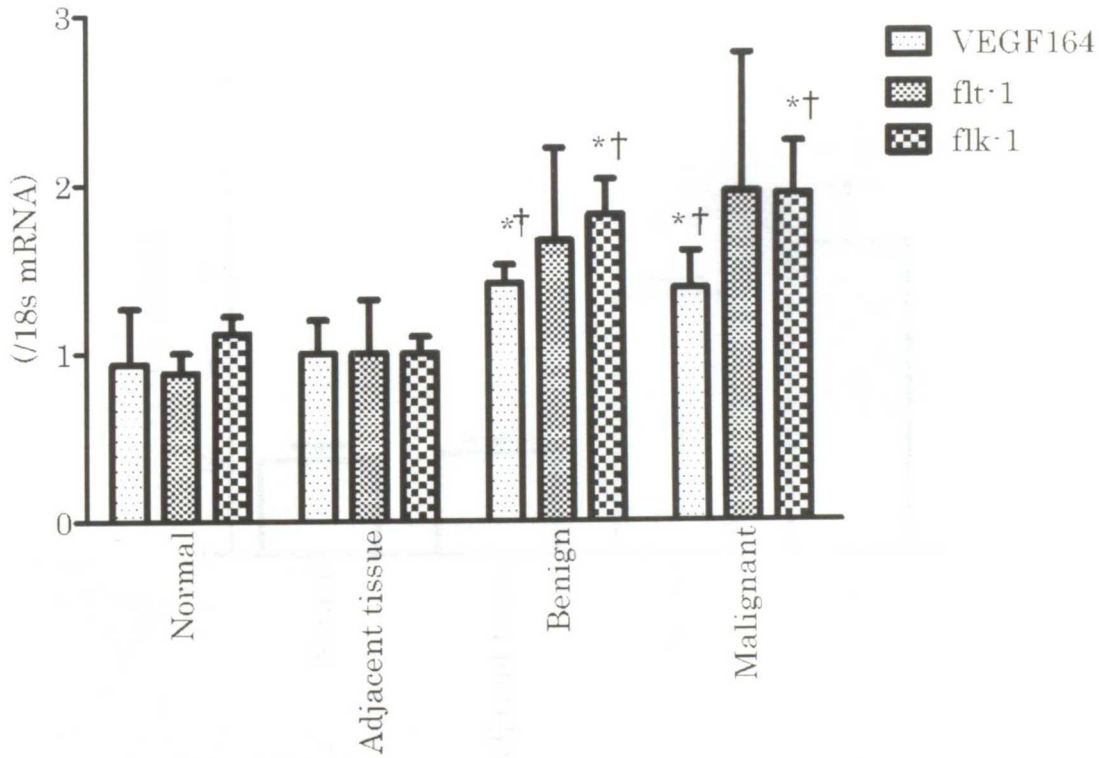
Semi-quantitative analysis of immunohistochemical staining for flt-1. There are no signififant correlation among normal, adjacent, and benign and malignant tumor tissues.

Figure 6



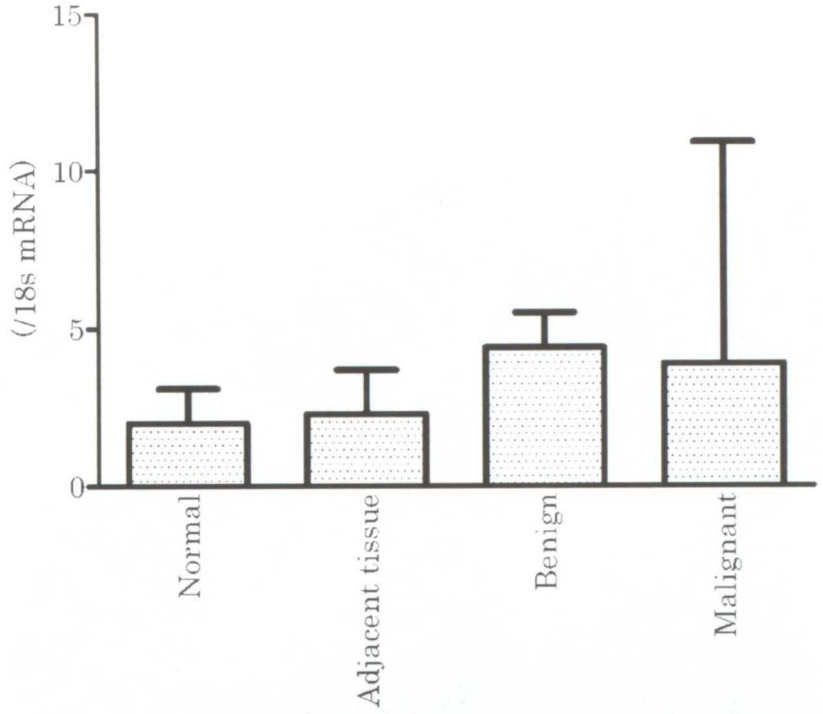
Semi-quantitative analysis of immunohistochemical staining for COX-2. * $p < 0.05$ vs normal, † $p < 0.05$ vs Adjacent tissues.

Figure 7



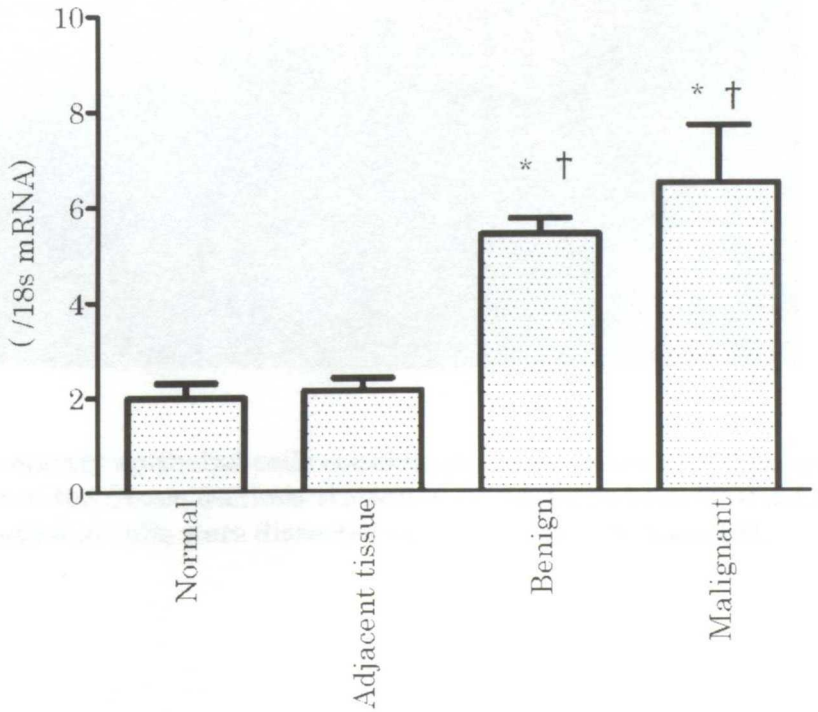
Quantitative analysis of gene expression for VEGF164, flt-1, and flk-1. * $p < 0.05$ vs normal, † $p < 0.05$ vs Adjacent tissues.

Figure 8



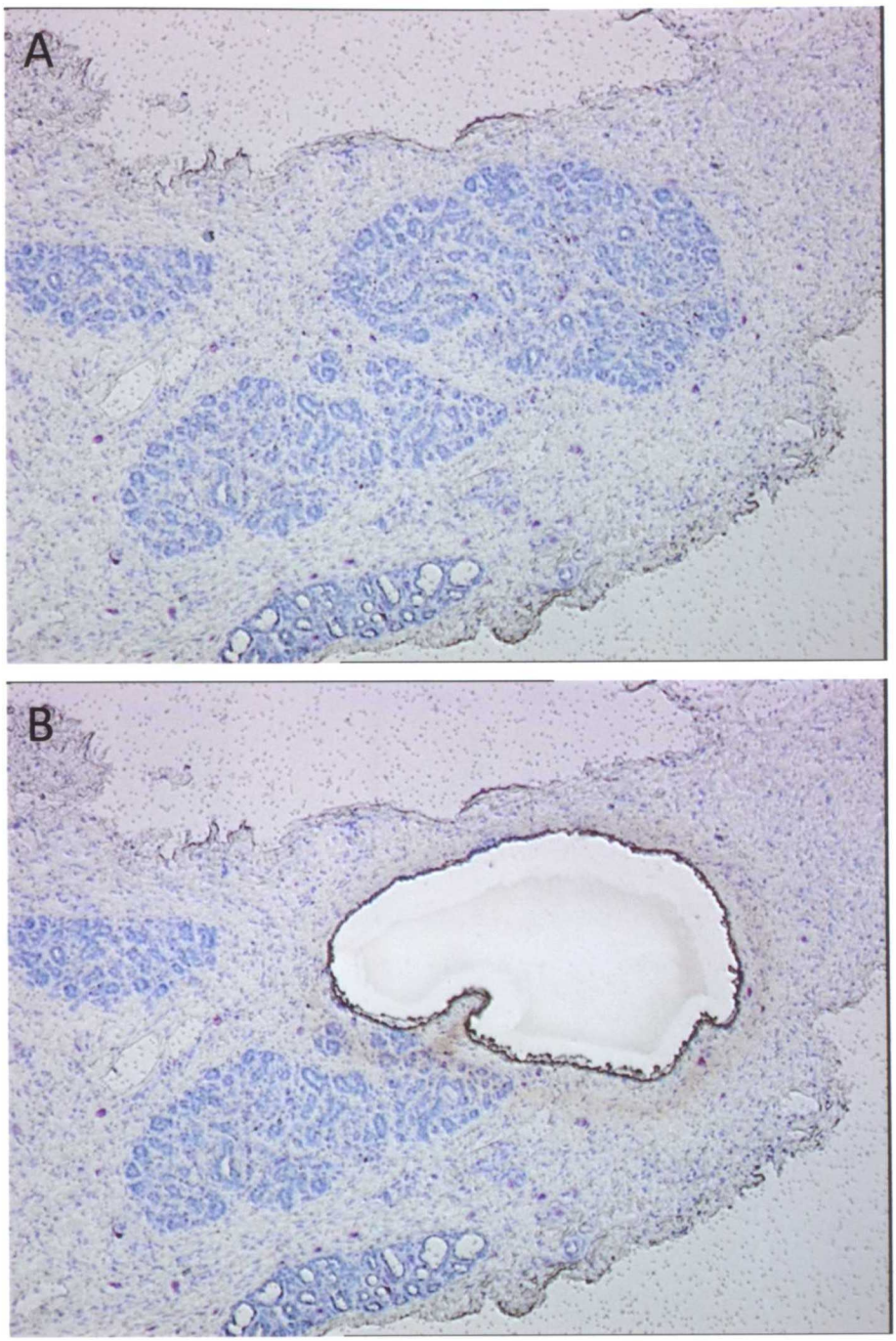
Expression levels of HIF-1α mRNA. There are no significant correlation among normal, adjacent, and benign and malignant tumor tissues.

Figure 9



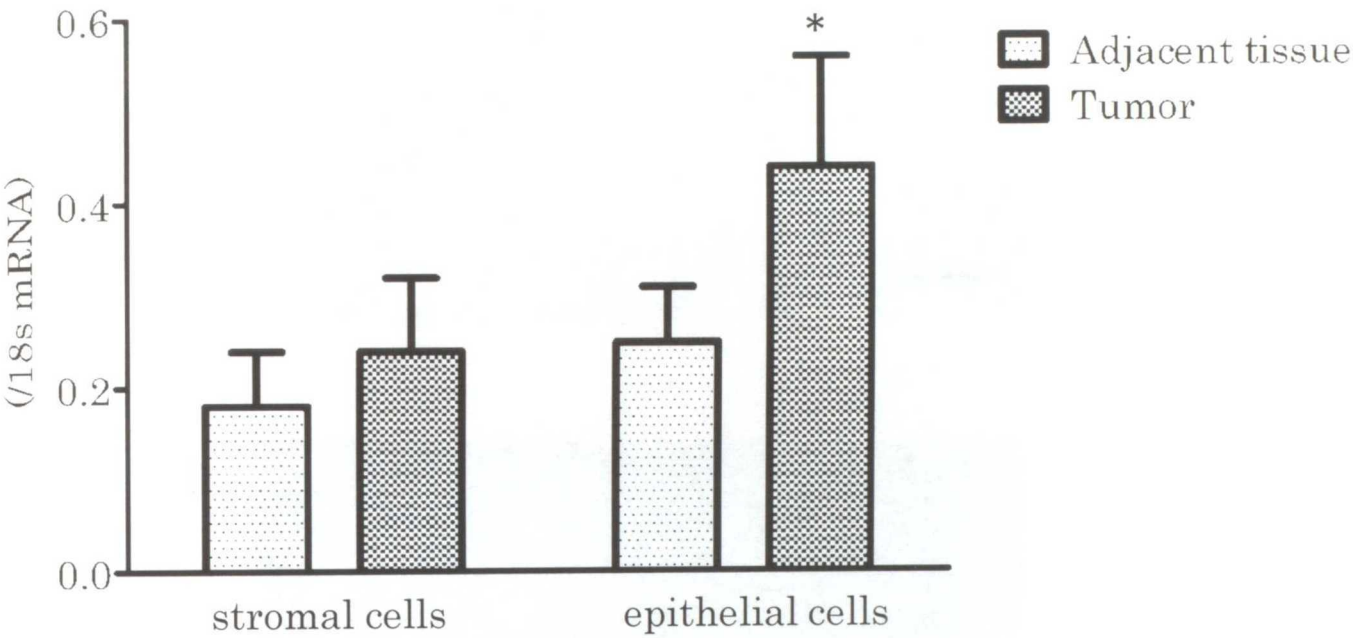
Expression levels of COX-2 mRNA. * $p < 0.05$ vs normal, † $p < 0.05$ vs adjacent tissues.

Figure 10



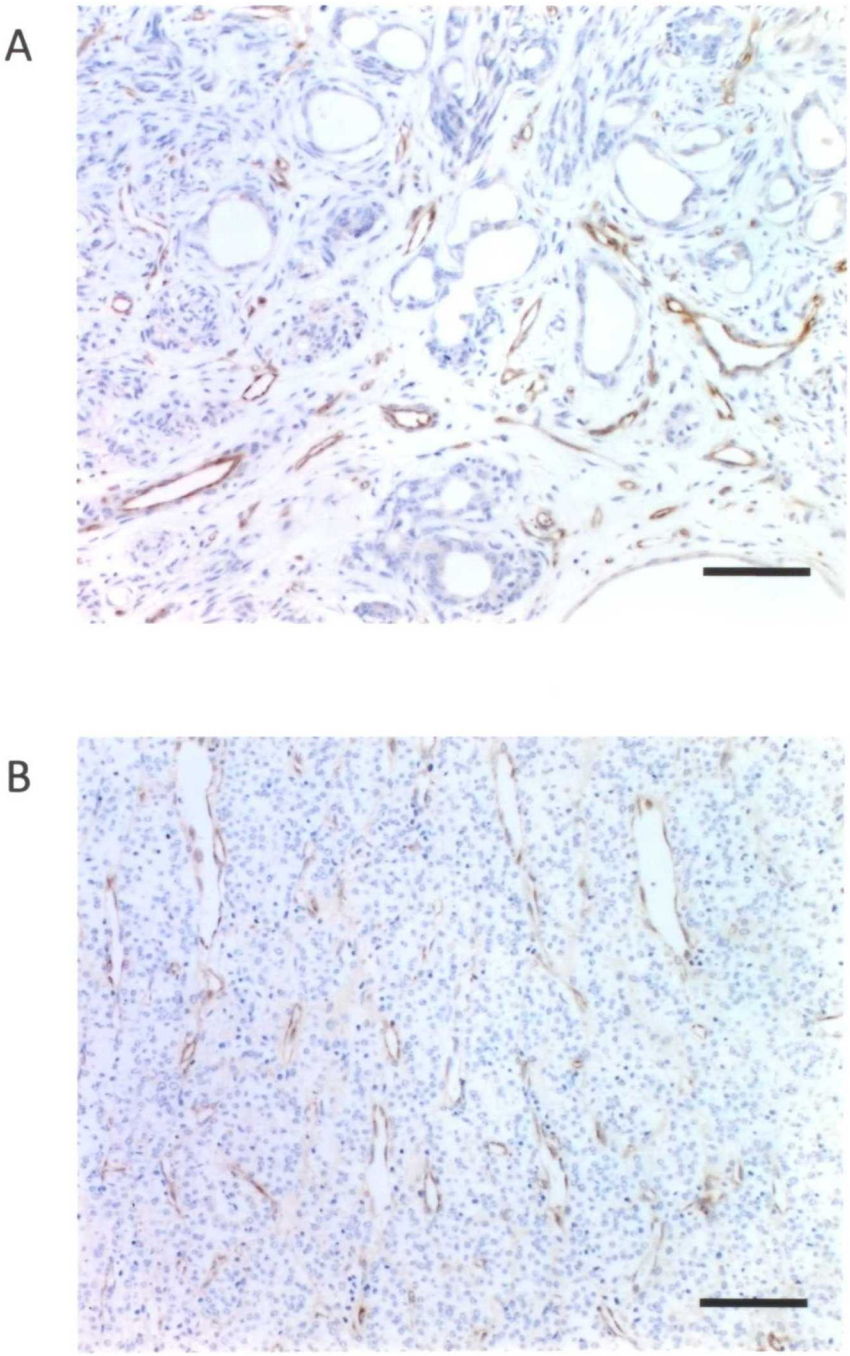
LMD method. The mammary epithelial cells could be observed easily and distinguished from other component in the frozen sections stained with toluidine blue (TB) solution (A). The mammary epithelial cells were dissected exactly by the UV laser (B).

Figure 11



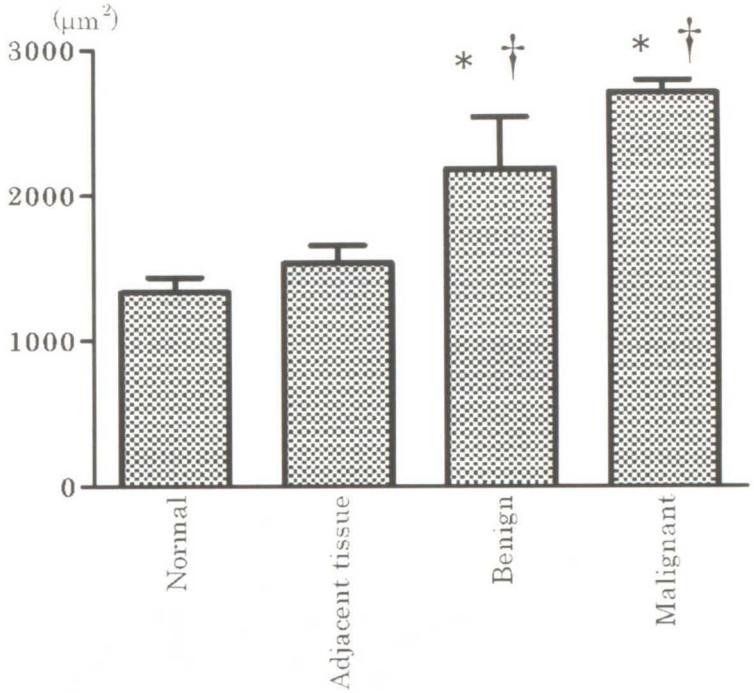
Real-time RT-PCR using LMD methods. VEGF164 mRNA was up-regulated in neoplastic epithelial cells. In contrast, there is no significant difference in stromal cells. * $p < 0.05$ vs adjacent tissue

Figure 12



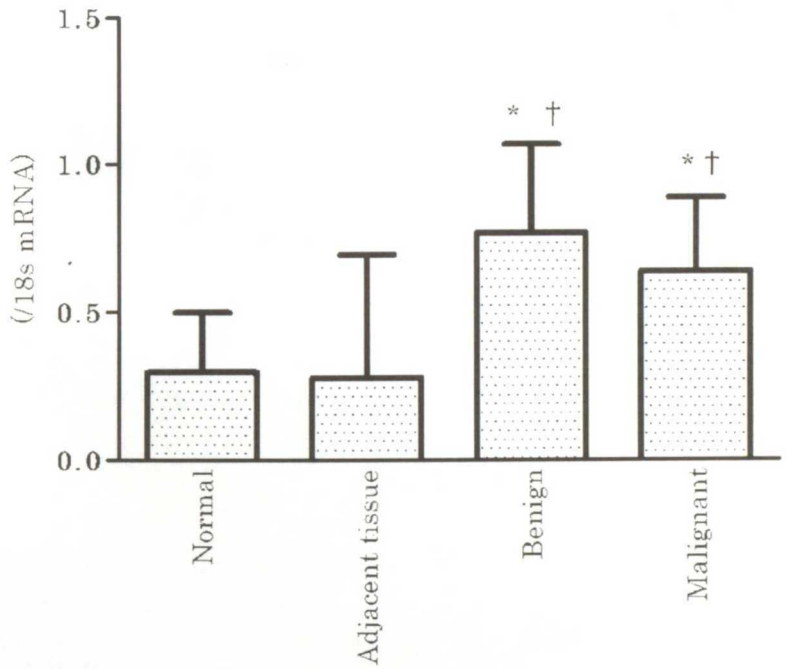
Immunohistochemical staining for vWF in benign tumor (A), and malignant tumor (B). Bar = 50 μm.

Figure 13



Analysis of IMVD using anti-vWF antibody.
* $p < 0.05$ vs normal, † $p < 0.05$ vs adjacent tissues.

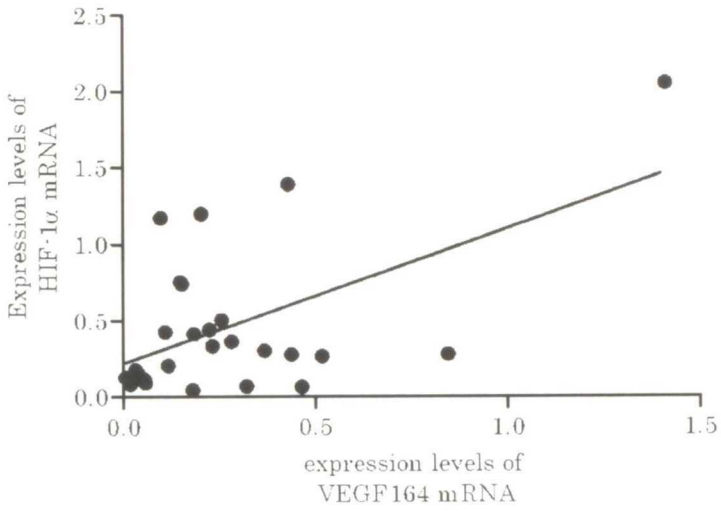
Figure 14



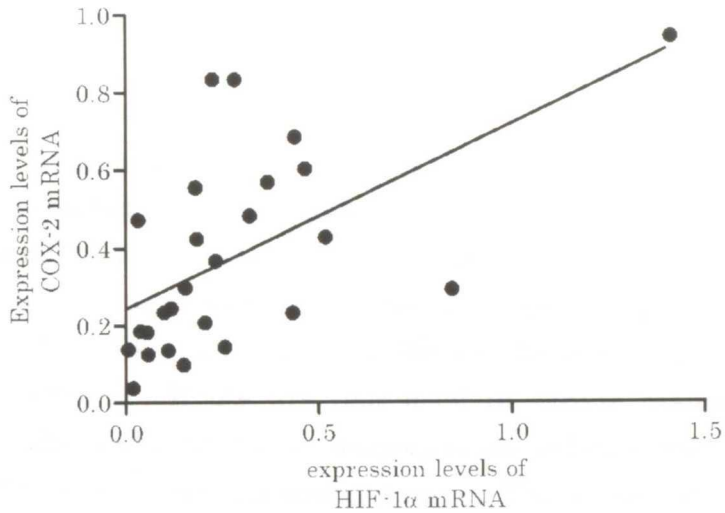
Expression levels of endoglin mRNA.
* $p < 0.05$ vs normal, † $p < 0.05$ vs adjacent tissues.

Figure 15

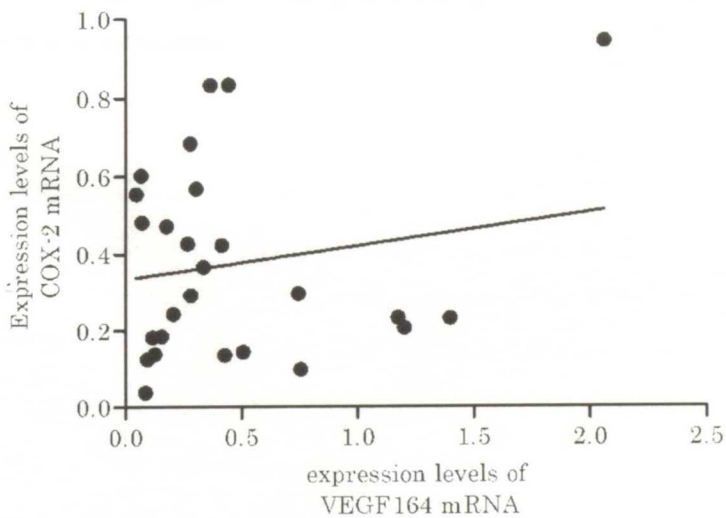
A



B



C



A) Correlation between the expression levels of HIF-1α and VEGF164 mRNAs. (Pearson's r=0.549, p=0.004)
B) Correlation between the expression levels of HIF-1α and COX-2 mRNAs (Pearson's r=0.709, p=0.027)
C) Correlation between the expression levels of VEGF164 and COX-2 mRNAs (Pearson's r=0.643, p=0.139)

Conclusions

This study shows the expression of angiogenic factors and vascular endothelial markers in canine mammary tumors both protein and gene levels. As the results, the author concluded as follows.

1. VEGF and its receptor, flt-1 and flk-1, are distributed in normal canine tissues and the expression levels of these factors among tissues tended to be similar.
2. endoglin, activated endothelial cell marker, is expressed in canine mammary tumors and has correlation with endothelial cell proliferation and expression of VEGF. Comparing the vWF, endoglin may be the better angiogenic marker to assess angiogenesis.
3. VEGF in canine mammary tumors is mainly expressed in tumor epithelial cells but not in stromal cells and suggests VEGF expressed in tumor epithelial cells binds flk-1. Although it is not shown the correlation between tumor aggressiveness and the gene expression levels of VEGF164, flt-1, flk-1, COX-2, and HIF-1α, the expression of VEGF164, flk-1, and COX-2 mRNAs are increased in canine mammary tumors. These results indicate these angiogenesis related factors plays an important role in canine mammary tumors. The author also suggests the COX-2/HIF-1α/VEGF pathway possibly playing an important role in canine mammary tumors.

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Japanese Abstract

腫瘍細胞は新生血管により酸素や栄養が供給されるため、血管新生は腫瘍の発達と転移に非常に重要である。血管新生には多くの因子が関与しており、これらの血管新生関連因子は腫瘍の予後因子として研究が行われている。その中でも血管内皮増殖因子 vascular endothelial growth factor (VEGF)は、そのレセプターである flt-1 や flk-1 と結合することにより血管新生の中心的な役割を担う。ヒトの多くの固形がんにおいては、腫瘍組織内の VEGF 発現は予後と関連していることが報告され、抗がん治療における重要なターゲットとされており、近年では抗 VEGF 薬を用いた併用療法が転移性乳癌に対する第一選択の治療法としてアメリカの FDA に迅速承認されている。

獣医学領域においては、いくつかのイヌの腫瘍で血管新生因子や微小血管密度、腫瘍のグレードの関連について研究が行われているものの、腫瘍における VEGF やそのレセプターを含む血管新生因子の発現や血管新生因子間の関連はまだよくわかっていない。ヒトの乳癌では血管新生に関して多くの研究が行われているが、イヌにおいても人と同様、乳腺腫瘍は頻繁に発生する腫瘍であり、良性と悪性の腫瘍が存在する。また、多くの症例で外科摘出時に非腫瘍部の乳頭部も共に摘出するため、同一の症例で腫瘍部と非腫瘍部の乳腺組織を比較することも可能であることから、VEGF や血管新生因子の腫瘍における動態を解析し、抗 VEGF 療法を適用出来るようになる可能性がある。このような背景から、本研究はイヌの乳腺腫瘍に対して抗血管新生療法を適用するための基礎的研究として、VEGF を中心とした血管新生因子発現の悪性度の指標としての評価と血管新生因子間および血管新生との関連を明らかにすることを目的とし、免疫組織化学的及び分子生物学的検索を行った。

第 1 章：正常イヌ組織における VEGF およびレセプターflt-1、flk-1 の発現と分布の検索

近年ではがんに対する治療として抗 VEGF 薬も用いられ始めているが、副作用も大きな問題となっている。これは VEGF が腫瘍の血管新生以外にも役割を持っていることによると考えられ、正常な組織における VEGF の作用が注目されてきている。近年ではイヌの腫瘍においても VEGF と悪性度や予後との関連が報告されてきているが、正常組織における VEGF の発現細胞はわかっていない。そこで本章では正常イヌの組織(6 例：肺、腎臓、心臓、副腎、肝臓、皮膚、甲状腺、腸管、膀胱、リンパ節、膵臓、脾臓、4 例：乳腺)における VEGF およびレセプターflt-1、flk-1 の発現と分布を明らかにすることを目的とした。

VEGF 及び flt-1 の免疫組織化学的発現は各臓器の様々な部位に陽性細胞がみられた。どの臓器にも存在する血管では、中膜平滑筋が VEGF、flt-1 ともに一部で陽性を示したが、内皮細胞に陽性像はみられなかった。また、動・静脈、血管サイズなど血管の種類による違いはみられなかった。RT-PCR により検索を行ったすべての正常組織で VEGF、flt-1、flk-1 の遺伝子発現が確認され、これらが正常な組織でも恒常的に発現していることが示された。VEGF については、複数のアイソフォームが確認され、その中でも最も生物学的活性が高いといわれる VEGF164 の発現量が最も多かった。また、定量的 RT-PCR では各組織における VEGF164 と flt-1、flk-1 遺伝子発現がほぼ同じ傾向を示した。以上の結果から、VEGF と flt-1、flk-1 は正常なイヌの組織においても発現していることが確認された。また、VEGF164 および flt-1 は、免疫組織化学的にタンパク質が発現している細胞の多かった肺や心臓で mRNA の発現も高く、発現細胞の少なかった脾臓などでは mRNA の発現も少なかったことから、VEGF と flt-1 は転写レベルで調節が行われていることが示唆された。

第 2 章：イヌの良性乳腺腫瘍における内皮マーカーの評価

第 1 章の結果から腫瘍組織においても VEGF の遺伝子発現量が血管新生を反映することが考えられる。この血管新生を評価する方法として、現在では微小血管密度や血管内皮マーカーの発現量が用いられており、多くの腫瘍で血管新生の評価と予後が関連していることが報告されている一方で、それらが関連しないという報告もある。この様な研究結果の不一致は、従来評価に用いられている von Willebrand factor (vWF)や CD31 などが汎血管内皮マーカーであり、新生血管のみならず既存の血管にも発現していることに起因することが考えられる。本研究で対象としている乳腺組織は正常でも血管が豊富に存在する組織であり、汎血管内皮マーカーを用いた評価では巻き込まれただけの既存の血管も評価に含まれ、血管新生の正確な評価が出来ない可能性がある。このような背景から、より正確に腫瘍組織における血管新生を評価し、その生物学的挙動との関連を探ることが必要である。近年では活性化された内皮細胞に強く発現する endoglin が注目されてきているが、イヌにおける endoglin の有用性は示されていない。そこで本章では、正常イヌの乳腺、良性乳腺腫瘍及び腫瘍罹患イヌの非腫瘍部の乳腺を用いて endoglin mRNA の発現を検索し、endoglin のイヌの乳腺腫瘍における新生血管マーカーとしての可能性を検討した。

RT-PCR により、endoglin mRNA の発現率は良性乳腺腫瘍では正常乳腺、非腫瘍部乳腺と比較して、有意に高いことが示された。免疫組織化学では、proliferating cell nuclear antigen (PCNA)が陽性となった血管内皮細胞の割合

およびVEGFが陽性となった血管の割合が、endoglin mRNAが発現していた組織で有意に高いことが示された。また、VEGF mRNAの発現量はvWF mRNA発現量よりもendoglin mRNA発現量と強い相関を示した。PCNAやVEGFに陽性を示す内皮は新生血管の内皮であると考えられることから、endoglin mRNAの発現量がイヌの乳腺腫瘍において血管新生のマーカーとして有用で、新生血管の程度を反映することが示された。

第3章：イヌの乳腺腫瘍におけるVEGFおよびその関連因子の発現

VEGFの発現調節には様々な因子が関与しているが、その中でも腫瘍組織内の低酸素が中心的な役割を担っていると考えられている。イヌの乳腺腫瘍にはしばしば壊死巣がみられ、この壊死巣は低酸素状態になっていると考えられることから、イヌの乳腺腫瘍においても低酸素がVEGFの発現に関与している可能性がある。低酸素誘導因子hypoxia-inducible factor (HIF)-1αは低酸素により細胞内の発現が増加する代表的な因子であり、低酸素条件下ではVEGFの転写を亢進させることが知られている。また、cyclooxygenase-2 (COX-2)の過剰発現は多くの腫瘍でみられるが、COX-2により誘導されるprostaglandinE_2 (PGE_2)がHIF-1αを介して血管新生を誘導するとの報告もある。本章では、正常イヌの乳腺と非腫瘍部乳腺、良性乳腺腫瘍、悪性乳腺腫瘍を用いてVEGFおよびそのレセプターの発現と第2章で血管マーカーとしての有用性が示唆されたendoglinとの関連および誘導因子としてのHIF-1αおよびCOX-2との関連を明らかにし、これら血管新生関連因子の悪性度の指標としての評価をすることを目的として検索を行った。

免疫組織化学的検索では、乳腺上皮細胞におけるVEGFの陽性細胞率は非腫瘍部乳腺、良性腫瘍、悪性腫瘍の順に有意に増加していたが、flt-1についてはこれらの間に有意差がみられなかった。COX-2発現は腫瘍性上皮細胞や血管内皮細胞、血管平滑筋細胞、マクロファージで確認され、非腫瘍部乳腺と比較して腫瘍組織で陽性細胞数が有意に多かったが、良性腫瘍と悪性腫瘍の間に有意な差は認められなかった。遺伝子発現量においてもVEGF164とflk-1、COX-2のmRNA発現量は非腫瘍部乳腺と比較して良性及び悪性腫瘍で有意な増加が認められたが、flt-1 mRNAについてはこれらの間に有意な差が認められなかった。また、HIF-1α mRNAについては良性腫瘍および悪性腫瘍での発現量は非腫瘍部乳腺よりも高い傾向がみられた。微小血管密度とendoglin mRNAの発現量は、非腫瘍部乳腺と比較して腫瘍組織で有意に増加していたが、良性腫瘍と悪性腫瘍の間に有意差はみられなかった。Laser microdissection(LMD)法を用いて腫瘍組織では腫瘍細胞と間質を、非腫瘍部では上皮組織と間質を分離し、非腫瘍

部乳腺組織と腫瘍組織の VEGF164 mRNA 発現量を比較した結果、腫瘍性上皮細胞については非腫瘍部乳腺上皮細胞よりも有意に高い発現を示したが、間質についてはこれらの間に差が認められなかった。また、得られたすべての結果において、正常イヌの乳腺と腫瘍罹患イヌの非腫瘍部乳腺の間に差はみられなかった。

以上の結果から、VEGF164 と flk-1、COX-2 の mRNA は腫瘍部において増加していることが明らかとなったが、悪性度との関連はみられなかった。また、flt-1 は腫瘍組織でも有意な増加が認められず、VEGF のレセプターとしては主に flk-1 が腫瘍組織内での血管新生に関与していることが示唆された。さらに、腫瘍組織における VEGF164 mRNA 発現の増加は間質細胞ではなく、主に腫瘍性上皮細胞に由来することが示唆された。今回の結果から、HIF-1α mRNA 発現は腫瘍組織において増加傾向がみられ、COX-2 および VEGF164 との相関も認められたため、イヌの乳腺腫瘍においても COX-2/HIF-1α/VEGF 経路が存在している可能性が考えられた。

以上、本研究により VEGF とそのレセプターflt-1、flk-1 の mRNA および VEGF と flt-1 のタンパク質がイヌの正常組織で恒常的に発現していることが示され、VEGF と flt-1 が転写レベルで調節されていることが示唆された。また、イヌの乳腺腫瘍における血管新生マーカーとして endoglin 遺伝子発現が有用である可能性を示した。イヌの乳腺腫瘍における VEGF の増加は主に腫瘍細胞に由来しており、flk-1 を介して血管新生を促進させることを示唆した。VEGF と COX-2 のタンパク質発現は血管新生の程度を反映する endoglin 遺伝子発現量と共にイヌの乳腺腫瘍で増加し、VEGF タンパク質は腫瘍化の指標となる可能性を示した。また、イヌの腫瘍の血管新生においても COX-2/HIF-1α/VEGF 経路が存在する可能性が示唆された。これらにより、血管新生関連因子の分布および因子間の関連はヒトの乳癌に類似していることが示され、ヒトの乳癌と同様にイヌの乳腺腫瘍でも抗血管新生薬が有用である可能性が示唆された。

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