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Growth Factors and COX2 Expression in Canine Perivascular Wall Tumors

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Abstract

Canine perivascular wall tumors (PWTs) are a group of subcutaneous soft tissue sarcomas developing from vascular mural cells. Mural cells are involved in angiogenesis through a complex crosstalk with endothelial cells mediated by several growth factors and their receptors. The evaluation of their expression may have relevance since they may represent a therapeutic target in the control of canine PWTs. The expression of vascular endothelial growth factor (VEGF) and receptors VEGFR-I/II, basic fibroblast growth factor (bFGF) and receptor Flg, platelet-derived growth factor B (PDGFB) and receptor PDGFRb, transforming growth factor b1 (TGFb1) and receptors TGFbR-I/II, and cyclooxygenase 2 (COX2) was evaluated on frozen sections of 40 PWTs by immunohistochemistry and semiquantitatively scored to identify their potential role in PWT development. Statistical analysis was performed to analyze possible correlations between Ki67 labeling index and the expression of each molecule. Proteins of the VEGF-, PDGFB-, and bFGF-mediated pathways were highly expressed in 27 (67.5%), 30 (75%), and 19 (47.5%) of 40 PWTs, respectively. Proteins of the TGFb1- and COX2-mediated pathways were highly expressed in 4 (10%) and 14 (35%) of 40 cases. Statistical analysis identified an association between VEGF and VEGFR-I/II (P ¼ .015 and .003, respectively), bFGF and Flg (P ¼ .038), bFGF and PDGFRb (P ¼ .003), and between TGFb1 and COX2 (P ¼ .006). These findings were consistent with the mechanisms that have been reported to play a role in angiogenesis and in tumor development. No association with Ki67 labeling index was found. VEGF-, PDGFB-, and bFGF-mediated pathways seem to have a key role in PWT development and growth. Blockade of tyrosine kinase receptors after surgery could represent a promising therapy with the aim to reduce the PWT relapse rate and prolong the time to relapse.

Keywords

basic fibroblast growth factor, canine, dogs, hemangiopericytoma, immunohistochemistry, platelet-derived growth factor, perivascular wall tumors, soft tissue sarcoma, tyrosine kinase receptor, vascular endothelial growth factor

Introduction

Canine perivascular wall tumors (PWTs), previously grouped under the term hemangiopericytoma, are a group of soft tissue sarcomas (STSs) developing from vascular mural cells (MCs), and they are characterized by variable differentiation, ranging from pericytic to myoid.[5,36] PWTs arise more commonly in the subcutaneous tissue of the extremities and are characterized by a low metastatic potential and an intermediate rate of local recurrence.[4,5,47] The major prognostic factors in canine PWTs are tumor size, completeness of margins, and site and depth of the lesion.[4]

MCs, from which PWTs arise, are involved in angiogenesis and blood vessel stabilization through a complex crosstalk with endothelial cells (ECs), a process mediated by several growth factors (GFs), including vascular endothelial GF (VEGF), basic fibroblast GF (bFGF), platelet-derived GF B (PDGFB), and transforming GF b (TGFb).[2] VEGF, PDGFB, and bFGF have been demonstrated to have a role in the development of several human STSs, including gastrointestinal stromal tumors, uterine leiomyosarcomas, and dermatofibrosarcoma protuberans,[13,32,43] and in canine STSs and feline vaccine-associated sarcoma in cats.[1,16,19,28] Based on in vivo and in vitro studies, these GFs have been regarded as targets for the therapy of STSs in humans[9,12,32] and dogs.[27,31,37] To the best of our knowledge, the involvement of GFs and their receptors in the pathogenesis of PWTs and their potential therapeutic value have not been investigated.

COX2 is an inducible enzyme involved in inflammation that is also responsible for pulmonary MC proliferation in hypoxic conditions, through TGFb-mediated modulation.[35,45] COX2 expression has been demonstrated in several canine and feline tumors, and its inhibition is considered a promising adjuvant therapy.[6,8,29,30,38,39–41]

The aims of this study are as follows:

to assess the expression of several GFs pathways— including VEGF, bFGF, PDGFB, and TGFb1 and their receptors VEGFR-I (Flt-1), VEGFR-II (Flk-1), PDGFRb, Flg, TGFbR-I, TGFbR-II, and COX2—to identify a high expression of molecules and their potential role as therapeutic targets;

to detect possible associations between GFs and GF receptor expression, similar to what occurs during the crosstalk between MCs and ECs throughout angiogenesis when expression of these proteins may be up- and downregulated (regulatory loops); and to assess the presence of associations between the high expression of GFs and Ki67 labeling index or mitotic index to identify a putative role of GFs in neoplastic cell proliferation.

Materials and Methods

Inclusion Criteria and Case Selection

All tumors included in the study had to fulfill the following criteria: available fresh tissue, origin from the skin and subcutis of dogs, and histopathologic features diagnostic for PWT (perivascular whorls, bundles from tunica media, staghorn vessels, and placentoid growth).[5] Cases expressing GFAP and/or S100 and negative for markers of myoid differentiation (smooth muscle actin, calponin, desmin, smoothelin, and myosin) were excluded.

Tissue Handling

One part of each excised tumor was fixed in10% neutral buffered formalin and routinely processed for histology, and 1 portion (diameter range of the sample, 5–10 mm) was snap-frozen in isopentane cooled at the freezing temperature in liquid nitrogen.

Histologic Evaluation

Histologic parameters assessed in each tumor included mitotic index, percentage of necrosis, and histologic grade, which were assessed according to the literature.[17]

Immunohistochemistry

Immunohistochemistry for Ki67 was performed on formalinfixed, paraffin-embedded tissues using the clone MIB-1 (Dako, Goldstrup, Denmark; dilution, 1:600) after heat-induced antigen retrieval (pressure cooker, citrate buffer, pH 5.6). Ki67 expression was evaluated as the labeling index and was defined as the percentage of Ki67-positive cells. The count of Ki67positive cells was performed in 10 high-power fields (400), with at least 1000 cells counted for each case, using the manual count tool of the Image Pro Plus 6.3 analysis software (Media Cybernetics Inc).

Immunohistochemistry was performed on cryostatic sections to exclude a peripheral nerve sheath tumor, to confirm a diagnosis of PWT, and to subtype canine PWTs, applying antibodies that identify S100, GFAP, vimentin, smooth muscle actin, calponin, desmin, smoothelin, and myosin, as previously reported.[5] Subtyping of PWTs was performed according with the following criteria[5]

Cases expressing only vimentin and characterized by perivascular whorls were classified as angiofibroma

Cases expressing vimentin and smooth muscle actin with predominant staghorn vessels were classified as hemangiopericytoma

Cases with a mature muscular phenotype (smooth muscle myosin and/or smoothelin positive) and any of the perivascular histological pattern were classified as angioleiomyoma or angioleiomyosarcoma based on the degree of atypia of neoplastic cells

Cases with intermediate myoid differentiation (smooth muscle actin, calponin, and variably desmin) and perivascular (pericapillary) whorls were classified as myopericytoma

Cases with intermediate myoid differentiation and perivascular (adventitial) whorls were classified as adventitial tumors.

For the evaluation of GF pathway and COX2 expression, 5-mm cryostatic sections were air-dried, fixed in cold acetone (3 minutes at 5), and stored at –70C. Dilution and source of primary antibodies are listed in Supplemental Table 1.

Sections were incubated at room temperature for 1 hour with the primary antibodies and 30 minutes with the appropriate biotin-linked secondary antibody (Dako, Goldstrup, Denmark; dilution 1:200).

The immunoreaction was visualized for all reactions with amino-9-ethyl-carbazole chromogen (AEC, Kit, Vector, Burlingame, CA, USA). Sections were counterstained with Mayer's hematoxylin and mounted with glycerine. As positive controls, sections of granulation tissue were used. The different cellular components of the wall of intratumoral vessel served as internal positive and negative controls. Negative controls consisted of the substitution of specific primary antibodies with an isotype-matched, irrelevant monoclonal antibody (for monoclonal primary antibodies), irrelevant polyclonal antibody (for polyclonal antibodies), or omission of the primary antibody. Scoring of the GFs pathways and COX2 was performed independently by 2 pathologists in a semiquantitative manner, examining the entire sample at intermediate magnification (200), and evaluating the percentage of positive neoplastic cells, according with the following scoring system: 0, negative; 1,

20%; 2, 21%–50%; 3, 51%–70%; 4, 70%. Since the majority of the GFs and GF receptors examined in MCs can be expressed during normal angiogenesis, these molecules were considered highly expressed if staining was present in >70% of neoplastic cells (score 4). On the contrary, since COX2 is seldom expressed by MCs, it was considered highly expressed when expressed by >20% of neoplastic cells according with scoring systems reported in the literature (score, 2).[41] Intensity of the staining and extracellular staining were not included in the scoring system. Variation of the staining intensity within a sample was recorded. Differences between the scores of the 2 pathologists were discussed jointly to achieve an agreement.

Statistical Analysis

The association between the expression of GFs and GF receptors and between COX2 and GFs was evaluated for each couple of variables by the odds ratio (OR) statistics estimated by the logistic regression model. OR is a measure of the strength of the association between 2 variables. In the case of independence, OR is equal to 1; OR values near to 1 indicate a weak association; and OR values further from 1 indicate the presence of a strong association. The strength of association was classified as follows: weak, >1.2 and 1.5; moderate, >1.5 and 3; strong, >3.[33] Because of the low number of tumors included in some of the categories (eg, COX2 high expression), only univariate analysis was performed.

The relationship between Ki67 labeling index and high expression of GF, GF receptors, and COX2 was evaluated by linear regression models. Residuals analysis was performed to evaluate the assumption underlying the regression parameters inference. Taking into account the sample size, only regression models with a maximum of 4 variables were considered to avoid unreliable results. KI67 was the dependent variable and the following models were considered: (1) VEGF þ VEGFR-I þ VEGFR-II, (2)

PDGFB þ PDGFRb þ flg þ bFGF, (3) COX2. In each model GF, GF receptors, and COX2 were included as dummy variables. A P value of .05 was considered significant and indicative of a putative association between variables. The association between GF high expression and PWT subtypes was not evaluated, because of the small number of cases included in some diagnostic categories.

Results

Diagnosis, Histologic Parameters, and Ki67 Labeling Index

We collected 40 cases of canine PWTs: 18 grade I, 18 grade II, and 4 grade III. Two cases were classified as hemangiopericytoma, 26 myopericytoma, 5 angioleiomyoma, 2 angioleiomyosarcoma, 2 adventitial tumors, and 3 angiofibroma. The major histologic pattern, the immunohistochemical phenotype, and specific diagnosis of each case are listed in Supplemental Table 2. Size (largest diameter) of the tumor was collected in 36 of 40 cases and ranged between 0.5 and 20 cm (median, 5 cm). Mitotic index evaluated in 10 high-power fields ranged between 0 and 48 (median, 4). Ki67 labeling index ranged between 0.8% and 36.2% (median, 7.20%). In 31 cases, necrosis was absent; in 7 cases, it was present in <50% of the tumor; and in 2 cases, it was present in >50% of the tumor.

Immunohistochemistry for GFs, Their Receptors, and COX2

The scores of the immunohistochemical expression of VEGF, VEGFR-I, VEGFR-II, PDGFB, PDGFRb, bFGF, Flg, TGFb1, TGFbR-I, TGFbR-II, and COX2 for each case are listed in Supplemental Table 3.

VEGF was highly expressed in 32 of 40 cases (80%; Fig. 1), VEGFR-I in 33 cases (82.5%; Fig. 2), and VEGFR-II in 32 cases (80%; Fig. 3). VEGF, VEGFR-I, and VEGFR-II were simultaneously highly expressed in 27 of 40 cases (67.5%). Cases negative for VEGF, VEGFR-I, and VEGFR-II were 1, 0, and 1, respectively. In intraneoplastic vessels, MCs were always VEGFR-II negative (Fig. 3). PDGFB was highly

expressed in 37 of 40 cases (92.5%; Figs. 4, 5) and PDGFRb in 32 cases (80%; Fig. 6). PDGFB and PDGFRb were simultaneously highly expressed in 30 of 40 cases (75%). Cases negative for PDGFB and PDGFRb were 0 and 3, respectively. In intraneoplastic vessels, MCs were PDGFB negative (Fig. 5) and PDGFRb positive (Fig. 6). bFGF was highly expressed in 29 of 40 cases (72.5%; Fig. 7) and Flg in 21 cases (52.5%; Fig. 8). bFGF and Flg were simultaneously highly expressed in 19 of 40 cases (47.5%). No cases were negative for bFGF and Flg. TGFb1 was highly expressed in 9 of 40 cases (22.5%) and had low expression in 31 cases (77.5%; Fig. 9). TGFbR-I was highly expressed in 16 of 40 cases (40%; Fig. 10) and TGFbR-II in 24 cases (60%; Fig. 11). TGFb1, TGFbR-I, and TGFbR-II were simultaneously highly expressed in 4 of 40 cases (10%). Cases negative to TGFb1, TGFbR-I, and TGFbR-II were 1, 1, and 0, respectively. COX2 was highly expressed in 14 of 40 cases (35%), while 21 cases were negative to COX2 (52.5%; Fig. 12). In a minority of cases, the intensity of the staining for VEGF, bFGF, PDGFB, TGFb1 and their receptors VEGFR-I, VEGFR-II, PDGFRb, Flg, TGFbR-I, TGFbR-II was more intense in a subgroup of neoplastic cells, ranging from 5% to 10% of positive cells. For each molecule evaluated, the expression was cytoplasmic. Graphs summarizing immunohistochemical results are depicted in Figure 13, 14, and 15.

Statistical Analysis

The odds of high expression of GF receptor was more than 3 in tumors with high GF expression relative to those with low GF expression ($P < .05$); specifically, a statistically significant and strong association was found between high expression of VEGF and VEGFR-I (OR ¼ 9.667; P ¼ .015), VEGF and VEGFR-II (OR ¼ 16.111; P ¼ .003), bFGF and Flg (OR ¼ 5.067; P ¼ .038), and bFGF and PDGFRb (OR ¼ 16.2; P ¼ .003). The odds of high expression of TGFb1 were 12 times greater in cases with high expression of COX2 relative to those with low expression of COX2 (P ¼ .006). Weak nonstatistically significant

associations were found between TGFb and TGFbR-I (OR ¼ 1.27, P ¼ .757) and between COX2 and bFGF (OR ¼ 0.92, P ¼ .911). Moderate nonstatistically significant associations were found between TGFb and TGFbR-II (OR ¼ 2.88, P ¼ .229), between PDGFB and Flg (OR ¼ 2.63, P ¼ .447), and between COX2 and VEGF (OR ¼ 1.80, P ¼ .511).

Concerning Ki67 labeling index, residuals analysis suggested the use of logarithmic scale. No significant relationship was found between Ki67 labeling index and VEGF, VEGFR-I, VEGFR-II ($F_{3,36}$ ¼ 0.6703, P ¼ .5758), between Ki67 and PDGFB, PDGFRb, flg, bFGF ($F_{4,35}$ ¼ 0.2989, P ¼ .8766), and between Ki67 and COX2 ($F_{1,38}$ ¼ 0.4791, P ¼ .4931).

Results of regression models suggested a weak association between Ki67 labeling index and the above-mentioned variables being near to 1.00 (from 0.8 to 1.3)—specifically, the estimated ratio between the average of Ki67 in high versus low expression of GFs and in high versus low expression of COX2 (see supplemental material).

Discussion

Most canine PWTs expressed molecules involved in VEGF-, PDGFB-, and bFGF-mediated signaling pathways. The statistically significant association between GFs and GF receptor expression observed in PWTs recapitulated the upregulatory loops involved in MCs and EC crosstalk described in vitro.[7,34] Immunohistochemistry revealed the expression of VEGF, VEGFR-I, and VEGFR-II in a high percentage of cells in most PWTs. VEGF, which is normally produced by MCs during angiogenesis, binds to VEGFR-I/II on ECs, stimulating EC proliferation.[42] Interestingly, PWT neoplastic MCs were characterized by the aberrant expression of VEGFR-II. VEGFR-II is normally absent in MCs, as reported in the literature[48] and as evidenced in the intratumoral vessels of tested cases. This finding can be related to the

concurrent activation of an autocrine and paracrine pathway stimulating proliferation of neoplastic cells and intratumoral angiogenesis. Furthermore, the association between the high expression of VEGF and VEGF receptors was statistically significant. This finding suggests the presence of an upregulatory pathway between VEGF and VEGFR-I/II that has been already demonstrated in vitro[7] and that, in canine PWTs, may cause the amplification of the autocrine/paracrine loop involved in neoplastic cell proliferation. This hypothesis has been suggested also for canine STSs and osteosarcomas, where VEGF and VEGFR-I/II expression by neoplastic cells and their presence in the serum of affected dogs have been reported.[15,16,19] As a consequence of these promising observations, therapies targeting VEGF have been applied to canine STSs utilizing vaccine and virotherapy approaches.[27,37]

The PDGFB-PDGFRb pathway seemed also involved in canine PWT development. During angiogenesis, PDGFB is produced by ECs and binds to PDGFRb that is expressed by MCs.[24,26] This process leads to MC recruitment and vascular wall stabilization.[24,26]

PDGFB is usually produced by ECs and not MCs,[24,26] a finding confirmed by intratumoral normal vessel expression pattern in this report. Interestingly, PWT neoplastic MCs highly expressed PDGFRb and PDGFB, suggesting the presence of an additional autocrine and paracrine pathway involved in PWT development and growth, characterized by aberrant expression of PDGFB. Upregulation of the PDGFB pathway has been demonstrated in canine hemangiosarcoma, feline injection site sarcoma,[3,28] and human sarcomas,[32] in which it has been also targeted for therapeutic purposes.[9,12]

The bFGF-Flg pathway was also highly expressed by canine PWTs. bFGF is a potent angiogenic factor that stimulates EC and MC proliferation.[21,46] While bFGF can directly stimulate EC proliferation, the presence of PDGFB is necessary for bFGF-mediated MC proliferation in vivo since bFGF and PDGFBB

upregulate PDGFRb on ECs and Flg on MCs, respectively.[34] The significant association between bFGF and PDGFRb high expression suggested a major involvement also of this upregulation loop in canine PWT development.

The fourth pathway investigated in this report involved TGFb1, TGFbR-I, and TGFbR-II. The majority of PWTs were characterized by the expression of TGFb1 by <70% of neoplastic cells. TGFb1 and its receptors are normally expressed in ECs and MCs and are involved in the induction of MCs from undifferentiated mesenchymal cells.[44] A further role of the TGFb superfamily is to regulate COX2 secretion in hypoxictissues;hypoxia itself represents anadditionaltrigger for MCs proliferation.[35,45] Simultaneous high expression of TGFb1 and the 2 corresponding receptors was evident in 4 of 40 PWTs. Based on these findings, the TGFb1 pathway seems to play a minor role in canine PWTs pathogenesis. Interestingly, in this study, TGFb1 expression was significantly associated with COX2 expression, supporting the presence of a link between these 2 molecules, as demonstrated by previous studies.[35,45]

COX2 was detected in a minority of tumors. This result was expected since COX2 expression has been reported mainly in epithelial tumors,[14,20] while its presence in mesenchymal neoplasm is inconstant and rarely has a prognostic relevance.[11,18,23,25]

Mitotic index and MIB-1 labeling index of PWTs were similar to what has been reported,[4,47] and a statistical association with the expression of GFs was not identified. This result, although deceiving, is not surprising, since neoplastic cell proliferation rate is likely the result of the interaction among several GFs, GF receptors, and other cell cycle regulators, but the number of cases included in this study did not allow the evaluation of association patterns of multiple GFs. An alternative hypothesis is that an alteration of the downstream intracellular signaling pathways induces an inefficient

expression of GF and GF receptors, hampering signal transduction. Evaluation of the expression levels of the molecules involved in signal transduction could better elucidate this hypothesis. As a last point, the expression of GFs involved in angiogenesis, such as bFGF, PDGFB, and VEGF, may derive from a hypoxic microenvironment (as often occurs for neoplastic lesions) inducing angiogenesis not directly correlated with neoplastic cell proliferation. Nevertheless, this latter hypothesis seems improbable given the distinctive lack of expression of specific GF receptors by MCs of intratumoral blood vessels. The association between TGFb and its receptors and between PDGFB and Flg was accounted for but surprisingly not detected in PWTs. For these cases, an OR >2 may be still suggestive of an association that nevertheless is not significant, likely as the consequence of the evaluation of a statistical insufficient number of cases in the modalities of the examined parameters.

Interestingly, the pathways with the highest expression in canine PWTs are mediated by tyrosine kinase receptors. This finding suggests that tyrosine kinase receptor blockade may represent a promising therapy to control canine PWT recurrence. If this hypothesis is substantiated by future clinical studies, results could parallel observations reported for some human STS subtypes[9,10,32] and in a few studies in veterinary medicine that attempted target therapies toward specific GFs.[27,31,37] Noteworthy, the efficacy and safety of the use of a commercially available tyrosine kinase inhibitor for canine mast cell tumor therapy has been already reported.[22]

However, based on the correlations between different GF and GF receptor expressions identified in this work, an approach aimed at multiple pathway inhibition (mainly VEGF, PDGFB, and bFGF) seems a possibly more promising approach for PWT control.

In summary, we have identified the high expression of 3 signaling pathways that may play a role in PWT pathogenesis and that represent good candidates for the control of local recurrence, which is the most common canine PWT relapse.[4,47]

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References

Al-Dissi AN, Haines DM, Singh B. Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor in canine cutaneous fibrosarcomas. J Comp Pathol. 2009;141:229–236.

Armulik A, Genove´ G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21:193–215.

Asa SA, Murai A, Murakami M, et al. Expression of platelet-derived growthfactor and its receptors in spontaneous canine hemangiosarcoma and cutaneous hemangioma. Histol Histopathol. 2012;27:601–607.

Avallone G, Boracchi P, Stefanello D, et al. Canine perivascular wall tumors: high prognostic impact of site, depth, and completeness of margins. Vet Pathol. 2014;51:713–721.

Avallone G, Helmbold P, Caniatti M, et al. The spectrum of canine cutaneousperivascular wall tumors: morphologic, phenotypic and clinical characterization. Vet Pathol. 2007;44:607–620.

Bardagı´ M, Fondevila D, Ferrer L. Immunohistochemical detection of COX-2 in feline and canine actinic keratoses and cutaneous squamous cell carcinoma. J Comp Pathol. 2012;146:11–17.

Barleon B, Siemeister G, Martiny-Baron G, et al. Vascular endothelial growthfactor up-regulates its receptor fms-like tyrosine kinase 1 (FLT-1) and a soluble variant of FLT-1 in human vascular endothelial cells. Cancer Res. 1997;57:5421–5425.

Belshaw Z, Constantio-Casas F, Brearley MJ, et al. COX-2 expression and outcome in canine nasal carcinomas treated with hypofractionated radiotherapy. Vet Comp Oncol. 2010;9:141–148.

Benjamin RS, Scho¨ffski P, Hartmann JT, et al. Efficacy and safety of motesanib, an oral inhibitor of VEGF, PDGF, and Kit receptors, in patients with imatinibresistant gastrointestinal stromal tumors. Cancer Chemother Pharmacol. 2010; 68:69–77.

Berdiaki A, Nikitovic D, Tsatsakis A, et al. bFGF induces changes in hyaluronan synthase and hyaluronidase isoform expression and modulates the migration capacity of fibrosarcoma cells. Biochim Biophys Acta. 2009;1790:1258–1265.

Carmody Soni EE, Miller BJ, Scarborough MT, et al. Cyclooxygenase-2 expression is not associated with clinical outcome in synovial sarcoma. Oncol Rep. 2011;26:1513–1517.

Chen YC, Chang CN, Hsu HC, et al. Sennoside B inhibits PDGF receptor signaling and cell proliferation induced by PDGF-BB in human osteosarcoma cells. Life Sci. 2009;84:915–922.

Cheng X, Yang G, Schmeler KM, et al. Recurrence patterns and prognosis ofendometrial stromal sarcoma and the potential of tyrosine kinase-inhibiting therapy. Gynecol Oncol. 2011;121:323–327.

Choi J, Chang H. The expression of MAGE and SSX, and correlation of COX2,VEGF, and survivin in colorectal cancer. Anticancer Res. 2012;32:559–564.

de Queiroz GF, Dagli ML, Meira SA, et al. Serum vascular endothelial growthfactor in dogs with soft tissue sarcomas. Vet Comp Oncol. 2013;11:230–235.

de Queiroz GF, Dagli MLZ, Fukumasu H, et al. Vascular endothelial growthfactor expression and microvascular density in soft tissue sarcomas in dogs. J Vet Diag Invest. 2010;22:105–108.

Dennis MM, McSporran KD, Bacon NJ, et al. Prognostic factors for cutaneousand subcutaneous soft tissue sarcomas in dogs. Vet Pathol. 2011;48:73–84.

Dickens DS, Kozielski R, Leavey PJ, et al. Cyclooxygenase-2 expression doesnot correlate with outcome in osteosarcoma or rhabdomyosarcoma. Pediatr Hematol Oncol. 2003;25:282–285.

Dvir E, Clift SJ. Evaluation of selected growth factor expression in canine spirocercosis (Spirocerca lupi)–associated non-neoplastic nodules and sarcomas. Vet Parasitol. 2010;174:257–266.

Ermert L, Dierkes C, Ermert M. Immunohistochemical expression of cyclooxygenase isoenzymes and downstream enzymes in human lung tumors. Clin Cancer Res. 2003;9:1604–1610.

348 Folkman J, Szabo S, Stovroff M, et al. Duodenal ulcer: discovery of a newmechanism and
349 development of angiogenic therapy that accelerates healing. Ann Surg. 1991;214:414–425.
350 Hahn KA, Ogilvie G, Rusk T, et al. Masitinib is safe and effective for the treatment of canine mast
351 cell tumors. J Vet Intern Med. 2008;22:1301–1309.
352 Hakozaki M, Tajino T, Konno S, et al. Overexpression of cyclooxygenase-2 inmalignant peripheral
353 nerve sheath tumor and selective cyclooxygenase-2 inhibitor-induced apoptosis by activating
354 caspases in human malignant peripheral nerve sheath tumor cells. PLoS One. 2014;9(2):e88035.
355 Hellstro¨m M, Kale´n M, Lindahl P, et al. Role of PDGF-B and PDGFR-beta in recruitment of vascular
356 smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse.
357 Development. 1999;126:3047–3055.
358 Herceg ME, Tsiatis AC, Halpern JL, et al. Cyclooxygenase 2 expression in softtissue leiomyosarcoma.
359 Anticancer Res. 2009;29:2913–2917.
360 Holmgren L, Glaser A, Pfeifer-Ohlsson S, et al. Angiogenesis during humanextraembryonic
361 development involves the spatiotemporal control of PDGF ligand and receptor gene expression.
362 Development. 1991;113:749–754.
363 Kamstock D, Elmslie R, Thamm D, et al. Evaluation of a xenogeneic VEGFvaccine in dogs with soft
364 tissue sarcoma. Cancer Immunol Immunother. 2007; 56:1299–1309.
365 Katayama R, Huelsmeyer MK, Marr AK, et al. Imatinib mesylate inhibitsplatelet-derived growth
366 factor activity and increases chemosensitivity in feline vaccine-associated sarcoma. Cancer
367 Chemother Pharmacol. 2004;54:25–33.
368 L'Eplattenier HF, Lai CL, Ham R, et al. Regulation of COX-2 expression incanine prostate carcinoma:
369 increased COX-2 expression is not related to inflammation. J Vet Intern Med. 2007;21:776–782.

370 Lavalle GE, Bertagnolli AC, Tavares WLF, et al. Cox-2 expression in caninemammary carcinomas:
371 correlation with angiogenesis and overall survival. Vet Pathol. 2009;46:1275–1280.
372 London CA, Hannah AL, Zadovoskaya R, et al. Phase I dose-escalating study ofSU11654, a small
373 molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. Clin Cancer
374 Res. 2003;9:2755–2768.
375 McCarthy CJ, O'Brien GC, Cummins RJ, et al. GIST with a twist: upregulationof PDGF-B resulting in
376 metachronous gastrointestinal stromal tumor and dermatofibrosarcoma protuberans. J
377 Gastrointest Surg. 2009;14:398–403.
378 Monson R. Occupational Epidemiology. 2nd ed. Boca Raton, FL: CRC Press Inc; 1990.
379 Nissen LJ, Cao R, Hedlund E-M, et al. Angiogenic factors FGF2 and PDGF-BBsynergistically promote
380 murine tumor neovascularization and metastasis. J Clin Invest. 2007;117:2766–2777.
381 Ohnaka K, Numaguchi K, Yamakawa T, et al. Induction of cyclooxygenase-2 by angiotensin II in
382 cultured rat vascular smooth muscle cells. Hypertension. 2000;35:68–75.
383 Palmieri C, Avallone G, Cimini M, et al. Use of electron microscopy to classifycanine perivascular
384 wall tumors. Vet Pathol. 2013;50:226–233.
385 Patil SS, Gentschev I, Adelfinger M, et al. Virotherapy of canine tumors withoncolytic vaccinia virus
386 GLV-1h109 expressing an anti-VEGF single-chain antibody. PLoS One. 2012;7(10):e47472.
387 Pereira PD, Lopes CC, Matos AJF, et al. COX-2 expression in canine normaland neoplastic mammary
388 gland. J Comp Pathol. 2009;140:247–253.
389 Poli A, Millanta F, Asproni P, et al. Immunohistochemical expression of COX2, mPGES and EP2
390 receptor in normal and reactive canine bone and in canine osteosarcoma. J Comp Pathol.
391 2012;147:153–160.

Prada J, Queiroga FL, Grego´rio H, et al. Evaluation of cyclooxygenase-2 expression in canine mast cell tumours. J Comp Pathol. 2012;147:31–36.

Queiroga FL, Pires I, Parente M, et al. COX-2 over-expression correlates withVEGF and tumour angiogenesis in canine mammary cancer. Vet J. 2011;189:77–82.

Reynolds LP, Grazul-Bilska AT, Redmer DA. Angiogenesis in the corpusluteum. Endocrine. 2000;12:1–9.

Sanci M, Dikis C, Inan S, et al. Immunolocalization of VEGF, VEGF receptors,EGF-R and Ki-67 in leiomyoma, cellular leiomyoma and leiomyosarcoma. Acta Histochem. 2011;113:317–325.

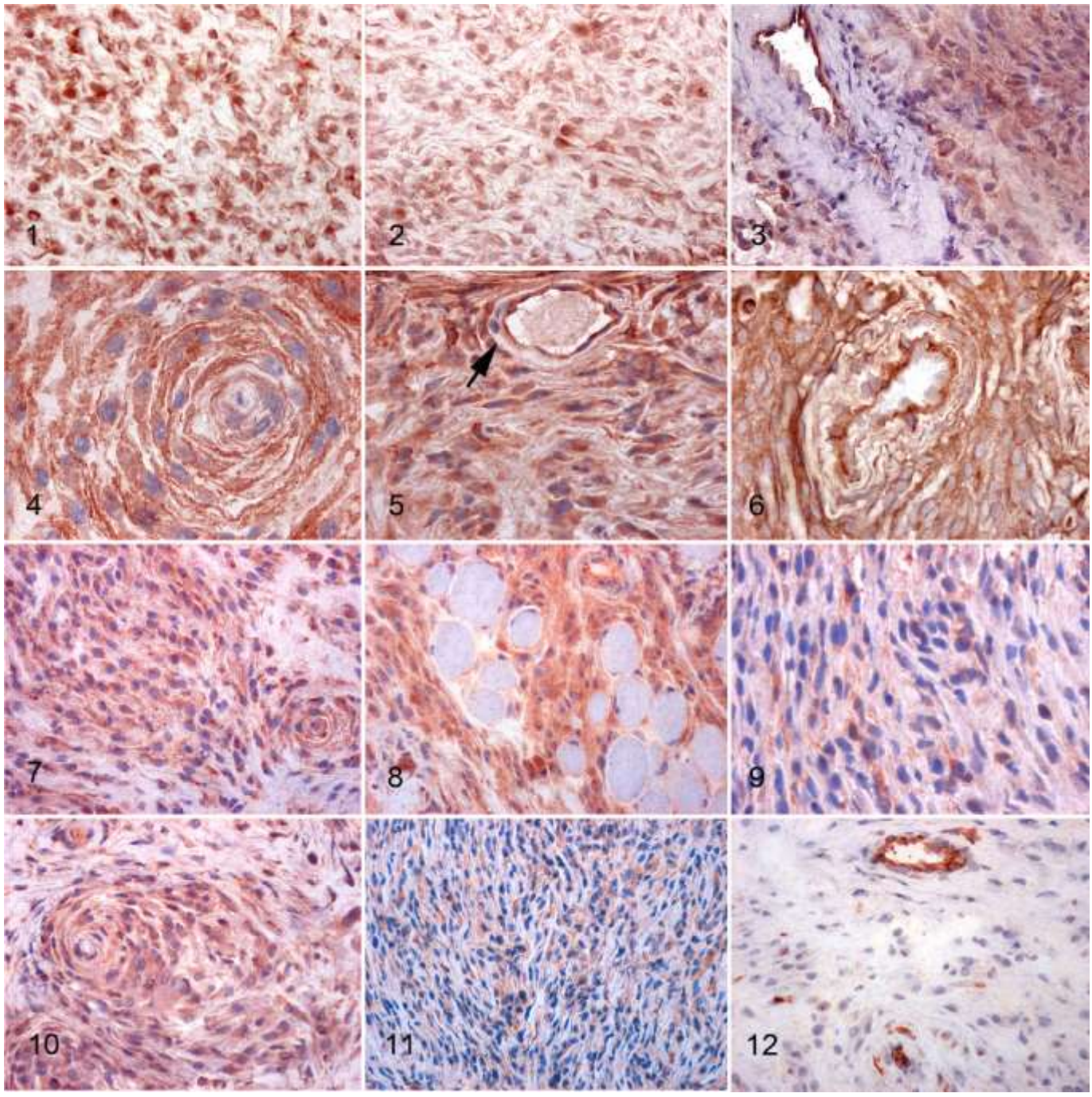
Sato M, Suzuki S, Senoo H. Hepatic stellate cells: unique characteristics in cellbiology and phenotype. Cell Struct Funct. 2003;28:105–112.

Sheares KK, Jeffery TK, Long L, et al. Differential effects of TGF-beta1 andBMP-4 on the hypoxic induction of cyclooxygenase-2 in human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol. 2004;287:919–927.

Shing Y, Folkman J, Sullivan R, et al. Heparin affinity: purification of a tumorderived capillary endothelial cell growth factor. Science. 1984;223(4642):1296–1299.

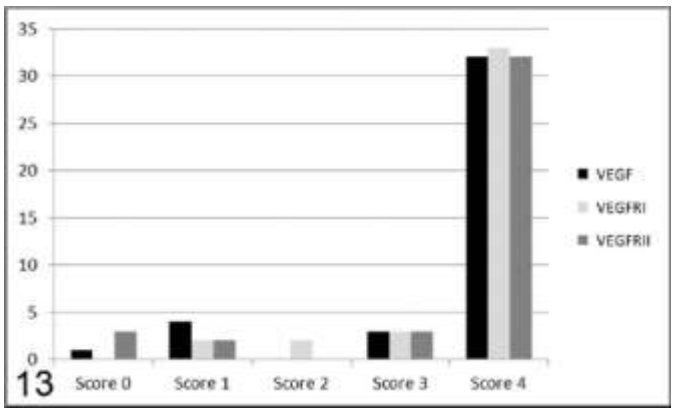
Stefanello D, Avallone G, Ferrari R, et al. Canine cutaneous perivascular walltumors at first presentation: clinical behavior and prognostic factors in 55 cases. J Vet Intern Med. 2011;25:1398–1405.

Yamashita J, Itoh H, Hirashima M, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature. 2000;408(6808):92–96.



Figures 1–12. Perivascular wall tumors, dog, subcutis. Immunohistochemistry for vascular endothelial growth factor (VEGF) and receptors VEGFR-I and VEGFR-II, platelet-derived growth factor B (PDGFB) and receptor PDGFRb, basic fibroblast GF (bFGF), Flg, transforming growth factor b1 (TGFb1) and receptors

TGFbR-I and TGFbR-II, and COX2. ABC method, AEC chromogen, hematoxylin counterstain. Figure 1. Intense and diffuse cytoplasmic expression of VEGF. Figure 2. Diffuse cytoplasmic expression of the majority of neoplastic cells for VEGFR-I. Note a subpopulation of cells are characterized by a more intense staining. Figure 3. Diffuse expression of VEGFR-II in neoplastic cells. Note that endothelium of intraneoplastic vessels is strongly positive, while nonneoplastic mural cells are negative (negative internal control). Figure 4. Diffuse expression of PDGFB in neoplastic cells that are arranged in perivascular whorls. Figure 5. The endothelium of intraneoplastic cells strongly expresses PDGFB, while nonneoplastic mural cells are negative (arrow; negative internal control). Figure 6. The majority of neoplastic cells strongly express PDGFRb as well as nonneoplastic mural cells of intraneoplastic vessels, while the endothelium is negative. Figure 7. Neoplastic cells diffusely and moderately express bFGF. Figure 8. Neoplastic cells infiltrating the skeletal muscle (negative internal control) diffusely and intensely express Flg. Figure 9. Less than 70% of the neoplastic cells intensely express TGFb1 and are admixed with negative cells. Figure 10. Diffuse and intense expression of TGFbR-I in neoplastic cells. Figure 11. Less than 70% of the neoplastic cells intensely express TGFbR-II and are admixed with negative cells. Figure 12. Neoplastic cells do not express COX2. Note the positive endothelium of newly formed intraneoplastic blood vessels (positive internal control)



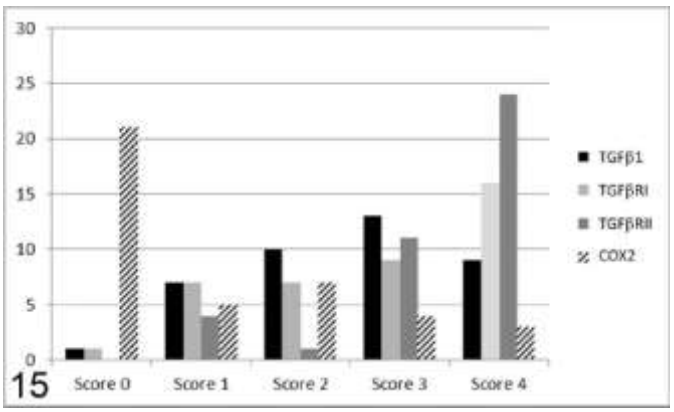


Figure 13. Graphs summarizing the number of cases (y-axis) in each score class (x-axis) for vascular endothelial growth factor (VEGF) and receptors VEGFR-I and VEGFR-II. More than 75% of cases received score 4 for these 3 markers.

Figure 15. Graphs summarizing the number of cases (y-axis) in each score class (x-axis) for transforming growth factor b1 (TGFb1) and receptors TGFbR-I and TGFbR-II, as well as COX2. More than 50% of the cases received score 4 for TGFbR-II. TGFb1 and TGFbR-I were less expressed, and COX2 was negative in the majority of cases.

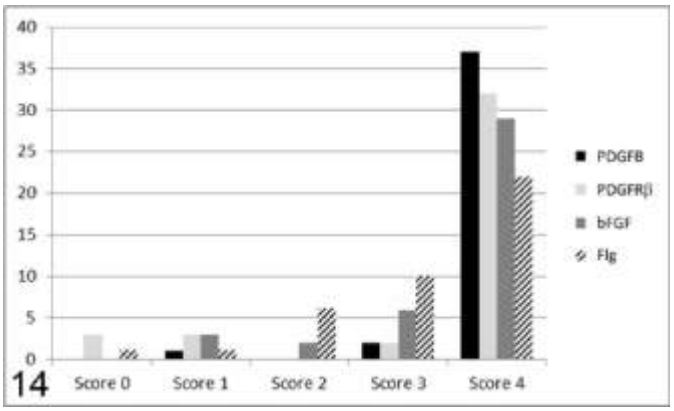


Figure 14. Graphs summarizing the number of cases (y-axis) in each score class (x-axis) for platelet-derived growth factor B (PDGFB) and receptor PDGFRb, as well as basic fibroblast GF (bFGF) and Flg. More than 75% of cases received score 4 for PDGFB and PDGFRb, and 50% of the cases received score 4 for bFGF and Flg. A statistically significant association was found between bFGF and PDGFRb.