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Vascular Endothelial Growth Factor Concentrations in Body Cavity Effusions in Dogs

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Vascular endothelial growth factor (VEGF) has potent angiogenic, mitogenic, and vascular permeability enhancing properties specific for endothelial cells. VEGF is present in high concentrations in inflammatory and neoplastic body cavity effusions and has been implicated in the pathogenesis of neoplastic and inflammatory effusion formation. In this study, VEGF was quantitated by solid-phase enzyme-linked immunoadsorbent assay (ELISA) in samples of pericardial, pleural, and peritoneal effusions (N = 38) from dogs (N = 35) with neoplastic and non-neoplastic diseases. VEGF was detected in 37 of 38 effusions (median, 754; range, 18–3,669 pg/mL) and was present in much higher concentrations than in previously established normal concentrations for canine plasma (median, <1 pg/mL; range, <1–18 pg/mL) or in those previously noted in the plasma of dogs with hemangiosarcoma (HSA; median, 17 pg/mL; range, <1–67 pg/mL). In 4 dogs with HSA, the concurrent plasma VEGF concentration was much lower than in the abdominal effusion ($P = .029$). No significant correlation was demonstrated between VEGF effusion concentration and effusion total protein content or nucleated cell count. Mean VEGF concentrations were significantly higher in pericardial (median, 3,533; range, 709–3,669 pg/mL) and pleural effusions (median, 3,144; range, 0–3,663 pg/mL) compared to peritoneal effusions (median, 288; range, 18–2,607 pg/mL; $P < .05$). There was no marked difference demonstrated between effusions associated with malignant and nonmalignant diseases. Further studies are necessary to elucidate the role of VEGF in body cavity effusion formation in dogs.

Key words: Angiogenesis; Ascites; Pericardial effusion; Vascular endothelial growth factor receptor; Vascular permeability.

Vascular endothelial growth factor (VEGF) is a dimeric heparin-binding glycoprotein with a molecular weight of approximately 45 kDa that has been shown to promote neovascularization.[1–5] VEGF is produced by numerous cell types including tumor cells, macrophages, platelets, megakaryocytes, lymphocytes, neutrophils, smooth muscle cells, keratinocytes, osteoblasts, astrocytes, and mesangial cells.[1–5] Two VEGF receptors have been identified: Flt-1 and Flk-1 kinase insert domain-containing receptor (Flk-1/KDR). The interaction between VEGF and Flk-1/KDR is thought to be important for tumor angiogenesis, increased vascular permeability, and effusion formation.[2,6–9] VEGF is a potent mitogen that is specific for endothelial cells, thereby promoting angiogenesis. It is 50,000 times more potent than histamine in its ability to increase vascular permeability.[9] VEGF production and release are stimulated by hypoxia, inflammatory mediators, and neoplasia.[1–9] VEGF is expressed by a variety of tumors, and high serum or plasma VEGF concentrations often are associated with a high tumor burden, the presence of metastasis, and a poor response to therapy in people.[10–18] VEGF is believed to be a causative factor in the pathogenesis of both neoplastic and non-neoplastic effusions; however, most studies have focused on neoplastic effusions.[19–34] Preclinical in vivo studies demonstrated the VEGF effusion concentration in human patients was directly correlated with progression of effusion.[19,20] Furthermore, blockade of VEGF activity in animal models by means of anti-VEGF antibodies or VEGF receptor tyrosine kinase inhibitors leads to a marked reduction in effusion volume.[20–22,24,25,27,31,33] Some studies in people demonstrate higher VEGF concentrations in neoplastic effusions compared to non-neoplastic effusions, but non-neoplastic infectious and inflammatory effusions also have high VEGF concentrations.[23–25,28–34] The presence of VEGF in non-neoplastic inflammatory effusions most likely is due to other inflammatory mediators and hypoxia stimulating the production and release of VEGF, which then contributes to further effusion accumulation.[24,28–30,32,33] Anti-VEGF agents block the biologic activity of VEGF in in vitro assays and have entered phase I clinical trials in human patients with cancer.[21,25]

If VEGF is shown to play a similar role in dogs, specific therapy may be developed against either VEGF or VEGF receptors to control effusion formation or, ideally, to limit tumor spread. To date, only a small number of studies have examined VEGF in dogs, and none have examined VEGF concentrations of effusions.[35–38] The main purpose of this study was to determine the concentration of VEGF in pericardial, pleural, and peritoneal fluid of dogs with various diseases. Secondary aims were to investigate whether any correlation existed between effusion VEGF concentration, total solids, and nucleated cell counts and to compare effusion VEGF concentrations between dogs with neoplastic and non-neoplastic disease.

Materials and Methods

Study Population and Sample Preparation

Thirty-eight body cavity effusion samples (pericardial, pleural, or peritoneal) were obtained from 35 client-owned dogs presented to the

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Veterinary Hospital of the University of Pennsylvania between August 1999 and April 2000. Medical records were reviewed retrospectively to establish the cause of effusion in each patient. Diagnostic testing was performed at the discretion of each clinician and included historical information, physical examination findings, clinical pathology tests (CBC, serum chemistry, urinalysis, coagulation panel, and bile acid analysis), microbiology, diagnostic imaging (radiography, echocardiography, and abdominal ultrasound), effusion fluid analysis (cytology and total protein content), and histopathological examination of surgical biopsy or postmortem specimens. Effusion samples were obtained via paracentesis or during surgery. Portions of the effusion samples (5 mL) were placed into glass tubes containing 3.8% sodium citrate at a 9:1 ratio[a] and immediately centrifuged at 3,000 rpm. Effusion supernatant was separated and stored at −80°C until assayed. This study was performed in accordance with the University of Pennsylvania's ''Use of Client-Owned Animals'' Protocol.

All samples were examined for total protein content, and 27 samples had cytologic analysis. Both protein content and cytologic analysis were performed before freezing the samples. Effusion fluid was categorized as neoplastic on the basis of cytologic or histologic findings. A diagnosis of neoplasia was made based on cytology in 6 dogs and on histopathology in 17 dogs (3 dogs were diagnosed by both methods). Effusions were designated non-neoplastic if a non-neoplastic cause was identified and the clinical evaluation indicated no evidence of neoplasia.[39] In 4 of the 8 dogs with hemangiosarcoma (HSA), concomitant plasma samples were obtained as part of this and another study.[37]

VEGF Assay

Effusion samples were assayed for VEGF by solid-phase enzyme-linked immunoadsorbent assay (ELISA) with antibodies that recognize secreted VEGF (VEGF 165).[b] One hundred fifty microliters of assay diluent was added to each well of the microtiter plate, which had been coated with anti-VEGF monoclonal antibody. Fifty microliters of effusion then was pipetted into each well and incubated for 2 hours at room temperature to bind VEGF in the sample to the antibody-coated plate. The plate was washed four times with buffer solution to remove unbound VEGF. Two hundred microliters of antibody-linked anti-VEGF polyclonal antibody was added to all wells and incubated for 3 hours at room temperature to allow the secondary anti-VEGF antibody to bind to the immobilized VEGF. The plate was washed again, 200 μL of antibody-linked enzyme substrate was added, and the preparation was incubated for 20 minutes at room temperature. Fifty microliters of a stop solution (2 M sulfuric acid) was added, and samples were analyzed on a microtiter plate reader at an optical density of 450 nm. All samples were run in duplicate, and calibration on the microtiter plate included standard serial dilutions of recombinant human VEGF. The optical density of the standard solutions was plotted against their corresponding concentrations to generate a standard curve and allow determination of all VEGF concentrations for all samples. All samples in this study were batched and analyzed at one time by an outside laboratory.[c] This assay has been validated previously for measurement of canine VEGF, and the inter- and intra-assay variability reported by the manufacturer is less than 10%.[35,40]

Statistical Analysis

Frequency distribution analysis initially was performed. For non-normally distributed data, VEGF concentrations are given as the median and range. VEGF concentrations from pleural, pericardial, and peritoneal effusions were compared by one-way analysis of variance and a Tukey test. The differences between plasma and abdominal VEGF effusion concentrations in HSA dogs were evaluated by the Mann-Whitney rank sum test. The Wilcoxon rank sum test was used to compare VEGF concentrations between dogs with neoplastic and non-neoplastic diseases. Spearman rank correlation was used to assess correlations between VEGF concentrations and nucleated cell counts and total protein concentrations. P values $< .05$ were considered significant. Statistical analysis was performed with 2 statistical software packages.[d,e]

Results

A total of 38 effusion samples from 35 dogs were analyzed for VEGF. The underlying causes (Table 1) were HSA (n = 8), congestive heart failure (CHF) (n = 5), pleural and peritoneal effusions secondary to cardiac tamponade (n = 3) due to idiopathic pericardial effusion (IPE), mesothelioma (n = 3), lymphoma (n = 3), septic peritonitis (n = 2) (perforated small intestine and bladder wall abscess), IPE (pericardial effusion sample) (n = 2), lung lobe torsion (n = 2), malignant histiocytosis (n = 2), bronchiogenic carcinoma (n = 2), hemothorax due to anticoagulant rodenticide intoxication (n = 1), fungal pericarditis (n = 1), liver disease (n = 1), idiopathic chylous effusion (n = 1), malignant melanoma (n = 1), and chemodectoma (n = 1). The 2 dogs with IPE were still alive with no signs of systemic disease or neoplasia 1.5 years after presentation. The pleural and peritoneal samples secondary to cardiac tamponade were obtained from the 2 dogs with IPE (the cause of the cardiac tamponade); however, these samples were listed under the etiology of cardiac tamponade and not IPE specifically to avoid confusion as to the site of the effusion and to more accurately group them according to their pathophysiologic cause. Of the 2 dogs with IPE, 1 had both pleural and peritoneal effusion (before pericardiocentesis), and 1 had only peritoneal effusion. Fourteen dogs had pleural effusions, 17 had peritoneal effusions, and 7 had pericardial effusions.

VEGF was detectable in 37 of 38 effusions analyzed. One dog with hemothorax due to anticoagulant rodenticide intoxication had an undetectable VEGF concentration in the pleural effusion. Effusion VEGF concentrations ranged from 18 to 3,669 pg/mL (median, 754; mean, 1,553).

Effusion VEGF concentrations varied widely, and no significant difference was demonstrable ($P = .3174$) between the neoplastic (median, 1,468 pg/mL; range, 18–3,663 pg/mL) and non-neoplastic effusion groups (median, 448 pg/mL; range, 0–3,669 pg/mL) (Table 1). Etiologies with 1 individual per group are listed as ''Miscellaneous'' for purposes of the table. Specifically, values for these groups are as follows: rodenticide intoxication (0 pg/mL), fungal pericarditis (3,631 pg/mL), idiopathic chylous effusion (705 pg/mL), malignant melanoma (3,663 pg/mL), chronic liver failure (88 pg/mL), and chemodectoma (3,648 pg/mL). Higher VEGF concentrations were noted in pericardial and pleural effusions compared to peritoneal effusions (mean, 2,773 ± 1,393, 2,062 ± 1,577, and 682 ± 851 pg/mL, respectively; $P < .05$). No correlation was found between VEGF concentration and total protein content or nucleated cell count in samples with cytologic examination (data not shown). In the 4 dogs with HSA with concurrent plasma (25, 54, <1, and 67 pg/mL) and effusion samples (218, 450, 18, and 3,459 pg/mL), effusion VEGF concentrations were significantly higher than those of plasma ($P = .029$).

Table 1. Diagnosis and effusion VEGF concentrations for 35 dogs with body cavity effusions.

Diagnosis	Site of Effusion (n)	Patients (n)	VEGF Concentration Median (pg/mL)	VEGF Concentration Range (pg/mL)
Congestive heart failure	Peritoneal (4) Pleural (1)	5	194	146–511
Cardiac tamponade[a]	Pleural (1) Peritoneal (2)		347	288–769
Lung lobe torsion	Pleural (2)	2	NA	349–3,645
Peritonitis	Peritoneal (2)	2	NA	2,392, 2,607
Idiopathic pericardial effusion	Pericardial (2)	2	NA	3,533, 3,669
Miscellaneous[b]	Pleural (3) Pericardial (2) Peritoneal (1)	6	3,631	0.0–3,663
Hemangiosarcoma	Pericardial (3) Peritoneal (5)	8	579	18–3,459
Mesothelioma	Pleural (3)	3	2,748	173–3,508
Lymphosarcoma	Peritoneal (2) Pleural (1)	3	2,148	824–3,652
Bronchogenic carcinoma	Pleural (2)	2	NA	2,757–3,144
Malignant fibrous histiocytoma	Peritoneal (1) Pleural (1)	2	NA	85–2,759

VEGF, vascular endothelial growth factor.

[a] Three effusion samples under this category were obtained from the 2 dogs because of idiopathic pericardial effusions.

[b] Contains individuals with effusions resulting from rodenticide intoxication, fungal pericarditis, idiopathic chylous effusion, malignant melanoma, chronic liver failure, or chemodectoma.

Discussion

Increased permeability of the small capillaries within body cavities may play an important role in effusion formation. Regardless of cause, increased permeability of vessels may enable extravasation of large proteins, which not only may produce an oncotic pull of fluid into the respective body cavity but also may impair lymphatic drainage and decrease fluid efflux.

The ability of soluble factors (such as VEGF) to produce fluid accumulation via increased permeability has been documented in animal models and in people.[20–34] VEGF can be produced by numerous cell types and is a powerful inducer of increased vascular permeability.[1–5] High VEGF concentrations have been noted in benign and malignant effusions in people.[28–32,41] VEGF also is believed to play a central role in effusion formation in murine tumor models and human ovarian cancer lines.[2,19,20,24,42] Much of this work has involved the use of mouse models in which tumors are transplanted into the peritoneal cavity. Within several days of tumor transplantation, small blood vessels located in tissues lining the body cavity and mesentery become hyperpermeable, leading to extravasation of a protein-rich exudate into the peritoneal cavity.[19] In these models, tumor cell number, hyperpermeability, VEGF concentration, and ascites volume were found to increase in parallel.[19] A recent study has further clarified the role of VEGF in effusion formation by means of a newly developed model for malignant pleural effusions associated with human lung adenocarcinoma cells.[43] Increased peritoneal effusion accumulation occurred in mice administered a cell line that highly expresses VEGF, compared to a low expression cell line from which little effusion accumulation was noted.[43] Finally, effusion formation and effusion VEGF concentrations decrease after administration of either anti-VEGF antibody or agents that interfere with VEGF signaling (recombinant human interferon γ, phosphokinase receptor inhibitors).[21,27,31] These studies suggest that VEGF is more likely to be the cause (or part of the cause) than the result of the effusions.

Clinically healthy dogs usually have plasma VEGF concentrations <1 pg/mL, and dogs with HSA have plasma VEGF concentrations <70 pg/mL.[37] Effusion VEGF concentrations were extremely high compared to plasma, regardless of the underlying cause. All but 2 samples had VEGF concentrations in excess of 70 pg/mL, with 16 exceeding 1,000 pg/mL and 10 exceeding 3,000 pg/mL. This phenomenon may represent local VEGF production. Similar results have been documented in people, in which VEGF effusion concentrations were 5- to 10-fold higher than those of matched serum samples.[29] In one study, VEGF effusion concentrations measured over a 9-week period demonstrated a continual steep rise compared to only a moderate increase in concentrations in matched serum samples.[29]

Low VEGF concentrations were noted in effusions from dogs with CHF, liver disease, rodenticide intoxication, cardiac tamponade, and idiopathic chylous effusions. The etiologies of the above-mentioned effusions, with the exception of idiopathic chylous effusion, often are not associated with inflammation or neovascularization, and low VEGF concentrations would be expected.

Concurrent plasma VEGF concentrations from 4 dogs with HSA were markedly lower than VEGF effusion concentrations. This difference may represent intratumoral concentrations of VEGF released during tumor rupture into the body cavity.[44] Alternatively, increased concentrations in these effusions also may represent local VEGF release from metastases within the body cavity.

High VEGF concentrations were observed in dogs with peritonitis, fungal pericarditis, and IPEs (pericardial samples), as well as in 1 dog with lung lobe torsion. High VEGF concentrations in effusions resulting from inflammatory conditions have been noted in several studies in people.[28–32,41] IPEs have been hypothesized to be the result of an inflammatory process.[45–47] Histologically, the presence of numerous thrombi in vessels is common in pericardial tissue of dogs with IPE, and these thrombi may serve as a stimulus for VEGF release.[45–47]

High VEGF concentrations were noted in most of the neoplastic effusions. Both samples from effusions associated with pulmonary carcinomas had high VEGF concentrations. This phenomenon also has been noted in effusions from human patients with lung carcinomas.[26,28,30,41] Although several of the other neoplastic effusions had high VEGF concentrations, the small sample size precluded comparisons among groups. Low VEGF concentrations were noted in a few neoplastic effusions. The origin of effusions in patients with neoplasia may be multifactorial. Tumor implants lining body cavities can produce changes in osmotic and oncotic pressures, leading to fluid accumulation.[48] Compression by tumors can cause lymphatic obstruction and tumor rupture, or invasion into blood vessels can yield a hemorrhagic effusion.[48] In dogs with low VEGF concentrations, it is possible that the effusions resulted from mechanisms other than fluid production by tumor cells. Cytokines other than VEGF may be involved in effusion formation. Interleukin-8, angiogenin, and tumor necrosis factor have been shown to increase vascular permeability.[49,50] The theory that localized hypoxia due to high intra-abdominal pressure from the effusion may have led to VEGF production in some effusions is plausible but unlikely, because many of the dogs with CHF had large effusions but low VEGF concentrations. It also is possible that thrombosis or inflammation may have led to the local production of VEGF in some effusions. Some studies have demonstrated a causative role for VEGF in effusion formation.[21,27,31,42,43] In the present study, without further testing, we are unable to determine whether VEGF was the result or the cause of the effusion.

A previous study in humans demonstrated no correlation between VEGF effusion concentration and effusion nucleated cell count.[28] Although not the primary goal of this study, it would have been interesting to compare VEGF concentrations and cytology for all samples rather than just a subset. Such a comparison may have identified differences not detected because of small sample size. Finally, it also would have been interesting to compare neoplastic cell counts (not performed on all samples) and VEGF concentrations to see if a correlation existed.

The main limitation of this study was the small sample size within each subgroup, which prevented elucidation of potential differences among etiologic categories. Failure to demonstrate a statistically significant difference could have been the result of inadequate power (type 2 error). With the observed variability, a sample size of 145 dogs per group would be necessary to determine whether these data (neoplastic versus non-neoplastic) represent a type 2 error. It is unlikely that the variability in data was a result of experimental error, because the inter- and intra-assay variation associated with this kit, reported by the manufacturer, is $<10\%$. The biologic activity of VEGF in the samples in this study was not determined, but several previous studies have demonstrated high biologic activity of VEGF in neoplastic effusions.[21,24,25]

VEGF was present in high concentrations in body cavity effusions in dogs, but concentrations were highly variable. The effects of VEGF on permeability likely contribute to the pathogenesis of some effusions (tumor associated and inflammatory). However, the process of effusion formation is complex and may involve multiple cytokines and growth factors. Further studies to determine the biologic activity of VEGF in effusions and the effect of VEGF inhibitors on effusion accumulation are needed to determine the role of VEGF in effusion formation. If a causative role is established for VEGF in effusion formation in dogs, novel anti-VEGF therapies may be useful.

Footnotes

[a] Glass tubes containing 3.8% sodium citrate, Becton Dickinson, Vacutainer Systems, Franklin Lakes, NJ
[b] ELISA test kit, R & D Systems, Minneapolis, MN
[c] Laboratory analysis, University of Delaware, Department of Animal and Food Science, Newark, DE
[d] STATA 6.0 for Windows, College Station, TX
[e] Sigma Stat, Version 2.0, Jandell Corporation, San Rafael, CA

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