Advances in Brief

Serum Vascular Endothelial Growth Factor Is Often Elevated in Disseminated Cancer[1]

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Abstract

In adults, marked angiogenesis takes place only during the female reproductive cycles, during wound healing, and accompanying some disease processes, such as tumor development. Vascular endothelial growth factor (VEGF) is a secreted, endothelial cell-specific growth factor, which is induced by tissue hypoxia and is angiogenic *in vivo*. We measured serum VEGF (S-VEGF) concentrations by ELISA in patients with a variety of types of cancer, as well as in healthy volunteers, and in patients with diabetes or rheumatoid arthritis. Elevated S-VEGF concentrations were found in patients with locoregional (n = 39; median, 158 pg/ml; range, 8–664 pg/ml) or disseminated (n = 58; median, 214 pg/ml; range, 17–1711 pg/ml) cancer in comparison to individuals without cancer (n = 113; median, 17 pg/ml; range, 1–177 pg/ml; $P < 0.0001$ for both comparisons). Values higher than 200 pg/ml were observed in 74% of patients with untreated metastatic cancer, and high serum levels were measured regardless of the histological type of cancer. S-VEGF levels were found to be higher in untreated patients with disseminated cancer than in those with local cancer ($P = 0.006$), and patients undergoing cancer therapy had lower values than those without cancer therapy ($P = 0.03$). The results indicate that both patients with locoregional cancer and patients with disseminated cancer may have elevated S-VEGF levels, regardless of the histological type of cancer, and that S-VEGF is often elevated in cancer with distant metastases.

Introduction

Angiogenesis, or the formation of new capillaries, is an important component of many biological processes, both physiological and pathological. In healthy adults, extensive angiogenesis occurs only during the female reproductive cycles. Angiogenesis may also take place in some pathological conditions, such as wound healing, rheumatoid arthritis, diabetic retinopathy, and tumor growth (reviewed in Ref. 1). Because other physiological or pathological conditions associated with angiogenesis are usually easily diagnosed, a serum marker for active angiogenesis may be of value in the diagnosis of cancer and in the follow-up of tumor response to cancer therapy. Tumor growth is largely dependent on angiogenesis, and a serum marker may therefore be useful, regardless of the histological type of cancer.

During tumorigenesis, as a result of disruption of the physiological balance between angiogenesis inhibitors and stimulators, normally quiescent or slowly growing vasculature can undergo rapid vascular proliferation (reviewed in Ref. 2). VEGF,[3] also called vascular permeability factor, is a secreted, dimeric protein (M_r 46,000) that is active as an endothelial cell-specific mitogen and as a vascular permeability factor (reviewed in Refs. 3 and 4). As a result of alternative splicing of VEGF mRNA, there exist at least three isoforms of VEGF encompassing 121, 165, and 189 amino acid residues (5). The two shorter forms are efficiently secreted from cells, whereas the longer one remains mostly cell-associated (3). Recently, two new putative growth factors structurally similar to VEGF have been characterized and named as VEGF-B and VEGF-C (6, 7).

VEGF has been shown to be hypoxia inducible *in vitro* in a variety of cultured cells (8–10) and *in vivo* in myocardium (11), retina (12), and lung (13). Hypoxia-induced expression of VEGF has been shown to stimulate the formation of new vessels in the retina (12). Elevated VEGF concentrations have been measured in the ocular fluid of patients with diabetic retinopathy and other retinal disorders (14, 15) and in the synovial fluid and synovial tissue of patients with rheumatoid arthritis (16).

VEGF expression has been detected in a large variety of malignant human tumors, including tumors of the breast, brain, lung, and gastrointestinal tract, and it has been concluded that VEGF plays an important role in tumor biology and in the process of tumor angiogenesis (reviewed in Refs. 17 and 18). Chinese hamster ovary cells or MCF-7 human breast carcinoma cells overexpressing VEGF from transfected DNA constructs showed enhanced tumor growth and an increase in associated vascular density *in vivo* in nude mice, but VEGF overexpression had no effect on tumor cell growth *in vitro* (19, 20). Similarly, expression of VEGF in transfected melanoma cells increased tumor growth, angiogenesis, and metastases when the cells were injected into nude mice (21). In agreement with these findings, anti-VEGF antibodies have been shown to inhibit the growth of tumor xenografts (22–24). Inhibition of glioma growth and angiogenesis has been observed in tumors in nude mice derived

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; S-VEGF, serum VEGF; CV, coefficient of variation.

from tumor cells transfected with an antisense VEGF sequence (25).

Because VEGF is a soluble, secreted growth factor and is expressed in cancers of many different histological types, it is of interest to measure S-VEGF concentrations in cancer patients and to compare them to those of healthy individuals. However, there are few data available on S-VEGF levels in healthy individuals or in pathological conditions. A few patients with primary breast cancer have been found to have elevated S-VEGF concentrations in comparison to healthy controls, and a large size of the primary breast tumor was associated with elevated S-VEGF concentrations (26). In addition, in this study, increased levels of S-VEGF were also found in patients with carcinoma of the gastrointestinal tract or lung.

In the current study, we have measured S-VEGF concentrations in presumably healthy subjects, in individuals treated for either diabetes or rheumatoid arthritis, and in patients with a variety of histological types of cancer. Our results suggest that patients with disseminated cancer often have highly elevated S-VEGF levels in comparison to individuals without cancer.

Patients and Methods

Subjects without Cancer. We recruited control subjects from volunteers, including patients, personnel, and students of Helsinki University Central Hospital. Sera were collected from 113 individuals without a known neoplasm, without recent trauma or surgery, and who were not pregnant. Eighty-one of these individuals were presumably healthy hospital personnel or students. Seven of these individuals were treated with insulin for diabetes mellitus and five were treated for rheumatoid arthritis. Ninety-four of these individuals were women and 19 were men, and their age ranged from 20 to 82. Particular care was taken to include young women in the series because menstruating women may have more ongoing physiological angiogenesis than men or postmenopausal women. However, personal menstruation histories were not collected.

Twenty of these 113 subjects without known cancer had been treated during the period from 1990 to 1996 for invasive ductal or lobular breast cancer with radical surgery and postoperative radiation therapy and had regular follow-up visits with no sign of recurrent disease. The original tumor-node-metastasis classification of their cancer ranged from T1N0M0 to T4N1M0. Three of these 20 individuals had also received postoperative adjuvant chemotherapy, and 2 had received adjuvant antiestrogen therapy for 3 years.

Subjects with Cancer. Serum samples were taken from 97 patients with histologically diagnosed cancer and 6 patients with low-grade (grades I and II) astrocytoma, admitted to the Department of Oncology, Helsinki University Central Hospital, in 1996. All 103 patients had tumors detectable clinically, in imaging examinations, or both. Fifty-eight of the patients were women and 45 were men, ranging in age from 23 to 85 years. The patients were selected at random for the study, except that individuals who had surgery or major trauma within 2 months prior to serum sampling were excluded. The latter were excluded from the study to avoid possibly elevated VEGF serum concentrations resulting from, *e.g.*, wound healing. The clinical and pathological reports were blindly reviewed, without knowledge of the S-VEGF immunoassay results. The patients with cancer were divided into three groups according to the stage of their disease. Twenty-five patients had local cancer without regional lymph node metastases (local cancer), 14 had regional lymph node metastases (regional cancer), and 58 had disseminated cancer. Forty-three patients were undergoing therapy for cancer at the time of sampling. Fifty-four of the patients were not on active cancer therapy, receiving only palliative treatment, such as nonsteroidal anti-inflammatory drugs, steroids, or morphine. The patients with low-grade (grades I and II) astrocytomas were not included in the group of cancer patients but were instead analyzed as a separate group.

Collection of Venous Blood Samples. Peripheral venous blood samples were collected in sterile test tubes using a Venoject blood collection system (Terumo, Leuven, Belgium) and a constricting tourniquet. All serum samples were collected between 8 and 12 a.m. To minimize serum protease activity and, therefore, possible proteolytic degradation of S-VEGF, venous blood samples were collected in sterile test tubes containing the protease inhibitor aprotinin (Bayer AG, Leverkusen, Germany) at 1000 Komberg international units per a 4-ml sample. The samples were placed on ice, allowed to coagulate at 4°C for a minimum of 30 min, centrifuged at 2000 × *g* for 10 min at 4°C, and then stored in aliquots at −70°C. After thawing, each serum aliquot was assayed only once. The study was approved by a medical ethical committee and informed consent to take the venous blood samples was obtained from all of the patients. Healthy volunteers gave an oral statement of permission.

S-VEGF Immunoassay. S-VEGF concentrations were determined as S-VEGF immunoreactivity, using a quantitative sandwich enzyme immunoassay technique (Quantikine R, R&D Systems, Minneapolis, MN). The system uses a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant human $VEGF_{165}$. According to the manufacturer, the assay is designed to measure natural human VEGF levels in serum or plasma; the CV of intra-assay determinations of S-VEGF varies from 4.5 to 6.7%, and the interassay CV varies from 6.2 to 8.8% at S-VEGF concentrations ranging from 50 to 1000 pg/ml.

For each analysis, 100 μl of serum were used. All analyses and calibrations were carried out in duplicate. In a separate control experiment, known amounts of recombinant human $VEGF_{165}$ were added to the serum samples of healthy subjects and cancer patients, and the concentrations measured were comparable to the projected values. The calibrations on each microtiter plate included recombinant human $VEGF_{165}$ standards. Optical densities were determined using a microtiter palate reader (Multiscan RC Type 351, Labsystems, Helsinki, Finland) at 450 nm. The blank was subtracted from the duplicate readings for each standard and sample. A standard curve was created using StatView 4.02 (Abacus Concepts Inc., Berkeley, CA) by plotting the logarithm of the mean absorbance of each standard *versus* the logarithm of the VEGF concentration. Concentrations are reported as pg/ml.

Statistical Analysis. S-VEGF distributions between different groups were compared using the Mann-Whitney test. All *P*s are two-tailed.

Table 1 S-VEGF concentrations in subjects with and without cancer

Group	No. of cases	S-VEGF (pg/ml), median (range)	P^a	S-VEGF ≥ 100 pg/ml, *n* (%)	S-VEGF ≥ 200 pg/ml, *n* (%)
Subjects without cancer, total	113	17 (1–177)		5 (4)	0 (0)
Presumably healthy	81	15 (1–177)			
Operated breast cancer, NED[b]	20	30 (3–172)			
Diabetes	7	16 (8–34)			
Rheumatoid arthritis	5	22 (7–30)			
Locoregional cancer					
No therapy	23	158 (29–664)	<0.0001	20 (87)	8 (35)
With therapy	16	170 (8–656)	<0.0001	9 (56)	7 (44)
Disseminated cancer					
No therapy	31	251 (17–1711)	<0.0001	28 (90)	23 (74)
With therapy	27	184 (21–557)	<0.0001	22 (81)	10 (37)

[a] When compared to subjects without cancer (n = 113); Mann-Whitney test.
[b] NED = no evidence of disease.

Results

Subjects without Cancer. S-VEGF concentrations ranged from 1 to 177 pg/ml (median, 15 pg/ml) in the presumably healthy individuals (n = 81; Table 1). Similarly, low serum levels were measured in individuals followed up after curative treatment for primary breast cancer (n = 20) and in individuals treated for diabetes or rheumatoid arthritis (n = 12; Table 1). Only 5 (4%) of the 113 subjects without cancer had a S-VEGF level higher than 100 pg/ml (Fig. 1; Table 1).

Subjects with Cancer or Low-Grade Astrocytoma. Cancer patients had elevated levels of S-VEGF (n = 97; median, 197 pg/ml; range, 8–1711 pg/ml) in comparison to the 113 subjects without cancer (median, 17 pg/ml; range, 1–177 pg/ml; $P < 0.0001$). Patients with locoregional or disseminated cancer had higher VEGF serum levels than subjects without cancer ($P < 0.0001$ for both comparisons; Table 1). Thirty-one (57%) of the patients with untreated cancer and as many as 23 (74%) of those with untreated metastatic cancer had a S-VEGF concentration higher than 200 pg/ml (Fig. 1; Table 1). This contrasts with the levels measured among the 113 individuals without cancer, all of whom had a S-VEGF concentration lower than 200 pg/ml. The median S-VEGF level of the patients who did not receive therapy for cancer was lower in local disease than in disseminated cancer (140 *versus* 251 pg/ml, respectively; P = 0.006).

Elevated serum levels were measured independently of the histological type of cancer (Table 2). S-VEGF concentrations over 200 pg/ml were observed in patients suffering from cancer of the breast, lung, prostate, esophagus, stomach, colon, rectum, brain, or head and neck or from melanoma, non-Hodgkin's lymphoma, mesothelioma, or angiosarcoma. However, low S-VEGF levels were consistently detected in patients with low-grade astrocytoma (n = 6; range, 15–54 pg/ml).

S-VEGF Concentration and Cancer Therapy. The S-VEGF concentrations of patients undergoing cancer therapy were also significantly higher than those of the 113 individuals without cancer (Table 1). This was the case for patients with locoregional cancer (median, 170 pg/ml; range, 8–656 pg/ml; $P < 0.0001$), and for patients with disseminated cancer (median, 184 pg/ml; range, 21–557 pg/ml; $P < 0.0001$). However, S-VEGF concentrations of the patients undergoing cancer therapy (median, 184 pg/ml; range, 8–656 pg/ml) were lower than those of the patients not under cancer therapy (median, 226 pg/ml; range, 17–1711 pg/ml; P = 0.03).

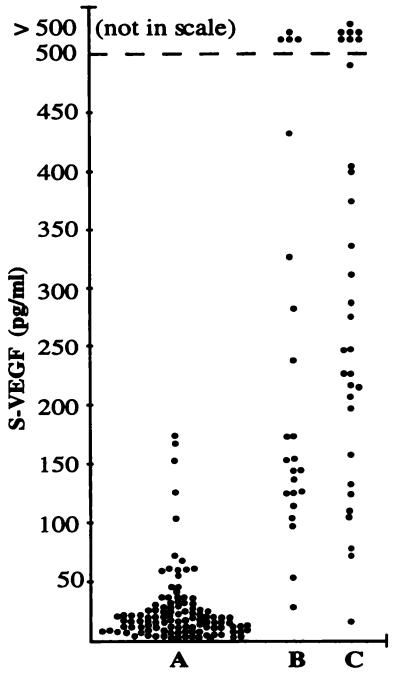

Fig. 1 A scatter plot of S-VEGF concentrations (pg/ml) in subjects without cancer and in cancer patients. *A*, subjects without cancer (n = 113); *B*, patients with locoregional cancer, no therapy for cancer (n = 23); *C*, patients with disseminated cancer, no therapy for cancer (n = 31).

Discussion

We observed elevated concentrations of VEGF in the sera of patients with cancer in comparison to individuals without cancer. Elevated S-VEGF levels were detected not only in cancer with distant metastases but also in locoregional disease. The highest concentrations were measured in patients with metastatic cancer who were not receiving cancer therapy. Pa-

Table 2 S-VEGF concentrations in patients with cancer (n = 97) or low-grade astrocytoma (n = 6) according to the site of origin

Site	No. of cases	S-VEGF (pg/ml)	
		Median	Range
Locoregional cancer			
Lung	9	289	41–664
Brain			
Grade I and II	6	28	15–54
Grade III and IV	9	95	14–319
Gastrointestinal tract	5	147	20–503
Prostate	5	132	29–298
Breast	3	150	132–244
Head and neck	3	440	8–656
Other cancers[a]	4		130–572
Disseminated cancer			
Breast	30	205	21–1347
Gastrointestinal tract	10	254	17–767
Lung	6	367	77–1711
Lymphoma	6	190	53–403
Prostate	3	125	33–250
Other cancers[b]	4		112–278

[a] Melanoma, mesothelioma, angiosarcoma, and cancer of the pancreas.

[b] Cancers of the kidney, thyroid, and gallbladder and melanoma.

tients with grade III and IV glioma also had elevated S-VEGF levels, whereas all six patients with grade I and II astrocytoma had low S-VEGF levels. Although we have not yet determined the origin of the VEGF measured from the serum, it is interesting to note that vascular proliferation is critical in the distinction between high- and low-grade gliomas.

Patients with disseminated cancer had higher S-VEGF concentrations than those with local cancer. In many types of human cancer, the cancer cells *in vivo* express VEGF (reviewed in Refs. 17 and 18), and it is a plausible hypothesis that at least part of S-VEGF is derived from cancer cells in such cases. However, lymphocytes infiltrating human cancers, peripheral blood T lymphocytes, and peritoneal macrophages have been shown to express VEGF (27, 28). Hence, the serum VEGF may originate not only from cancer cells, but from tumor-infiltrating inflammatory cells and circulating peripheral blood cells as well.

Our recent results also indicate that platelets contain VEGF, which can be released when blood samples are subjected to prolonged incubation.[4] However, we estimate that normal levels of platelet-derived VEGF cannot be responsible for the highest S-VEGF concentrations observed in cancer patients. The half-life of VEGF in the circulation has been reported to be approximately 3 min (1). One possibility is that an excess of VEGF secretion by hypoxic cells of solid tumors escapes to the circulation, where most of it is initially absorbed by a rapid clearance mechanism, but continued high-level production causes accumulation of VEGF in the circulation or in blood cells, such as platelets. Our ongoing studies now address whether VEGF determinations from platelet-deficient plasma allow us to better distinguish between VEGF of blood cell and tumor origin.

In this study, elevated S-VEGF concentrations were observed in patients with many different histological types of cancer, such as cancers of the breast, prostate, lung, and gastrointestinal tract, malignant glioma, melanoma, and lymphoma. Because tumor blood vessels may be derived from normal stromal vasculature upon secretion of VEGF by the tumor cells, it is possible that elevated S-VEGF levels are detectable in patients with many kinds of malignant tumors undergoing angiogenesis, regardless of their histological type. On the other hand, in this study, nine patients (17%) with disseminated cancer and not undergoing cancer therapy had low levels of S-VEGF, comparable to levels found in healthy individuals, indicating that low S-VEGF may be found in patients with untreated metastatic cancer. We also have preliminary data suggesting that patients with small local breast carcinomas may typically have S-VEGF concentrations that do not significantly differ from those of healthy individuals.[4]

The biological role of S-VEGF in the metastatic process and cancer progression may be important. Local and perhaps even circulating VEGF may not only stimulate proliferation of tumor blood vessels but also increase vascular permeability, contributing to tumor cell extravasation and metastasis formation. Also, VEGF has recently been found to inhibit maturation of dendritic cells, important antigen-presenting cells (29). Hence, prolonged exposure of the immune system to high levels of VEGF may play a role in allowing tumors to avoid induction of an immune response. Therefore, not only may high serum levels of VEGF reflect a large tumor mass or a rapid rate of angiogenesis but tumors that produce high levels of VEGF may also disseminate more effectively due to greater vascular permeability or immune defects.

Dirix *et al.* (30) have recently found that high serum basic fibroblast growth factor and VEGF levels are associated with a short tumor volume-doubling time in advanced colorectal cancer. Our data on elevated S-VEGF in the majority of disseminated cancers, regardless of the histological type of cancer, suggest that S-VEGF determinations may find clinical applications in the follow-up of cancer therapy. However, diagnostic studies and studies with longitudinal patient follow-up are required to determine the value of S-VEGF measurement in detection of recurrent cancer.

In summary, our results indicate that S-VEGF levels are often elevated in cancer patients and that the majority of patients with disseminated disease have higher levels of S-VEGF than individuals without cancer. Furthermore, we found lower S-VEGF concentrations in patients undergoing cancer therapy than in untreated patients. Although these data are encouraging, further studies are required to investigate the origin of S-VEGF, as well as its levels in conditions such as benign tumors, trauma, and infectious and inflammatory states.

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[4] Unpublished data.

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