Advances in Brief

Leukocytes and Platelets of Patients with Cancer Contain High Levels of Vascular Endothelial Growth Factor[1]

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Abstract

Vascular endothelial growth factor (VEGF) is a secreted endothelial cell-specific mitogen and permeability factor. Malignant human tumors have been shown to produce VEGF. Elevated levels of VEGF have been detected in sera of cancer patients, but its origin is unsettled. We analyzed VEGF concentrations in serum, plasma, whole blood, and peripheral blood mononuclear cells (PBMNCs) and platelets in 56 cancer patients and 52 healthy controls using ELISA. The VEGF concentrations in the lysed whole blood samples [blood VEGF (B-VEGF)] were higher in cancer patients than in healthy controls (median, 464 *versus* 298 pg/ml; $P < 0.0001$). The highest B-VEGF values were found in disseminated cancer. In cancer patients, a high B-VEGF concentration was associated with a high peripheral blood leukocyte count ($P = 0.0012$) and platelet count ($P = 0.019$). In healthy individuals, a high B-VEGF was associated with a high leukocyte count ($P = 0.0001$) but not with the platelet count ($P > 0.1$). The cancer patients regularly had higher B-VEGF concentrations than healthy individuals with comparable leukocyte or platelet counts. The VEGF content of isolated PBMNCs and platelets was severalfold higher in cancer patients than in healthy controls (median, 10.6 *versus* 0.9 pg per 10^6 PBMNCs, and median, 1.6 *versus* 0.5 pg per 10^6 platelets; $P < 0.0001$ and $P = 0.0008$, respectively). Serum VEGF and B-VEGF correlated strongly ($P < 0.0001$). Very little or no VEGF was found in the plasma. The results indicate that VEGF in the bloodstream is transported by blood cells, including leukocytes and platelets. The blood cells of cancer patients contain greatly elevated amounts of this major angiogenic growth factor, and this reservoir of VEGF may have a role in tumor angiogenesis and metastasis formation. VEGF in serum samples originates from blood cells, and the use of VEGF of whole blood or of isolated blood cells may improve the clinical value of VEGF measurements.

Introduction

During tumorigenesis, the vasculature becomes activated to grow new capillaries. This process, which is called angiogenesis, is essential for the growth of all solid tumors [reviewed by Folkman (1)]. VEGF,[3] which is also called vascular permeability factor, is a soluble, dimeric glycoprotein of M_r 34,000–45,000. VEGF is a strong endothelial cell specific mitogen and also a potent vascular permeabilizing agent. Expression of VEGF increases angiogenesis and tumor growth, and anti-VEGF antibodies inhibit tumor growth and reduce experimental metastasis in nude mice [reviewed by Dvorak *et al.* (2)].

Recently, patients with various histological types of cancer have been found to have elevated S-VEGF concentrations in comparison to healthy controls (3–8). We recently determined the VEGF concentrations in serum samples of 82 patients with non-Hodgkin's lymphoma taken before treatment and found that patients with lower than median S-VEGF at diagnosis had a 71% 5-year survival rate, in comparison to only 49% among those with a higher than the median S-VEGF, suggesting that a high S-VEGF is associated with unfavorable prognosis in non-Hodgkin's lymphoma (9). A similar relationship was also observed in patients with small cell lung cancer, and furthermore, a high pretreatment level of S-VEGF was also associated with poor response to chemotherapy (10).

However, the origin of the elevated levels of VEGF measured in the serum samples obtained from cancer patients remains unsettled. It is well documented that, in several types of human cancer, the cancer cells express VEGF mRNA and polypeptides [reviewed by Dvorak *et al.* (2)]. Also, tumor-infiltrating inflammatory cells have been shown to express VEGF in several histological types of cancer (11, 12). Hence, it is an attractive hypothesis that VEGF found in the sera of cancer patients originates from the tumor. However, peripheral blood cells, including platelets, B- and T-lymphocytes, granulocytes, and monocytes, also express VEGF (11, 13, 14). Consequently, the elevated amounts of VEGF detected in the serum samples of cancer patients could also be liberated from the peripheral blood cells. To investigate the origin of S-VEGF, here we measured VEGF in serum, plasma, and whole blood as well as in some blood cell fractions in healthy individuals and in patients with cancer.

Materials and Methods

Patients. Peripheral venous blood samples were collected from 52 randomly selected patients with histologically

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; S-VEGF, serum VEGF; PBMNC, peripheral blood mononuclear cell; B-VEGF, blood VEGF.

diagnosed cancer admitted to the Department of Oncology, Helsinki University Central Hospital in 1997. Thirty (58%) of the patients were male, and the age range for all patients was 31–88 years. Nineteen (37%) patients had locoregional cancer, and 33 had disseminated cancer. Ten of the patients were treated for cancer (chemotherapy or hormonal therapy) at the time of sampling, whereas the rest did not have any cancer therapy or were receiving only palliative treatment, such as nonsteroidal anti-inflammatory drugs, steroids, or morphine. In addition to the 52 cancer patients, samples were also collected from four patients with surgically treated glioma and four patients with lymphoma who were clinically in a complete remission with no evidence of disease at the time of sampling. The median follow up time for these eight patients was 36 months (range, 12–96 months).

Healthy Controls. Venous blood samples were also collected from 56 presumably healthy volunteers, including personnel and students of Helsinki University Central Hospital. Twenty-six (46%) of the healthy volunteers were male, and the age range for all patients was 18–62 years.

Collection of Venous Blood Samples. Peripheral venous blood samples were collected using a Venoject blood collection system (Terumo, Leuven, Belgium). The serum samples were collected in sterile test tubes, and the plasma and the whole blood samples were collected in sterile test tubes containing sodium citrate as an anticoagulant. After sampling, the samples were incubated at +4°C for 60–240 min. The serum and plasma samples were centrifuged at 2000 × *g* for 10 min at +4°C and then stored in aliquots at −70°C. The cells of the whole blood samples were lysed by adding 2 volumes of sterile aqua, and subsequently freeze-thawing the samples twice. A medical ethical committee approved the study, and informed consent to take the venous blood samples was obtained from all patients. Healthy volunteers gave an oral statement of permission.

Isolation of Peripheral Blood Platelets. A platelet suspension was prepared from the venous blood as described by Muszbek *et al.* (15). Peripheral venous blood samples were collected in sterile test tubes anticoagulated with acid-citrate-dextrose and containing 0.18 μM prostaglandin E_1 (Sigma Chemical Co., St. Louis, MO). Platelet-rich plasma was obtained by centrifugation (120 × *g* for 20 min, +37°C). Platelet-rich plasma was collected and transferred to new tubes, and the cell counts were determined using a differential cell counter Technicon H2 (Bayer, Leverkusen, Germany). In every isolation, platelets from one cancer patient and at least one healthy control were isolated simultaneously to minimize potential errors due to sample handling between the study groups. Prior to the VEGF immunoassay, the platelets were lysed by adding two volumes of sterile water and subsequently freeze-thawing the samples twice.

Isolation of PBMNCs. Peripheral venous blood samples were collected in sterile test tubes containing sodium citrate as an anticoagulant. A PBMNC suspension was prepared using density gradient centrifugation on a mixture of Ficoll and sodium metrizoate. Blood anticoagulated with sodium citrate was diluted with an equal volume of PBS and layered on top of Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 400 × *g* at +18°C for 30 min. The PBMNC layer was then collected, washed three times with PBS containing 10% fetal bovine serum (Sigma), and resuspended in sterile BPS. The identity of cells was then confirmed, and the cell counts were determined using a differential cell counter Technicon H2. In every isolation, cells from one cancer patient and at least one healthy control were isolated simultaneously to minimize potential errors due to sample handling between the study groups. Prior to the VEGF immunoassay, the cells were lysed as described above.

VEGF Immunoassay. VEGF concentrations were determined as S-VEGF immunoreactivity, essentially as described previously (9), using a quantitative sandwich enzyme immunoassay technique (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, MN). The system uses a solid-phase monoclonal and an enzyme-linked polyclonal antibody raised against recombinant human VEGF. For each analysis, 100 μl of sample were used. All analyses and calibrations were carried out in a duplicate. The calibrations on each microtiter plate included recombinant human VEGF standards. Optical densities were determined using a microtiter plate 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. VEGF concentrations were determined without any knowledge of the clinical data except age and sex of the individuals, which could be found on the test tube labels.

Statistical Analysis. The Mann-Whitney *U* test and Spearman rank correlation test were used to compare different groups. The scattergrams with regression lines (Fig. 1) and the box plots (Fig. 2) were calculated and plotted using StatView 4.02 (Abacus Concepts Inc., Berkeley, CA). All *P*s are two-tailed.

Results

Comparison of S-VEGF and Plasma VEGF Concentrations. The plasma samples of the healthy controls ($n = 23$) and the cancer patients ($n = 36$) contained only very low amounts of VEGF ranging from below the detection limit (9.0 pg/ml) to 109 pg/ml (median 15 pg/ml). In contrast, the S-VEGF concentrations of the same 23 healthy controls ranged from 12 to 492 pg/ml (median, 66 pg/ml), and those in the sera of the same 36 cancer patients ranged from 29 to 1260 pg/ml (median, 343 pg/ml). In a separate control experiment, known amounts of recombinant human $VEGF_{165}$ were added to the plasma samples of healthy subjects and cancer patients, and the concentrations measured were comparable to the projected values, thus confirming that recovery of VEGF from plasma samples was complete.

Whole B-VEGF Concentrations in Subjects without Cancer. VEGF concentrations in lysed whole blood samples (the total B-VEGF) ranged from 92 to 554 pg/ml (median, 298 pg/ml) in the presumably healthy individuals ($n = 56$; Table 1). No association was found between age and B-VEGF ($P > 0.1$; Mann-Whitney *U* test). Low B-VEGF levels were measured also in individuals who were having regular follow-up visits with no evidence of disease after potentially curative treatment for lymphoma or glioma (median, 285 pg/ml; range, 217–465 pg/ml; $n = 8$). When the B-VEGF values of the healthy controls were compared to the S-VEGF values of the same individuals collected at the same time (median, 125 pg/ml; range, 17–492

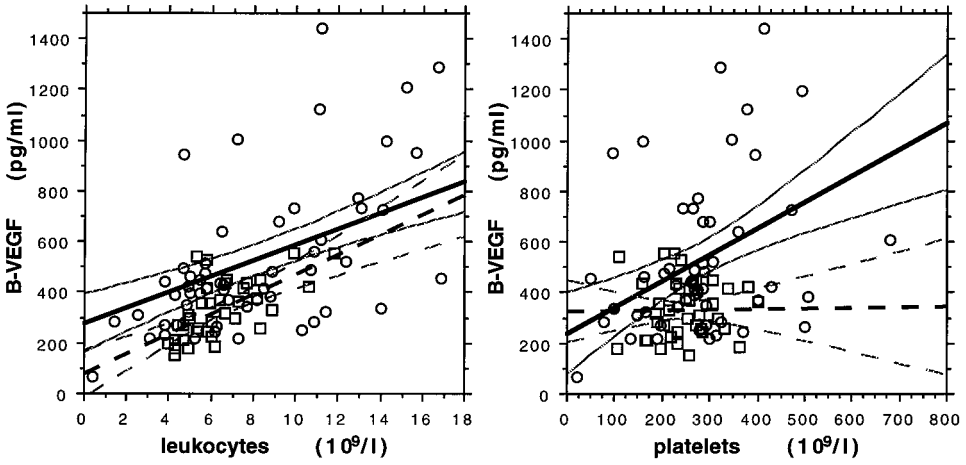

Fig. 1 B-VEGF (pg/ml) against the peripheral blood leukocyte and platelet counts in 36 healthy controls (□) and 47 cancer patients (○). The regression lines and the 90% confidence intervals for the mean are shown separately for healthy controls (- - - -) and cancer patients (——).

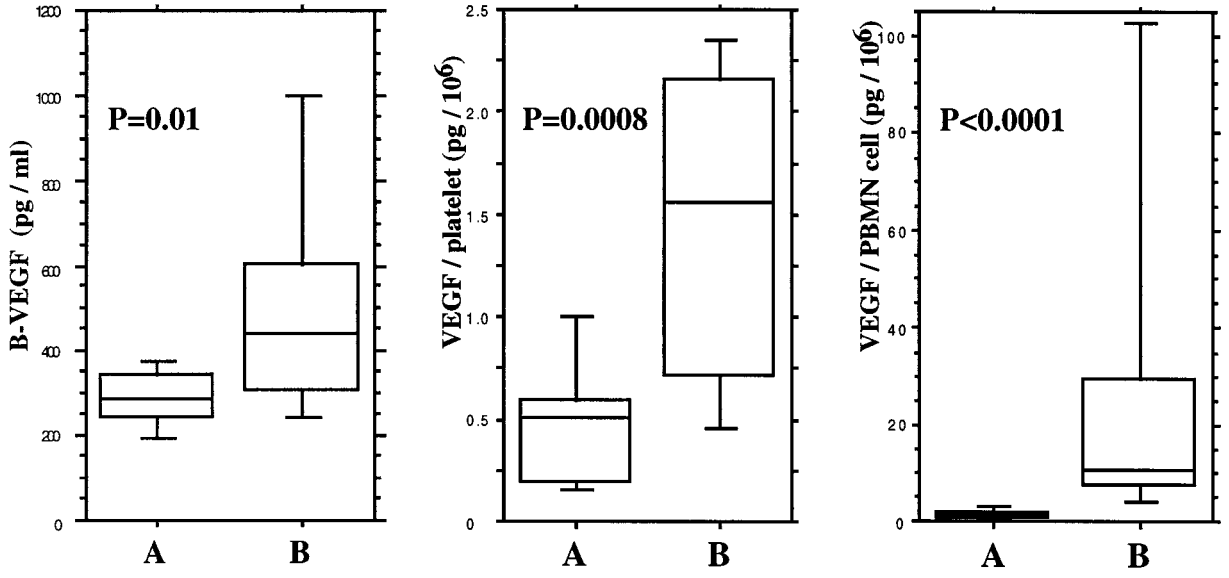

Fig. 2 Comparisons of 15 healthy controls (*columns A*) and 14 cancer patients (*columns B*) for B-VEGF (pg/ml), VEGF (pg) per 10^6 platelets, and VEGF (pg) per 10^6 PBMNCs. The *horizontal lines* indicate 10th, 25th, 50th (median), 75th, and 90th percentiles. The Mann-Whitney *U* test demonstrated that the differences are significant.

pg/ml; $n = 54$), a strong correlation was found ($P < 0.0001$; Spearman rank correlation).

B-VEGF in Subjects with Cancer. The B-VEGF (whole blood) concentrations of cancer patients were usually clearly elevated (median, 464 pg/ml; range, 69–1531 pg/ml; $n = 52$; Table 1), compared to the 56 healthy controls ($P < 0.0001$; Mann-Whitney *U* test). The highest B-VEGF levels were found in patients with disseminated cancer. Of them, 29 (89%) had B-VEGF higher than 298 pg/ml (the median B-VEGF of the healthy controls), and 15 (45%) had a B-VEGF value higher than any of the controls. S-VEGF measured from the same patients ranged from 29 to 1999 pg/ml (median, 343 pg/ml; $n = 48$). No association was found between the age and B-VEGF ($P > 0.1$, the Mann-Whitney test). Similar to the healthy individuals, the B-VEGF and S-VEGF values measured from the same cancer patient's samples showed a strong correlation ($P < 0.0001$; Spearman rank correlation).

B-VEGF and Peripheral Blood Leukocyte and Platelet Counts. To study whether the higher B-VEGF concentrations in cancer patients could simply result from peripheral blood leukocytosis or thrombocytosis, we compared the leukocyte and platelet counts of 47 cancer patients and 36 healthy controls to his or her B-VEGF level. The scattergrams with regression lines are shown in Fig. 1. In cancer patients, a high B-VEGF was associated with both a high leukocyte count ($P = 0.0012$, Spearman rank correlation) and a high platelet count ($P = 0.019$). In healthy individuals, a high B-VEGF concentration was associated with a high peripheral blood leukocyte count ($P = 0.0001$; Spearman rank correlation), whereas in contrast to cancer patients, B-VEGF and platelet count were not associated

Table 1 VEGF concentrations (pg/ml) in the blood samples of healthy controls ($n = 56$), patients treated for cancer ($n = 8$), and patients with cancer ($n = 52$)

Group	No. of cases	B-VEGF (pg/ml)		P^a
		Median	Range	
Healthy controls	56	298	108–554	
Patients with treated cancer, NED[b]	8	285	217–465	0.70
Treated glioma, NED	4	231	226–465	
Treated lymphoma, NED	4	263	217–418	
Patients with cancer, all	52	464	69–1531	<0.0001
Glioma	11	446	218–772	0.0051
Lymphoma				
Locoregional lymphoma	3	221	221–413	
Disseminated lymphoma	11	397	69–1441	0.011
Other locoregional cancers[c]	5	393	263–725	0.055
Other disseminated cancers[d]	22	563	253–1531	<0.0001

[a] Compared to healthy controls ($n = 56$); Mann-Whitney U test.
[b] NED, no evidence of disease.
[c] Cancers of the lung, thyroid, or prostate.
[d] Cancers of the lung, breast, colon, jejunum, esophagus, kidney, thyroid, testis, bladder, or gallbladder; sarcomas; or melanomas.

($P > 0.1$). The cancer patients regularly had higher B-VEGF concentrations than healthy individuals with comparable leukocyte or platelet counts. The results indicate that the high B-VEGF levels found in cancer patients cannot be explained only by the presence of leukocytosis or thrombocytosis.

VEGF in the PBMNC and Platelet Fractions. VEGF of the PBMNC and platelet fractions was assessed in 15 healthy controls and 14 cancer patients. The PBMNCs of the cancer patients contained as much as 12 times more VEGF than those of the healthy controls (median, 10.6 pg per 10^6 PBMNCs; range, 3.6–198.9 pg per 10^6 PBMNCs *versus* median, 0.9 pg per 10^6 PBMNCs; range, 0.3–15.5 pg per 10^6 PBMNCs, respectively; $P < 0.0001$; Mann-Whitney U test; Fig. 2). Similarly, platelets of the cancer patients contained ~3 times more VEGF than those of healthy individuals (median, 1.6 pg per 10^6 platelets; range, 0.3–2.6 pg per 10^6 platelets *versus* median, 0.5 pg per 10^6 platelets; range, 0.1–1.6 pg per 10^6 platelets, respectively; $P = 0.0008$; Fig. 2). The blood cell VEGF content turned out to be a much better discriminator between cancer patients and controls than the total B-VEGF, although the difference in the B-VEGF values between the two groups was also significant (median, 285 pg/ml; range, 117–544 pg/ml *versus* median, 438 pg/ml; range, 69–1531 pg/ml, respectively; $P = 0.01$; Fig. 2).

Discussion

Here, only very low concentrations of VEGF could be detected in plasma of both healthy controls and cancer patients. In contrast to this, elevated levels of VEGF were observed in the lysed whole blood samples of cancer patients in comparison to healthy controls. Even when the leukocyte and platelet counts were taken into account, the levels of B-VEGF were generally higher in patients with cancer than in healthy individuals. In particular, we found large differences in VEGF concentrations in isolated PBMNCs and platelets between cancer patients and healthy individuals.

In previous studies, both we and others have reported that high S-VEGF levels in cancer patients are associated with various unfavorable clinical parameters. These include short tumor volume doubling time (5), progressive disease (6, 16), extensive disease (16–19), poor patient survival (9, 10, 20), and poor response to chemotherapy (10). Elevated S-VEGF levels have also been associated with pregnancy and preeclampsia, which both are conditions accompanied with endothelial cell activation (21, 22). Plasma does not contain significant quantities of VEGF, indicating that VEGF measured from serum samples is released from the blood cells during the coagulation process. It should, therefore, be noted that variations in sample handling may affect the blood cell activation and, thus, the release of VEGF to serum. For example, activated platelets release VEGF in a rapid discharge reaction (13, 14). This creates a potential hazard for systematic errors inside and between different study groups when VEGF concentrations are measured from serum samples. Consequently, B-VEGF appears to be a more reliable indicator for circulating VEGF than S-VEGF. As shown in Fig. 2, the amount of VEGF as calculated per a blood cell was large in cancer patients with only little overlap between the cancer patients and controls. This suggests that especially the use of VEGF of isolated PBMNCs might improve the clinical value of VEGF measurement as compared to standard serum samples.

The elevation of circulating VEGF in cancer patients is due to a rise of VEGF in blood cells. In addition to hypoxia, various cytokines such as epidermal growth factor, transforming growth factor-α, transforming growth factor-β, and platelet-derived growth factor have been shown to induce the expression of VEGF in cultured cells [reviewed by Dvorak *et al.* (2)]. Therefore, several factors in the tumor microenvironment might be responsible for up-regulation of VEGF biosynthesis in the blood cells traveling in the tumor vasculature. Megakaryocytes have been shown to contain both VEGF mRNA and protein (14) so that at least a part of the VEGF in the platelets is endogenously synthesized in megakaryocytes. However, in addition to synthesizing various proteins, megakaryocytes and platelets also endocytose and concentrate circulating plasma proteins and later transport them to α-granules (23). Channels of the canalicular system serve as the pathway for transport of substances into the platelets as well as for the discharge of α-granule proteins secreted during the platelet release reaction (24).

The reservoir of VEGF in the blood cells of cancer patients may have a role in tumorigenesis. Recent data indicate that the amount of VEGF may be crucial for its function, because studies

with heterozygous and homozygous VEGF-deficient transgenic mice suggest a tight dose-dependent regulation of embryonic vessel development by VEGF (25, 26). Besides stimulating proliferation of tumor blood vessels, VEGF also increases vascular permeability [reviewed by Dvorak *et al.* (2)], possibly contributing to tumor cell extravasation and metastasis formation. Because VEGF has been found to inhibit maturation of dendritic cells (27), exposure of the immune system cells to high levels of VEGF in the tumor microenvironment and in the circulation could aid tumors in avoiding induction of an immune response. Leukocytes transporting VEGF might even play a role in presenting this growth factor to its target cells, thus contributing to the progression of cancer, and VEGF released from activated platelets may also play an important role in tumor angiogenesis and metastasis formation. It is interesting to speculate that a part of the anticancer effect of myelotoxic drugs could result from a reduction of the number of VEGF containing leukocytes and platelets.

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