Advances in Brief

Concentrations of Vascular Endothelial Growth Factor in the Sera of Normal Controls and Cancer Patients

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Abstract

Vascular endothelial growth factor (VEGF) is known to play crucial roles in tumor angiogenesis. We have investigated the circulating level of VEGF in sera from cancer patients as well as from healthy normal controls using a sensitive enzymatic immunoassay. Immunoreactive VEGF proteins were detectable in normal sera, and the cutoff level was determined to be 180 pg/ml. In examined patients with all types of cancer, including breast, gastrointestinal, hepatobiliary, and lung cancer, an aberrant increase in the circulating level of VEGF was detected. For example, in 137 primary breast cancer patients, 12 (8.8%) showed an aberrant increase in VEGF levels. This aberrant expression of VEGF in sera was significantly associated with the progression of the disease, and with VEGF protein expression in tumor tissues. In addition, a Western blot analysis confirmed the presence of the VEGF165 form in sera from a patient with recurrent breast cancer.

It was concluded that VEGF was detectable in normal sera, and its level was increased in some populations of cancer patients. A positive angiogenesis regulator, VEGF might function as an endocrine growth factor, particularly for solid tumors.

Introduction

Angiogenesis, the formation of new vessels from a preexisting network of vessels, is essential for the progression of solid tumors (1–3). The process of neovascularization is multistep, involving endothelial cell migration, proliferation, and differentiation in addition to proteolysis of extracellular matrix (4). Many angiogenic factors including the fibroblast growth factor family (5), VEGF[2] (6), TGF-α (7), platelet-derived endothelial cell growth factor (8), and hepatocyte growth factor (9) have been identified and demonstrated to play crucial roles in tumor angiogenesis. Among these novel angiogenic factors, VEGF is thought to be one of the most important (10, 11). VEGF was first isolated from the culture supernatant and the ascites of rodent tumors as a potent vascular permeability factor (12). Independently, VEGF was also purified from the conditioned medium of cultured bovine pituitary folliculo stellate cells as a potent endothelial mitogen (13). Subsequent molecular analyses confirmed that the two forms were identical (14, 15). VEGF includes four alternative splicing variants (VEGF121, VEGF165, VEGF189, and VEGF209), but the four forms possess signal sequences and similar functions involving endothelial proliferation, migration *in vitro*, and permeability *in vivo* (16). Many reports showed that VEGF expression was observed in a variety of tumor cell lines and also in many types of human solid tumors, including breast cancer (17), central nervous system neoplasms (18), gastrointestinal cancer (19), urinary tract cancer (20), and ovarian cancer (21). In particular, in breast cancer tissues, we found that VEGF expression was associated significantly with the amount of MVD determined by the immunocytochemical analysis using antiendothelial monoclonal antibody (17). In addition, a recent report documented that the transfection of this growth factor gene into a tumor cell line expressing a low level of VEGF proteins altered tumor cells more angiogenic and more progressive in the nude mouse model without the change of cell growth rate *in vitro* (11, 22). Thus, it is evident that VEGF is responsible for the neovascularization as a paracrine factor in many types of tumors. However, still little is known about its behavior in the circulating level. In this study, we measured VEGF concentrations in sera from cancer patients as well as from healthy individuals with a sensitive EIA for VEGF. The present result will show the existence of VEGF, a positive regulator of angiogenesis, in the circulating level.

Patients and Methods

Healthy Controls and Cancer Patients. One hundred eighty-four healthy individuals (132 males, 52 females) without any evidence of diseases (*e.g.*, liver dysfunction, diabetes, etc.), 9 individuals without cancer but with liver dysfunction, and 286 cancer patients were enrolled in this study. Cancer patients included 175 breast (137 primary, 38 recurrent), 66 gastric (58 primary, 8 recurrent), 15 hepatobiliary and pancreas (8 hepatocellular carcinomas, 4 cholangiocellular carcinomas, and 3 pancreas carcinomas), 12 colorectal, 6 lung, and 3 esophageal

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[2] The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; MVD, microvessel density; EIA, enzymatic immunoassay; ICA, immunocytochemical assay; TGF-α, transforming growth factor α.

cancer patients treated in Tokyo Metropolitan Komagome Hospital from 1993 to 1994. Patients with liver dysfunction due to hepatitis B and C virus infection were excluded from this study, except hepatocellular carcinoma patients. In breast cancer patients, we examined the correlation between serum VEGF concentrations and clinicopathological parameters determined by the rules of the Japanese Breast Cancer Society, which were based on Union International Contre Cancer criteria. Also, in 73 primary breast cancers, intratumoral VEGF expression was examined by immunostaining using anti-VEGF monoclonal antibody and compared with serum VEGF levels.

Samples. Venous blood samples were drawn into a tube and centrifuged at 3000 rpm for 10 mins, and the serum samples were stored at −20°C until they were used for determinations of VEGF. Primary tumors were frozen immediately after the surgical removal and stored at −80°C until processing.

Chemiluminescence EIA for VEGF. For the VEGF-EIA, an antihuman VEGF polyclonal antibody was prepared from rabbit serum immunized with recombinant human VEGF121-glutathione-*S*-transferase fusion protein. Ninety-six-well microtiter plates (Microfluorblack plate, Dynatech Laboratories, Chantilly, VA) were coated with 10 mg/ml of the purified anti-VEGF antibody in 0.1 M NaCl and 0.25 M carbonate buffer (pH 9.0), then blocked with 1% BSA, 0.2 M carbonate buffer (pH 9.5), 0.1 M NaCl, and 0.1% NaN_3. For the assay, 100 μl of samples and serially diluted VEGF (standards) were added to the wells, and incubated for 1 h at 22°C. After washing the wells six times, 100 μl of alkaline phosphatase-conjugated Fab' of the anti-VEGF antibody was added to each well and incubated for 1 h at 22°C. Wells were washed eight times, and then the enzyme reaction was carried out at 37°C for 1 h with 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxethane disodium salt (Lumi-phos 530; Wako Pure Chemicals, Inc., Osaka, Japan) as a substrate. The chemiluminescence of each well was measured with a plate luminometer, and the VEGF content of the samples was estimated from the standard curve determined from the serially diluted VEGF121. The analytical sensitivity of this assay was assessed by measuring serially diluted recombinant human VEGF121 (23) ranging from 1587.3 pg/ml to 0 pg/ml. The minimal detectable concentration, defined as +2 SD above the zero standard, was 1 pg/ml. The intra-assay and interassay coefficients of variation were less than 10% and 15% throughout the range, respectively. Because the thawing caused a loss of VEGF immunoreactivity in the serum by maximally 50%, repeated frozen samples were not used in this study.

MVD, VEGF Immunostaining, and Hormone Receptors. Intratumoral microvessels were identified by immunostaining using antihuman factor VIII related-antigen monoclonal antibody (DAKO Japan, Tokyo, Japan), and MVD was assessed as the count of endothelial deposits/mm^2 in the areas that were considered to be most active for neovascularization, as described previously.

VEGF expression was also examined by immunostaining using an antihuman VEGF121 monoclonal antibody (24). VEGF status was characterized as negative or positive according to its staining intensity in tumor cells. These evaluations were done completely blinded from any information of the patient characteristics.

Estrogen receptor and progesterone receptor were measured by EIA. Tumors with more than 5 fmol/mg protein were defined as positive.

Western Blot Analysis. Fifty ml serum from a breast cancer patient were size fractionated through Sephadex G-100 column chromatography. The positive fractions in the VEGF-EIA were pooled and subjected to an affinity column in which MV303, an antihuman VEGF monoclonal antibody (25), was immobilized to Sepharose 4B resin (Pharmacia, Uppsala, Sweden). The eluate from the column was precipitated by acetone and fractionated through a SDS-PAGE gradient (4–20%) under nonreducing conditions. Samples were blotted from the gel to a nylon membrane with an electroblotter. The membrane was blocked by 2% BSA and then incubated for 2 h with the mixture of the MV101 and MV303 antihuman VEGF monoclonal antibodies (25) at a final concentration of 10 μg/ml for each antibody. The antibody-VEGF interactions were located by incubating the membrane for 1 h with 0.2 μg/ml of phosphatase-labeled goat antimouse IgG (H + L) absorbed with human serum (Kirkegaard & Perry, Gaithersburg, MD), followed by the enzyme reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates.

Statistical Analysis. The Mann-Whitney *U* test was used for analyses of two unpaired samples. For other statistical analyses, we used programs included in the Statistical Analysis System Package (Beccel Mark-II; Beccel Co., Ltd., Tokyo, Japan).

Results

Normal Controls and Individuals with Liver Dysfunction. Circulating levels of VEGF in healthy controls varied from under the detection limit to 227.5 pg/ml (mean, 77.0; Fig. 1). There was no significant difference between ages (from 21–59 years old; Fig. 2) and between men and women. In a premenopausal volunteer, serum VEGF levels were measured every 3 or 4 days for a month to see the effect of the menstrual cycle; however, only a limited change within the cutoff level was shown. Cutoff level of serum VEGF concentration was determined to be 180 pg/ml, which was the mean concentration + 2 SD for normal controls. In individuals without cancer but with liver dysfunction, serum VEGF concentrations varied from 49.3 to 340.4 pg/ml (mean, 201.3 pg/ml). Four of nine cases showed increased VEGF concentrations over the cutoff level.

Cancer Patients. Serum VEGF concentrations in cancer patients were shown in Fig. 1. In all types of cancer patients, several cases showed an abnormal increase in VEGF levels in sera. The positive rate exhibiting over the cutoff level was 13.1 and 19.7% in breast cancer and stomach cancer patients, respectively.

In 137 primary breast cancer patients, the serum VEGF-positive rate was notably higher in stage III patients compared with stage I or stage II patients (Table 1). The Mann-Whitney *U* test showed a significant difference in serum VEGF concentrations between stage I and II patients and stage III patients ($P = 0.0012$). A significant difference was also observed between tumors less than 2 cm and tumors more than 2 cm ($P = 0.0289$). However, there was no significant difference with respect to menopausal status, nodal status, and hormone receptor status.

In addition, the serum VEGF-positive rate was increased in association with the increments of increase of the MVD. In patients with less than 100 counts/mm^2 MVD, the serum VEGF-positive rate was only 2.9%, which was considerably lower than

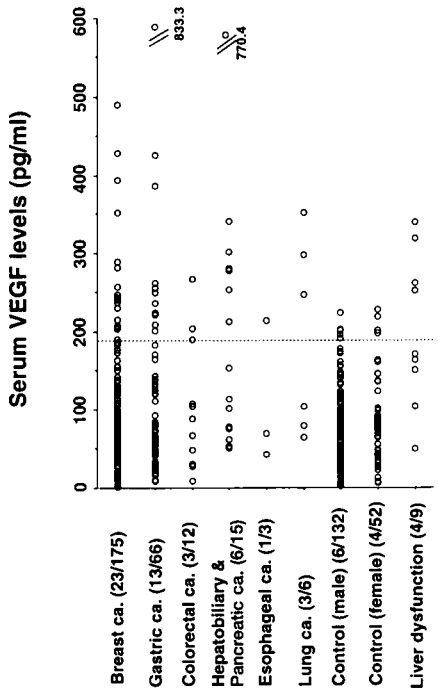

Fig. 1 The circulating levels of VEGF in normal controls, individuals without cancer but with liver dysfunction, and cancer patients. Healthy controls exhibited the detectable VEGF levels up to 227.5 pg/ml (mean, 77.0 pg/ml). The cutoff level of serum VEGF concentrations was decided to be 180 pg/ml, which was calculated as the mean + 2 SD of normal controls. Aberrant expression of VEGF in sera was found in a variety of carcinomas, including breast, stomach, colorectal, hepatobiliary, esophageal, and lung cancer. The highest level (833.3 pg/ml) was seen in gastric cancer patients with peritoneal metastases.

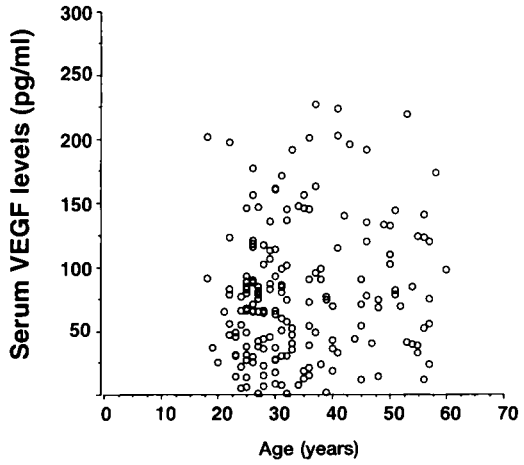

Fig. 2 Age and serum VEGF concentrations in normal controls. No significant difference was seen between age groups (age range, 21–59).

Table 1 Serum VEGF levels and clinicopathological characteristics in breast cancer patients

	No. of cases	Mean ± SD (pg/ml)	*P*-value	Positive rate (>58 pg/ml)
Menopause				
Pre-	77	70.6 ± 74.3	NS[a]	5.2%
Post-	60	84.4 ± 76.4		13.3%
Stage				
I	28	70.9 ± 57.8		6.9%
II	85	68.0 ± 73.7	0.0012	7.1%
III	24	113.7 ± 89.6		16.7%
(Recurrence)	(38)	(148.2 ± 86.6)		(28.9%)
Tumor size				
<2 cm	26	63.4 ± 87.1		7.7%
2–5 cm	88	76.2 ± 59.6	0.0289	8.0%
>5 cm	23	93.2 ± 109.0		13.0%
No. of nodal metastases				
−	63	63.1 ± 54.6		6.3%
+	74	88.1 ± 87.9	0.0938	10.4%
Estrogen receptor				
−	62	74.8 ± 75.3		4.8%
+	47	89.6 ± 80.9	NS	14.3%
Progesterone receptor				
−	70	75.3 ± 75.0		5.7%
+	47	88.8 ± 83.8	NS	14.9%
Microvessel density				
<100	34	69.0 ± 56.2		2.9%
101–149	36	109.8 ± 83.2	0.0216	19.4%
(>150)	(7)	(148.3 ± 106.3)		(42.9%)
VEGF ICA				
−	31	65.2 ± 48.4		0%
+	42	108.5 ± 81.8	0.0152	19.0%

[a] NS, not significant.

normal control levels; however, in those with more than 100 counts/mm^2 MVD, the positive rate was 19.4%. Particularly, in patients with more than 150 counts/mm^2 MVD, the positive rate was 42.9% (Table 1).

On the other hand, in 73 patients whose intratumoral VEGF expression was examined by ICA, serum VEGF concentrations were significantly higher in VEGF-ICA-positive patients than in VEGF-ICA-negative patients ($P = 0.0152$). The serum VEGF-positive rate also differed between VEGF-ICA-positive patients and VEGF-ICA-negative patients (Fig. 3).

In six primary breast cancers, serum VEGF levels were examined sequentially between pre- and postoperation. Five of six patients showed a decrease in the serum VEGF concentrations in sera (Fig. 4). Particularly, in a case that showed a level of 193.1 pg/ml before the operation, a marked decrease at 1 month after the operation was observed, suggesting that the increased serum VEGF level was associated with the presence of primary tumor. One case showing no significant reduction in serum VEGF level after the operation had distant metastases. Of six patients who had relapses after the primary operation, three showed a significant increase in the serum VEGF levels (Fig. 5).

Western Blot Analysis. To examine the molecule that is immunoreactive to the VEGF antibody in the circulating blood, we carried out Western blot analysis by using a serum sample from a breast cancer patient. Although the phosphate-labeled antimouse IgG antibody (secondary antibody) used in this study was absorbed with human serum, we learned that the antibody produced significant nonspecific signals in the human serum. Thus, we compared the patterns obtained by incubating the membrane with or without the antihuman VEGF monoclonal antibodies (Fig. 6, *A* and *B*, respectively) to identify VEGF-specific signals. The recombinant human VEGF121 and VEGF165 were detected only in the blot incubated with the

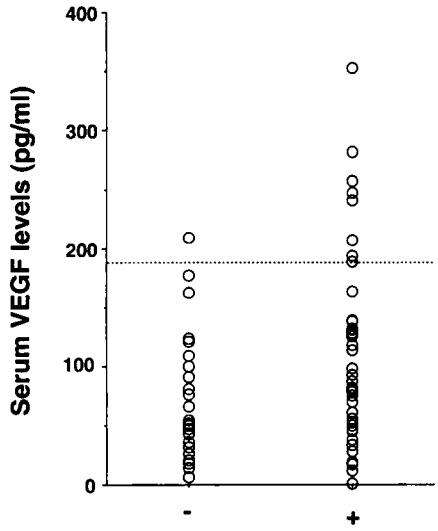

Fig. 3 Serum VEGF levels and intratumoral VEGF expression determined by ICA. The serum VEGF-positive rate was significantly higher in VEGF-ICA-positive patients than in VEGF-ICA-negative patients.

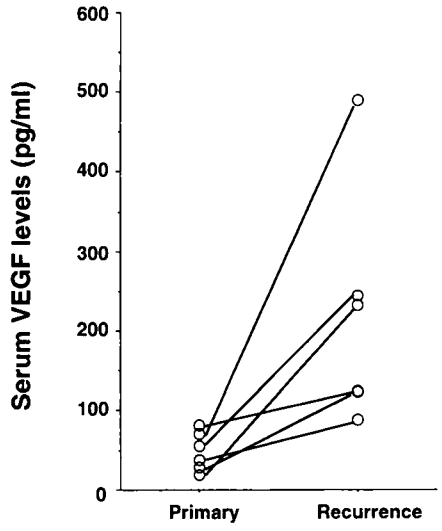

Fig. 5 Increase in the circulating levels of VEGF after the relapse. Three of six patients whose cancer recurred showed a marked increase in the serum VEGF levels after the relapse compared with preoperative serum VEGF levels.

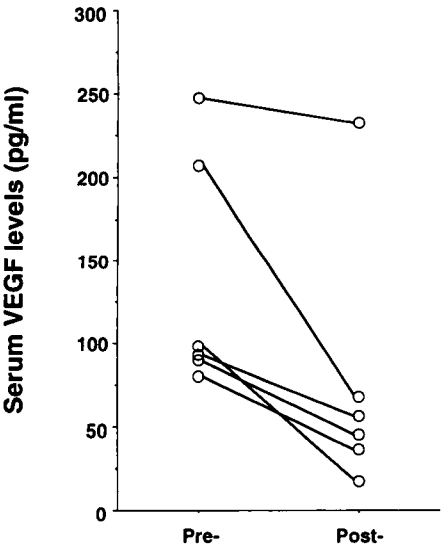

Fig. 4 Decrease in the circulating levels of VEGF after removal of the primary tumor. A patient whose VEGF level was not altered by the resection of the primary tumor had distant metastases.

antihuman VEGF monoclonal antibodies, ensuring the specificity of the assay. In the serum of the patient, a notable difference was observed on two signals (Fig. 6, *triangles 1* and 2). Band 1 is bold and strong in Fig. 6*A* and weak in Fig. 6*B*. Band 2 is clearly specific for VEGF in Fig. 6*A*. The migration ratio of band 1 corresponded to that of the recombinant human VEGF165, suggesting the possibility that VEGF165 was circulating in the blood of the patient. Band 2 was definitely specific for VEGF antibody but smaller than VEGF121, the shortest isoform of human VEGF, implying that a degradative product of human VEGF might also have been in the circulating blood.

Discussion

One of the important findings in recent angiogenesis research is the endocrine function of angiogenesis regulators. There is evidence that many angiogenic growth factors and inhibitors act in a paracrine or autocrine fashion between endothelial cells and tumor cells *in situ*; however, recently, the expression of angiogenesis regulators in the circulating level was documented. In a mouse model using Lewis lung carcinoma, Holmgren *et al.* (25) demonstrated that a negative angiogenesis regulator produced by primary tumors plays an important role in the growth of metastatic tumors, particularly for keeping metastatic tumors dormant. In fact, a potent angiogenesis inhibitor, angiostatin was identified from the urine and serum of mice with Lewis lung carcinoma (26). On the other hand, Lewis lung carcinomas are also known to produce VEGF proteins, suggesting the importance of the net balance between positive and negative regulators for tumor growth.

Recent studies have revealed the existence of VEGF in various biological fluids such as pleural and peritoneal effusions (27, 28) of cancer patients. In this study, we have determined serum VEGF concentrations in human tumors as well as healthy individuals, and analyzed them to address the biological significance of this factor. Using a sensitive chemiluminescence EIA, which can detect 1.0 pg/ml human recombinant VEGF121, we found the presence of detectable VEGF levels in sera in healthy individuals (similar results are obtained by Hanatani *et al.* (29). Although we have not confirmed the characteristics of these immunoreactive VEGF proteins, it seems very interesting that a certain VEGF level is kept normally in human sera. A VEGF level of less than 50 pg/ml may not have to induce its various biological activities in the quiescent endothelium (30); however, it may function in the endothelium already sensitized endothelium by some factors, including growth factors, cytokines, and altered physiological environments as in abnormal conditions

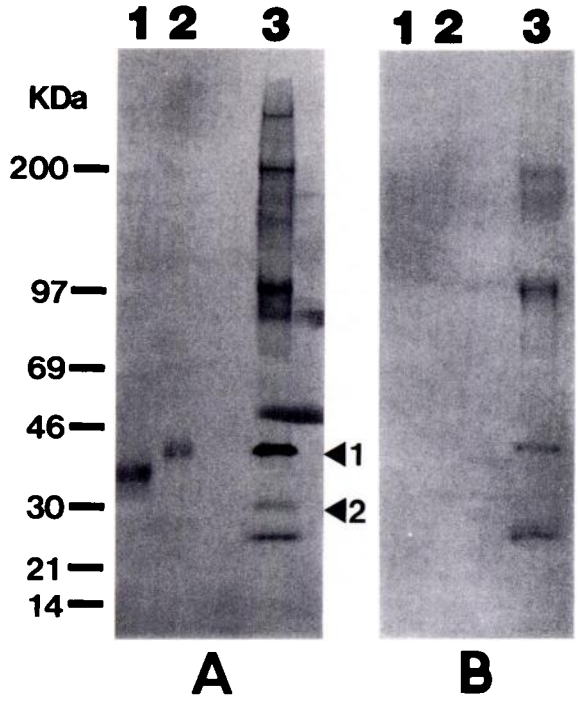

Fig. 6 Western blot analysis of VEGF in serum from a breast cancer patient who donated her serum to the investigation. The blots were incubated with or without the monoclonal antibodies to human VEGF (*A* and *B*, respectively) and then incubated with an enzyme-labeled antimouse IgG antibody to locate VEGF-specific signals. Two specific signals are indicated in *A* (◀). *Lane 1*, recombinant human VEGF121; *Lane 2*, recombinant human VEGF165; *Lane 3*, partially purified serum sample.

such as organ damage. In fact, an increase in serum VEGF levels in patients with liver dysfunction was observed, suggesting that serum VEGF level is up-regulated in response to an event that requires neovascularization for organ repair. In a preliminary study, we also found a significant increase in the circulating VEGF levels in a patient with massive skin infections (data not shown). Thus, VEGF proteins existing in the control sera seem to imply the necessity of circulating VEGF for maintaining the normal vascular system. Recently, it was documented that the VEGF-induced angiogenesis pathway depends on the expression of distinct vascular cell integrins, including $\alpha_v\beta_5$ (31), suggesting that the mere presence of VEGF cannot necessarily elicit its biological activity. Additional investigation on the serology of VEGF in normal controls should be done.

In cancer patients, the aberrant increase in the level of VEGF was detected in some subpopulations in each type of tumor. In breast cancer, only 13% of examined cases showed VEGF concentrations of more than 180 pg/ml in sera; however, the incidence of the aberrant increase of VEGF levels was elevated in relation to tumor progression. Serum VEGF concentrations were significantly higher in advanced stage cancer patients compared with early stage patients. Approximately 30% of recurrent breast cancer patients exhibited increased serum VEGF levels. This tendency, that the increase in serum VEGF levels was associated with tumor progression, was also observed in gastric cancer patients, although statistical significance was not demonstrated.

With respect to the source of increased serum VEGF in cancer patients, much remains unproven. However, according to two of our results, that an aberrant increase in serum VEGF concentration was seen frequently in VEGF-ICA-positive tumors compared with VEGF-ICA-negative tumors and that removal of the tumor decreased serum VEGF concentrations, tumor tissues (tumor cells) are likely to produce and secrete VEGF proteins in sera. In a previous analysis, we demonstrated that at least the VEGF121 and VEGF165 forms of the four splicing variants were expressed in breast cancer cells (17). These two forms are thought to be largely free to diffuse in tissues (32). In addition, the present study also showed a positive correlation between the increment of intratumoral MVD and serum VEGF concentrations.

The chemiluminescence VEGF-EIA used in this study was very sensitive to VEGF proteins. One pg/ml VEGF121 was detectable in a standard curve analysis. Originally, the anti-VEGF polyclonal antibody was designed based on VEGF121 (28). Recent studies have indicated that the VEGF165 form, as well as the VEGF121 form, was expressed in many types of human tumors (33). Also, we detected the aberrant increase in the circulating levels of VEGF in cancer patients and the presence of the VEGF165 form in sera from a patient with recurrent breast cancer with the use of a Western blot analysis.

In recent studies, several angiogenic factors, including bFGF, TGF-α, and hepatocyte growth factor have been demonstrated to be present in the circulating levels or in urine. Several authors showed that serum bFGF levels were elevated in gastric cancer (34), renal cell carcinoma, brain tumors, lung cancer (35), and breast cancer (36) patients. In addition, bFGF has been also detected in the urine of a variety of cancer patients (37). The increase in the circulating levels of TGF-α was also reported in breast cancer patients (38). Furthermore, we found an increase in human growth factor levels in the sera of breast cancer patients. Particularly, serum hepatocyte growth factor levels were elevated markedly in patients with liver metastases (39). On the other hand, the role of negative angiogenesis regulators, as well as positive angiogenesis regulators, in the circulating level has also been a focus. Therefore, the behavior of VEGF, a potent positive angiogenesis regulator, in the circulating levels seems to be crucially important for considering the net balance between positive and negative angiogenesis regulators. This study suggests strongly that VEGF might have to function as an endocrine endothelial growth factor in some populations of solid tumors. Recently, many antiangiogenesis drugs have been investigated in clinical trials (40). Some of them were reported to inhibit heparin-binding growth factor activity in sera. The monitoring of VEGF might be also useful for assessing the antiangiogenic activity of angiogenesis inhibitors.

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