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Original Paper

Clinical Significance of Plasma Vascular Endothelial Growth Factor in Gastrointestinal Cancer

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Circulating vascular endothelial growth factor (VEGF) was measured in gastric and colorectal cancer patients using an enzyme-linked immunosorbent assay (ELISA). Firstly, serum and plasma samples were collected from 20 normal controls to compare the values of VEGF and to determine which specimen type was most suitable for detecting circulating VEGF. Seventeen of 20 normal controls had plasma VEGF levels under the limit of detection (15 pg/ml) and the levels of the remaining three controls were 21, 22 and 38 pg/ml. In contrast, all serum samples indicated high levels of VEGF (mean 238 pg/ml), ranging from 44 to 450 pg/ml. In a time-course test of two normal controls serum VEGF values increased markedly between 30 and 60 min and remained high, whilst plasma VEGF values were low up to 480 min. Thus, plasma samples are more suitable for the measurement of circulating VEGF. Next, plasma VEGF levels were examined in 44 patients with gastric cancer (8 early, 7 advanced without remote metastasis and 29 metastatic), 13 with colorectal adenoma (2 with focal cancer) and 26 with colorectal carcinoma (8 advanced without metastasis and 18 metastatic) before treatment. An extremely high plasma concentration of VEGF was seen in some cancer patients with metastasis. To discriminate these patients, a cut-off level was determined, considering both the distribution of the sample concentration and the upper limit of 95% confidence area of VEGF in the cancer patients without metastasis. The cut-off value was 108 pg/ml and most cancer patients without metastases had VEGF levels below the cut-off value. In 11 of 29 metastatic gastric cancer patients (38%) and 9 of 18 metastatic colorectal cancer patients (50%), plasma VEGF levels were higher than the cut-off value. Survival was also analysed in the patients with metastasis. It was significantly longer in the patients with low VEGF levels (below the cut-off) than in those with high VEGF levels (logrank test, $P = 0.042$). 34 patients with metastasis (19 gastric cancer and 15 colorectal cancer) were treated with systemic chemotherapy, and their pretreatment levels of plasma VEGF and conventional tumour markers (CEA and CA19-9) were evaluated in relation to response. The response to chemotherapy was significantly higher in patients with low VEGF levels (≤ 108 pg/ml) than in those with high VEGF levels ($P = 0.047$). Such a difference was not observed with CEA/CA19-9. In conclusion, plasma VEGF is a useful marker for tumour metastasis and patient survival, and a possible predictive factor for the response of patients with gastrointestinal cancer to chemotherapy.

Key words: vascular endothelial growth factor, enzyme-linked immunosorbent assay, gastrointestinal cancer, chemotherapy
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INTRODUCTION

VASCULAR ENDOTHELIAL growth factor (VEGF) is the most potent angiogenesis factor and has been detected in many human tumours [1]. VEGF has been reported to relate closely to tumour progression and metastasis [2]. It is a glycosylated dimeric polypeptide which is abundantly expressed and secreted by most human tumours, and exerts important effects on endothelial cells. It increases the permeability of microvessels to circulating macromolecules, with well-documented hyperpermeability of tumour blood vessel [3]. Furthermore, VEGF potently promotes endothelial cell proliferation [4]. VEGF has also been reported to alter endothelial gene expression and increase several proteinases including interstitial collagenase [5]. There is abundant evidence showing the important role of VEGF in tumour angiogenesis [6]. It has been demonstrated that systemic treatment of tumour bearing mice with a neutralising antibody for VEGF reduced tumour growth and this effect correlated with tumour vascularity [7]. Many preclinical angiogenesis inhibitors have emerged and some are already in clinical trials [8–11].

Tumour vasculature has been reported to be a significant prognostic and haematogenous metastatic predictor in gastric and colorectal cancer, and VEGF expression has been recognised in these tumours [12–14]. Recently, serum VEGF has been measured by an enzyme-linked immunosorbent assay (ELISA) in patients with various types of cancer and increased levels of VEGF were demonstrated in most patients with cancer [15–17]. This VEGF elevation in sera is suggested to relate closely to tumour progression and metastasis. However, recently, it has been revealed that VEGF is produced and secreted from megakaryocytes and platelets associated with blood coagulation [18], so serum samples may not be suitable for the measurement of circulating VEGF levels, because the clot formation in the collecting process of serum induces platelet activation and subsequent abundant cytokine release into sera [19].

We have been using plasma but not serum for the measurement of circulating VEGF, because the results were more reliable, reproducible and stable from plasma than serum. A preliminary report of some of our results on plasma VEGF levels in gastrointestinal cancer has been previously published [20]. Here, we describe the reliability of the measurement of circulating levels of VEGF both in plasma and in sera, and report the relationship of circulating VEGF levels to disease stage, prognosis and chemotherapy sensitivity in patients with gastrointestinal cancer.

PATIENTS AND METHODS

Circulating VEGF in normal controls

Circulating VEGF levels were examined in plasma and serum samples using an ELISA kit with antibodies which recognise $VEGF^{165}$ (Quantikine human VEGF, R&D Systems, Minneapolis, Minnesota, U.S.A.). Venous blood samples were obtained from 20 normal controls. For plasma collection, blood samples were drawn into a vacutainer containing sodium ethylenediamine tetra-aceteic acid (EDTA-Na) and the plasma was immediately prepared by centrifugation at 3,000 rpm. For serum sample collection, blood samples without EDTA-Na were centrifuged after clot formation after 20–30 min at room temperature, and then the serum was removed. Both samples were stored at $-70^{\circ}C$ until the measurement of VEGF.

A time-course test was performed on plasma and serum. Blood samples with EDTA-Na for plasma collection and without it for serum collection were drawn from two normal controls. Each whole blood sample was divided into six fractions. One fraction with EDTA-Na was centrifuged immediately, and each of the others, with or without EDTA-Na, was left at room temperature for 30, 60, 120, 240, and 480 min and then centrifuged for plasma and serum collection, respectively. VEGF was measured in each plasma and serum sample.

Patients

After obtaining informed consent, plasma was collected as described above from 44 gastric cancer patients, 13 colorectal adenoma patients (including 2 patients with focal cancer in adenoma) and 26 colorectal cancer patients before treatment and was stored at $-70^{\circ}C$ until use. Serum carcinoembryonic antigen (CEA) and CA19-9 were measured using conventional enzyme immunoassays. The cancer patients were divided into groups using UICC TNM classification as follows; early cancer patients who showed stage I, advanced cancer patients without distant metastasis who showed stage II/III and metastatic cancer patients who showed stage IV. Patients' characteristics are shown in Table 1.

Of 47 metastatic cancer patients, 34 (19 gastric cancer and 15 colorectal cancer) were treated with systemic chemotherapy using various regimens such as 5-fluorouracil (5-FU), 5-FU plus methotrexate, 5-FU plus cisplatin and irinotecan. Their pretreatment levels of plasma VEGF and tumour markers (CEA, CA19-9) were evaluated in relation to tumour response. The response to chemotherapy was judged by WHO tumour response criteria [21].

Statistical analysis

Statistical analysis was performed using the Statview version 4.0 software application (Abacus Concepts). Survival

Table 1. *Patients' characteristics*

Disease	No. of patients	Age (mean ± SD)	Sex (M/F)	PS (0–2/3–4)	Metastatic site
Normal control	20	29 ± 3.9	10/10		
Gastric	44				
Early cancer (stage* I)	8	70 ± 7.2	6/2	8/0	
Advanced cancer (stage II/III)	7	72 ± 8.3	3/4	6/1	2 (LN)
Metastatic cancer (stage IV)	29	61 ± 13	23/6	26/3	10 HEP; 15 PER; 3 OSS; 2 OTH
Colorectal	39				
Polyp (adenoma)	13†	61 ± 10	5/8	13/0	
Advanced cancer (stage II/III)	8	60 ± 15	2/6	8/0	3 (LN)
Metastatic cancer (stage IV)	18	66 ± 9.8	10/8	11/7	16 HEP; 5 PER; 3 PUL; 1 OSS; 1 OTH

PS, performance status; M, male; F, female; HEP, hepatic; PER, peritoneal; PUL, pulmonal; OSS, osseous; LN, lymph node; OTH, others; SD, standard deviation. *Stage was determined by UICC TNM classification. †Including 2 patients with focal cancer.

curves were plotted according to the Kaplan–Meier methods and statistical differences were analysed by the logrank test and the Gehan–Wilcoxon test. The difference in response frequency was examined by the chi-square test. Unless otherwise noted, *P* values less than 0.05 were considered significant.

RESULTS

Circulating VEGF in normal controls

Seventeen of the 20 normal controls had plasma VEGF levels under the limit of detection (15 pg/ml) and the plasma VEGF levels of the other three controls were 21, 22 and 38 pg/ml. In contrast, all serum samples from the 20 normal controls had high levels of VEGF (mean ± standard deviation (SD) 238 ± 125 pg/ml) ranging from 44 to 450 pg/ml.

Plasma and serum VEGF levels in the time-course test are shown in Figure 1. Serum VEGF values increased markedly between 30 and 60 min, whilst plasma VEGF values remained low, with no significant change up to 480 min.

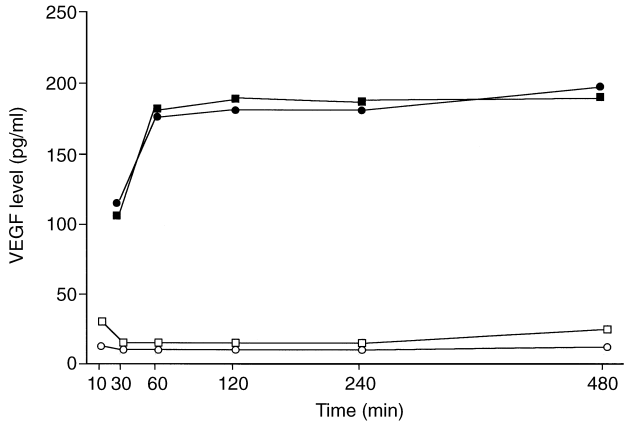

Figure 1. Plasma (open symbols) and serum (solid symbols) vascular endothelial growth factor (VEGF) levels of two normal controls in a time-course test. Serum VEGF values increased markedly between 30 and 60 min, whilst plasma VEGF values remained low and no significant change was seen up to 480 min.

Table 2. Plasma levels of vascular endothelial growth factor (VEGF) and serum levels of carcinoembryonic antigen (CEA) and CA19-9

Disease	VEGF (pg/ml) Mean ± S.D. (median)	CEA (ng/ml) Mean ± S.D. (median)	CA19-9 (ng/ml) Mean ± S.D. (median)
Normal control	26 ± 8.2		
Gastric			
Early cancer (stage I)	55 ± 37	1.8 ± 0.93	14 ± 11
Advanced cancer (stage II/III)	99 ± 81	77 ± 150	14 ± 8.1
Metastatic cancer (stage IV)	169 ± 181 (113)	387 ± 1746 (5.9)	1316 ± 3163 (35)
Colorectal			
Polyp	54 ± 35	2.9 ± 1.2	4.1 ± 3.8
Advanced cancer (stage II/III)	35 ± 19	20 ± 37	78 ± 131
Metastatic cancer (stage IV)	122 ± 81 (105)	417 ± 985 (51)	3115 ± 9805 (75)

S.D., standard deviation.

Plasma VEGF in gastrointestinal cancer

The results of plasma VEGF, serum CEA and serum CA19-9 in the patients are summarised in Table 2 and plasma VEGF levels of each patient are plotted in Figure 2. All three parameters tended to increase markedly in the cancer patients with metastasis. An extremely high plasma concentration of VEGF was seen in some patients with metastasis. To discriminate these patients, a cut-off level of VEGF was determined, taking into account both the distribution of the sample concentration and the upper limit of 95% confidence area in the cancer patients without metastasis. The cut-off value was 108 pg/ml (shown as a dotted line in Figure 2). Most cancer patients without metastases were included in the area below the cut-off value. 11 of 29 metastatic gastric cancer patients (38%) and 9 of 18 metastatic colorectal cancer patients (50%) showed higher levels of VEGF than the cut-off value (Figure 2).

Survival was analysed in 47 patients with metastatic gastric or colorectal cancer (Table 3). The median survival time of 26 patients showing lower VEGF levels than the cut-off value

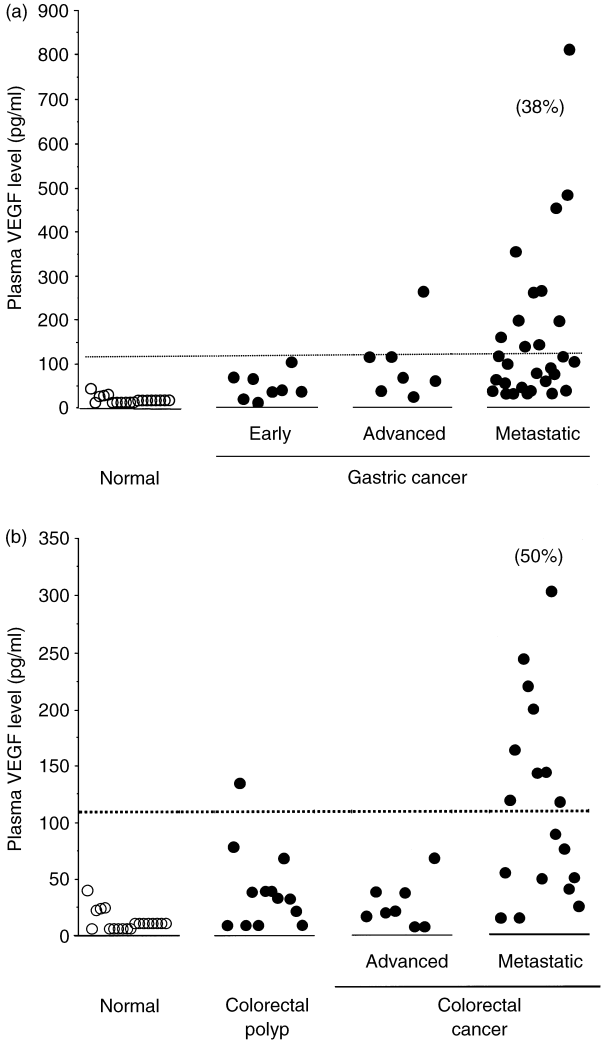

Figure 2. Plasma vascular endothelial growth factor (VEGF) level of each normal control and patient with gastric cancer (a) and with colorectal polyp or cancer (b). An extremely high plasma concentration of VEGF was seen in some cancer patients with metastasis. The dotted line indicates the cut-off level of 108 pg/ml.

was 214 days and that of 21 patients showing higher VEGF levels than the cut-off value was 130 days. The survival time was significantly longer in the former patient group (logrank test, $P = 0.042$; Figure 3), although by 18 months the two survival curves had converged.

From the viewpoint of chemotherapy for the metastatic cancer patients, 8 of 19 patients (42%) whose pretreatment plasma VEGF levels were lower than the cut-off value achieved a partial response (Figure 4), whilst only 1 of the 15 patients (7%) whose pretreatment plasma VEGF levels were higher than the cut-off value achieved a partial response ($P = 0.047$). Such a difference in the response rate to chemotherapy was not observed for CEA/CA19-9 (Figure 5).

DISCUSSION

The results of this study confirm the unsuitability of serum for measuring VEGF. The values of serum VEGF in drawn blood samples increased during clot formation. This increase in serum VEGF may be caused by release from platelets as recently reported *in vitro* [18]. In contrast, blood samples with added EDTA-Na were stable for 480 min and plasma was suitable for the measurement of circulating VEGF. In the time-course test for serum, VEGF levels increased up to 60 min and then reached a plateau, whilst for plasma the VEGF levels remained very low for 480 min. Recently, VEGF has been shown to be induced and released from activated platelets *in vitro* [18]. Our time-course data suggest that VEGF is released from platelets into serum during clot formation and its release ceases at the end of platelet aggregation. During clot formation, platelets are activated and many cytokines are released. However, EDTA-Na added into the drawn venous blood acts as a chelating agent of divalent cations and suppresses platelet activation and aggregation. Therefore, limited if any, VEGF can be released into the plasma from the platelets. From our results, plasma rather than serum should be considered suitable for the measurement of circulating VEGF. Previous reports on circulating VEGF using serum samples [15–17] may be inaccurate. Our results confirm those recently reported, indicating VEGF levels are 5–8-fold higher in serum samples than plasma samples [19]. Taken together with our present observations from the time-course test, serum samples tend to be unreliable for VEGF measurement.

The growth of solid tumours and the formation of metastases depend on neoangiogenesis [22, 23]. Many reports, suggesting a close relationship between tumour vascularity

Table 3. Survival analysis of gastrointestinal cancer patients with distant metastasis according to plasma vascular endothelial growth factor (VEGF) level

	No. of patients	MST (day)	Survival analysis (*P* value) Logrank test	Breslon–Gehan–Wilcoxon test
All patients				
VEGF < 108 pg/ml	26	214	0.042	0.0089
VEGF ≥ 108 pg/ml	21	130		
Gastric cancer				
VEGF < 108 pg/ml	17	185	0.11	0.00083
VEGF ≥ 108 pg/ml	12	94		
Colorectal cancer				
VEGF < 108 pg/ml	9	556	0.034	0.08
VEGF ≥ 108 pg/ml	9	260		

MST, median survival time.

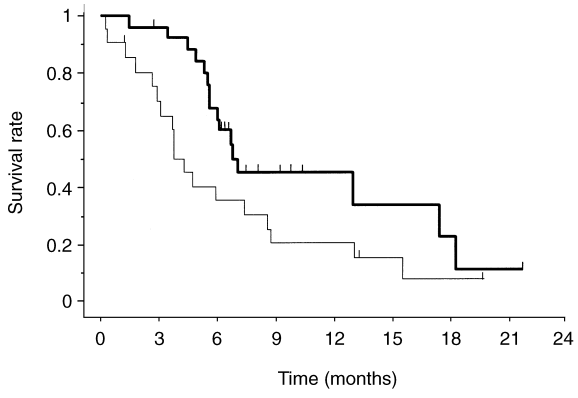

Figure 3. Survival curves for 26 metastatic gastric or colorectal cancer patients whose plasma vascular endothelial growth factor (VEGF) levels were less than 108 pg/ml (—) and 21 metastatic gastric or colorectal cancer patients whose plasma VEGF levels were higher than 108 pg/ml (–) (logrank test, *P*=0.042).

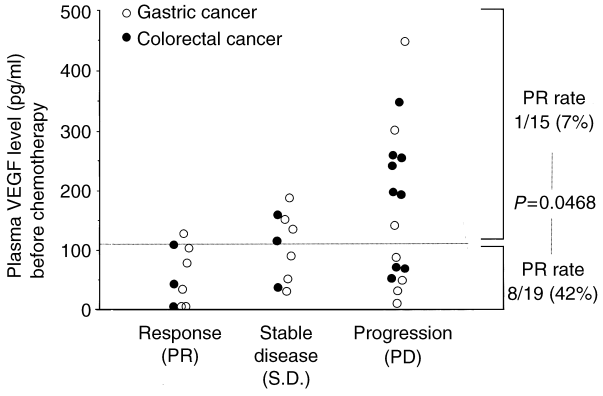

Figure 4. Response to chemotherapy in 34 patients with metastatic gastrointestinal cancer and their plasma vascular endothelial growth factor (VEGF) levels before treatment. 8 of 19 patients who had lower plasma VEGF levels than the cut-off value achieved a partial response (42%). 1 of 15 patients who had higher plasma VEGF levels than the cut-off value achieved a partial response (7%). The response rate was significantly higher in the former group (*P*=0.047).

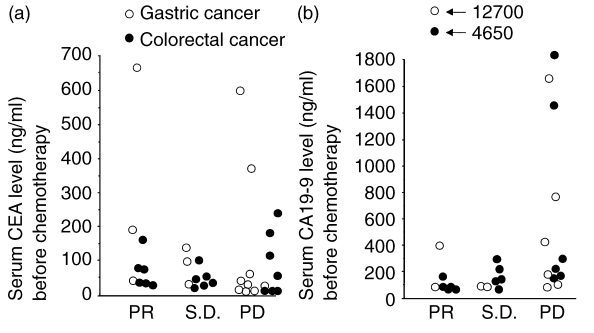

Figure 5. Response to chemotherapy in 34 patients with metastatic gastrointestinal cancer and their serum (a) carcinoembryonic antigen (CEA) and (b) CA19-9 levels before treatment. No relationship was observed between the pretreatment levels of these markers and the response to treatment. ○ Gastric cancer; ● colorectal cancer.

and tumour progression/metastasis in gastrointestinal cancer have been published [12–14]. In our present study, plasma VEGF increased in gastrointestinal cancer patients with metastases, and the increase was closely related to a more advanced stage of disease. However, it should be noted that VEGF sensitivity in detecting metastatic disease (38% for gastric cancer and 50% for colorectal cancer) was not high, so it may not be an easy means of discriminating patients with metastatic disease. It is clinically important to search for the metastasis radiologically in patients with high plasma VEGF levels.

Metastasis is known to be accompanied by platelet aggregation and activation. At metastatic sites, it has been suggested that platelets aggregate due to factors released from metastatic cells, resulting in microthrombosis, tumour adhesion and growth [24, 25]. In this step, VEGF would be released from activated platelets and this released VEGF would induce the neovascular formation for tumour cell invasion and growth. Therefore, in advanced cancer patients, the sources of circulating VEGF are tumour cells themselves and platelets activated in and adjacent to metastatic (or primary) tumours. The expression of VEGF in tumour tissues has been implicated as a poor prognostic factor [26, 27], and, our results for survival of patients with metastases indicate that high levels of plasma VEGF are related to poor prognosis.

Finally, in our study we have shown that the response rate to systemic chemotherapy was significantly higher in patients with low plasma VEGF levels than in those with high plasma VEGF levels (> 108 pg/ml). A similar result has been reported in a recent study on serum VEGF and basic fibroblast growth factor [17]. VEGF-mediated angiogenesis is considered to play an essential role in cancer progression and metastasis [1, 2]. High circulating VEGF levels in cancer patients might indicate that the cancer is too advanced to treat with chemotherapy, and would relate to factors such as tumour burden, the increased permeability of tumour vessels and the activation of many proteinases. However, it is currently unknown how VEGF relates to the sensitivity of chemotherapy. Our findings were retrospective, and adapted-chemotherapy regimens were different, so the role of VEGF levels for predicting the chemosensitivity should be confirmed in a prospective clinical trial.

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