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Platelet number and interleukin-6 correlate with VEGF but not with bFGF serum levels of advanced cancer patients

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Summary We have compared the platelet number and the serum concentration of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and interleukin-6 (IL-6) in 80 blood samples of 50 patients with advanced cancer. We have also measured the mitogenic effect of patient sera on endothelial cells in vitro in order to estimate the biological activity of serum VEGF. Serum VEGF concentration correlated with platelet number ($r = 0.61$; $P < 10^{-4}$). Serum IL-6 levels correlated with platelet count ($r = 0.36$; $P < 10^{-3}$), with serum VEGF levels ($r = 0.55$; $P < 10^{-4}$) and with the calculated load of VEGF per platelet ($r = 0.4$; $P = 3 \times 10^{-4}$). Patients with thrombocytosis had a median VEGF serum concentration which was 3.2 times higher ($P < 10^{-4}$) and a median IL-6 serum level which was 5.8 times higher ($P = 0.03$) than in other patients. Serum bFGF did not show an association with any of the other parameters. Patient sera with high VEGF and bFGF content stimulated endothelial cell proliferation significantly more than other sera ($P = 4 \times 10^{-3}$). These results support the role of platelets in the storage of biologically active VEGF. Platelets seem to prevent circulating VEGF from inducing the development of new blood vessels except at sites where coagulation takes place. IL-6, besides its thrombopoietic effect, also seems to affect the amount of VEGF stored in the platelets. This is in accordance with the indirect angiogenic action of IL-6 reported previously. The interaction of IL-6 with the angiogenic pathways in cancer might explain the stimulation of tumour growth occasionally observed during IL-6 administration. It also conforms to the worse outcome associated with high IL-6 levels and with thrombocytosis in several tumour types and benign angiogenic diseases.

Keywords: angiogenesis; interleukin-6; blood platelets; VEGF

An interim conclusion of the ongoing translational research concerning tumour angiogenesis is that obtaining quantitative information of this process in cancer patients seems to add prognostic and predictive power to the currently used tumour staging systems (Gasparini, 1996). Angiogenesis is an element of the stromal reaction to tumour cell populations and supports their growth (Folkman, 1990), invasion (Skobe et al, 1997) and metastasis (Liotta and Saidel, 1974). Although counting individual microvessels on immunostained tissue sections is feasible and informative (Vermeulen et al, 1996), a more observer-independent and dynamic method of quantifying angiogenesis in cancer patients is based on the assessment of circulating angiogenic factor concentrations.

Serum levels of basic fibroblast growth factor (bFGF) predicted accelerated growth of lung metastases in patients with renal cell carcinoma (Duensig et al, 1995). In advanced colorectal cancer, elevated serum bFGF and serum vascular endothelial growth factor (VEGF) levels were closely associated with short estimated volume doubling times of the metastases (Dirix et al, 1996). Serum angiogenic factor levels might also be used to select patients for a specific treatment. In metastatic renal cell carcinoma patients, elevated serum levels of bFGF predicted response to interferon alpha (IFN-α) treatment (Vermeulen et al, 1997). In colorectal cancer, preoperative serum VEGF measurements correlated with T stage and the extent of nodal involvement (Kumar et al, 1998). Evaluation of response to treatment might be another application of assessing circulating angiogenic factors in cancer patients. Patients with response to treatment had less frequently elevated bFGF and/or VEGF serum levels than patients with progressive tumours of various histological types (Dirix et al, 1997). Survival of cancer patients has been estimated using pretreatment serum angiogenic factor measurements. High VEGF concentrations were associated with shorter survival in non-Hodgkin's lymphoma (Salven et al, 1997) and in small-cell lung cancer (Salven et al, 1998). Elevated serum bFGF predicted a worse outcome in renal cell carcinoma patients (Dosquet et al, 1997).

Recently, preliminary evidence for VEGF storage by platelets in cancer patients has been described (Verheul et al, 1997; Banks et al, 1998). Serum VEGF concentrations correlated with platelet numbers in breast cancer patients during chemotherapy. After induction of platelet aggregation, the VEGF content in the supernatant of the platelet suspension was significantly increased (Verheul et al, 1997). Comparable results have been reported by Banks et al (1998). Activated platelets release VEGF together with β-thromboglobulin, suggesting that VEGF resides in the α-granules of platelets (Wartiovaara et al, 1998). Although megakaryocytes in the bone marrow produce VEGF (Möhle et al, 1997), the presence of VEGF mRNA and VEGF protein has been demonstrated to be up-regulated in several tumour types (Brekken

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Table 1 Correlation coefficient *r* and *P*-value of the correlation analysis of the different sets of variables

	VEGF *r*/*P*	IL-6 *r*/*P*	Platelet *n* *r*/*P*
bFGF	0.06/0.6	0.03/0.8	0.12/0.3
VEGF	–	**0.55/<10^{-4}**	**0.61/<10^{-4}**
IL-6	–	–	**0.36/10^{-3}**

Platelet n = platelet number. Significant correlations are printed in bold.

et al, 1998). Therefore, an important source of circulating VEGF in cancer patients is presumably the tumour tissue. The hypothesis is that fast-growing tumours, besides producing angiogenic factors such as VEGF, also release thrombopoietic cytokines. An increase in the number of circulating platelets could then, by the uptake of free VEGF, focus the effect of this angiogenic factor to sites where coagulation takes place, e.g. a wound or a tumour.

Both thrombopoietin (Kaushansky, 1995) and interleukin-6 (IL-6) (Ishibashi et al, 1989) potently stimulate platelet production. Recombinant human thrombopoietin and IL-6 proteins have been used in clinical trials in cancer patients (Weber et al, 1993; Vadhan-Raj et al, 1997). Transient stimulations of tumour growth have been observed during IL-6 treatment, which disappeared immediately upon cessation of IL-6 treatment (Ravoet et al, 1994). IL-6 is produced in malignant tumours and in inflammatory tissues (Rak et al, 1996). IL-6 is up-regulated by hypoxia (Yan et al, 1995), like VEGF and other angiogenic factors. IL-6 expression is elevated in healing wounds (Mateo et al, 1994). Response elements for IL-6 signalling are present upstream to the transcription initiation site of VEGF (Cohen et al, 1996). Thrombocytosis is a clinical sign of malignant mesothelioma progression and has been associated with high levels of circulating IL-6 (Nakano et al, 1998). In inflammatory bowel disease, independent studies have shown that exacerbation of disease coincided with high serum levels of angiogenic factors (Bousvaros et al, 1997), with high serum levels of IL-6 (Bross et al, 1996) and with high platelet numbers (Lake et al, 1978).

Given these arguments, we compared the serum VEGF, bFGF and IL-6 concentrations with the platelet numbers in 80 samples from 50 patients with various types of metastatic cancer. To estimate the biological meaning of serum angiogenic factor levels we also assessed whether high serum VEGF and bFGF concentrations concurred with a mitogenic effect on human endothelial cells in vitro.

PATIENTS AND METHODS

Patients

Of advanced cancer patients, 10 ml of venous blood were drawn into a serum separator tube (type vacutainer code 607213, Becton-Dickinson) and immediately centrifuged at 3000 r.p.m for 10 min. Thereafter the serum was separated and aliquoted in 1.0-ml fractions and stored at –80°C. Eighty samples were randomly selected from this serum collection, related to 50 patients. Twenty-six patients had a metastatic breast adenocarcinoma, seven a metastatic renal carcinoma, six a metastatic ovarian adenocarcinoma, five a metastatic colorectal adenocarcinoma, two a high-grade lymphoma, one a high-grade testis tumour, one a metastatic small-cell lung tumour, one a malignant fibrous histiocytoma and one had a metastatic carcinoid tumour.

Methods

Serum levels of bFGF and VEGF were determined using two enzyme-linked immunosorbent assay (ELISA) kits of R&D Systems (Minneapolis, MN, USA; Quantikine High Sensitivity human FGF basic and Quantikine human VEGF). Within-assay reproducibility has been tested before (Dirix et al, 1997). Due to exhaustion of the serum aliquots, bFGF could not be measured in four patients.

Serum levels of Il-6 were determined with an ELISA kit of R&D Systems (Quantikine human IL-6). The assay employs a quantitative sandwich enzyme immunoassay technique combining a bound monoclonal antibody specific for IL-6 with an unbound enzyme-linked polyclonal antibody specific for IL-6. Within-assay reproducibility was evaluated on duplicates. When 40 serum samples from apparently healthy individuals were evaluated by R&D Systems, 83% had an IL-6 content of less than 3.13 pg ml^{-1} or the detection limit. Comparably, IL-6 was undetectable, or was at the limit of detection of the assay, i.e. 4 pg ml^{-1}, in the serum of normal subjects in the study of Nakano et al (1998). Thrombocytosis was defined as a platelet number above 400×10^9 l^{-1}. In four patients, platelet counts were not performed on a sample taken at the time of serum sampling and were therefore not used.

Endothelial cell proliferation assay

Human umbilical vein endothelial cells (HUVEC) were isolated according to Jaffe et al (1973) and seeded onto gelatin-coated plastic. They were maintained in M199 medium supplemented

Table 2 The comparison of bFGF, VEGF and IL-6 serum levels in patients without thrombocytosis versus patients with thrombocytosis and according to the median platelet number (platelet *n*)

Platelet *n* (10^9 l^{-1})	bFGF (pg ml^{-1})	VEGF (pg ml^{-1})	IL-6 (pg ml^{-1})
≤ 400 (*n* = 62)	5.43 ± 6.68 (2.52)	351 ± 297 (233)	8.67 ± 22.35 (1.50)
> 400 (*n* = 14)	9.02 ± 9.19 (6.72)	822 ± 464 (739)	28.54 ± 47.51 (8.72)
P-value	0.10	< 10^{-4}	0.03
≤ median (*n* = 40)	5.81 ± 7.12 (2.98)	254 ± 186 (191)	5.78 ± 7.13 (1.50)
> median (*n* = 36)	6.46 ± 7.57 (4.06)	642 ± 431 (562)	19.60 ± 40.96 (3.96)
P-value	0.83	< 10^{-4}	0.08

Mean concentrations ± standard deviation (median) are given. Mann–Whitney *U*-test.

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Table 3 The comparison of bFGF and VEGF serum levels according to platelet number (platelet *n*) and IL-6 serum levels

	bFGF (pg ml^{-1})	VEGF (pg ml^{-1})
Platelet *n* low **and** IL-6 low (*n* = 23)	5.31 ± 7.70 (1.39)	161 ± 81 (180)
Platelet *n* high **or** IL-6 high (*n* = 30)	5.73 ± 5.67 (4.54)	384 ± 263 (304)
Platelet *n* high **and** IL-6 high (*n* = 23)	7.38 ± 8.70 (3.09)	785 ± 423 (760)
P-value	0.52	< 10^{-4}

For platelet number the cut-off value was the median. For IL-6 the cut-off value was the detection limit. Mean concentrations ± standard deviation (median) are given. Kruskal–Wallis test.

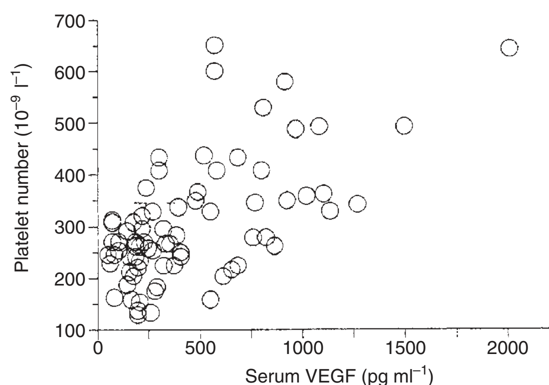

Figure 1 Correlation between platelet number and serum VEGF concentration (*n* = 76; *r* = 0.61; *P* < 10^{-4})

with 20% fetal calf serum (FCS), ECGS 0.01 mg ml^{-1} (Sigma), heparin 1 U ml^{-1} and antibiotics. First to second passage cells were used. All cultures were maintained in a humidified 5% carbon dioxide incubator at 37°C. Subcultures were obtained by enzyme treatment (0.05% trypsin, 0.2% EDTA).

For the proliferation assay, 20 000 cells were put into one well (24-well plate) and allowed to adhere and proliferate for 24 h. The medium was then changed. In the control wells the standard medium was put with 20% FCS. In the other wells, the FCS was replaced by patient serum (20%). After 24 h the endothelial cells were harvested and the viable cells were selected by trypan blue dye exclusion and counted in a haematocytometer. All experiments were performed in triplicate. The results were expressed as the ratio of the number of HUVEC in the wells with the patient serum versus the number of HUVEC in control wells (percentage). Twenty serum samples of cancer patients and five samples of healthy volunteers were randomly taken from the serum collection of our laboratory. Serum bFGF and VEGF were determined as described before.

Statistical analysis

Statistical analysis was performed with the Statview 4.51 software application (Abacus Concepts) on an Apple Macintosh personal computer. The relation between continuous variables was analysed by a correlation analysis. Comparisons of continuous variables in different subgroups were performed by Mann–Whitney *U*-test and Kruskal–Wallis test. The differences in fractions of patients/samples were analysed by a χ^2 test. A *P*-value < 0.05 was considered to be significant.

RESULTS

Forty-two (53%) serum samples from 35 (70%) patients had a measurable IL-6 content, which was higher than the detection limit of 3.12 pg ml^{-1}. The mean coefficient of variation of the duplicate analysis of these samples was 5.7% (*n* = 42; range 0.53–29.50). When IL-6 was not detectable in a serum sample, the value of 1.5 pg ml^{-1} was entered for further analysis, i.e. approximately 50% of the detection limit.

Mean bFGF level was 5.96 pg ml^{-1} (standard deviation (s.d.) 7.20; median 3.07; range 0.11–33.0; *n* = 76). Mean VEGF level was 453 pg ml^{-1} (s.d. 381; median 314; range 40–2000; *n* = 80). Mean IL-6 level was 13.74 pg ml^{-1} (s.d. 30.42; median 3.42; range 1.50–170.40; *n* = 80). Mean platelet number was 307 10^{9} l^{-1} (s.d. 115; median 276; range 132–655; *n* = 76). Fourteen of 76 (18%) samples from 13 of 46 (28%) patients showed thrombocytosis.

Results of the correlation analysis of the different sets of variables are given in Table 1. The strongest correlations were present between serum VEGF and platelet number (*n* = 76; *r* = 0.61; *P* < 0.0001) (Figure 1) and between serum VEGF and IL-6 (*n* = 80; *r* = 0.55; *P* < 0.0001). A correlation was also found between platelet number and serum IL-6 levels (*n* = 76; *r* = 0.36; *P* < 0.0011). There was no correlation between bFGF and any of the other variables.

The comparison of bFGF, VEGF and IL-6 serum levels in patients without thrombocytosis (*n* = 33) versus patients with thrombocytosis (*n* = 13) is given in Table 2. Median VEGF serum levels were 3.2 times higher in the latter group (*P* < 0.0001). Median IL-6 serum levels were 5.8 times higher when thrombocytosis was present (*P* = 0.03). The difference of mean bFGF serum levels was not significant. Comparable results were obtained when median platelet number, i.e. 276 10^{9} l^{-1}, was taken as the cut-off value (Table 2).

When the samples were categorized according to the median serum VEGF level, i.e. 314 pg ml^{-1}, according to the median platelet number, and according to the detection limit for serum IL-6, the following distributions of samples were found. Of 40 samples with low serum VEGF, 30 also had a low platelet number. Of the 36 samples with high serum VEGF, 26 also had a high platelet number (*n* = 76; χ^2 value = 16.95; *P* < 0.0001). Of the 40 samples with low serum VEGF, 30 also had undetectable low levels of IL-6. Of the 40 samples with high serum VEGF, 33 had serum IL-6 levels above the detection limit (*n* = 80; χ^2 value = 26.60; *P* < 0.0001). Of the 40 samples with a low platelet number, 23 also had undetectable low levels of IL-6. Of the 36 samples with a high platelet number, 23 also had serum IL-6 levels above the detection limit (*n* = 76; χ^2 value = 3.48; *P* = 0.0622).

Platelet number and IL-6 serum levels co-determined VEGF serum levels but not bFGF serum levels (Table 3). Serum VEGF levels were higher (mean serum VEGF of 785 pg ml^{-1}) when both platelet number and IL-6 serum levels were high, i.e. above the median value and above the detection limit, respectively, than when only platelet number or only IL-6 was high (mean serum VEGF of 384 pg ml^{-1}) (*P* < 0.0001). When serum bFGF was analysed in this way, no differences were observed (Table 3).

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Table 4 HUVEC proliferation expressed as the ratio of the number of HUVEC in the wells with the patient serum versus the number of HUVEC in the control wells with fetal calf serum

HUVEC proliferation (% of control)	bFGF (pg ml^{-1})	VEGF (pg ml^{-1})
bFGF high and VEGF high (*n* = 8)		
228	10.30	763
200	17.00	813
184	17.00	1490
160	17.00	796
160	32.70	670
152	11.60	767
152	7.51	957
126	11.80	632
(170 ± 32; 160)a		
bFGF low and/or VEGF low (*n* = 12)		
155	0.49	206
150	17.00	389
137	0.51	105
136	0.55	185
135	0.49	109
134	1.85	172
134	0.87	226
129	1.01	1130
126	0.31	115
113	4.39	1650
97	33.00	244
97	1.30	1160
(128 ± 18; 134)a		

The mean proliferation assay result for healthy persons (*n*= 5) was 152% (s.d. 31). Results printed in bold are above this mean value. [a]Between brackets, the mean ± s.d.; median values for HUVEC proliferation (%) are given. Mann–Whitney *U P*-value: 0.0037.

If, as suggested by Banks et al (1998) the platelets are the major source of VEGF in the serum, the theoretical VEGF load of platelets can be calculated by the ratio of VEGF serum concentration and the platelet number. A mean VEGF load of 1.37 pg VEGF 10^{-6} platelets was found (s.d. 0.39; range 0.16–3.64; median, 1.16; n = 76). A significant correlation was found between VEGF load and serum IL-6 concentration (r = 0.4; P = 0.0003) (Figure 2). When only samples with a platelet number lower than the median of 276 10^9 l^{-1} were analysed, this correlation became stronger (r = 0.67; P < 0.0001). The respective coefficient r for the samples with a high platelet number was 0.39 (P = 0.0171).

When patient sera with a bFGF and a VEGF content higher than the cut-off values we have previously used, i.e. 7.5 pg ml^{-1} and 500 pg ml^{-1} respectively (Dirix et al, 1996; Dirix et al, 1997), were tested, in most cases (five of eight samples) the HUVEC number after 24 h was higher than when the sera of apparently healthy volunteers were used. The opposite was found when patient sera with low bFGF and/or low VEGF content were assayed: only one sample out of 12 resulted in a high HUVEC number in the proliferation assay (P = 0.018). The mean proliferation assay result for the healthy persons was 152%, and was 170% for the patient sera with elevated bFGF and elevated VEGF as compared to 128% for the patient sera with low serum bFGF and/or low serum VEGF content (P = 0.0037) (Table 4).

DISCUSSION

This study supports the role of platelets in the storage of circulating VEGF, as suggested by Verheul et al (1997), Banks et al (1998) and Wartiovaara et al (1998). A strong correlation was found between platelet count and serum VEGF concentration in a cohort of patients with different types of advanced cancer. The cytokine IL-6 correlated with the total amount of VEGF in the serum and also with the calculated VEGF content of the platelets, indicating a more complex indirect angiogenic action of IL-6 than just the thrombopoietic effect.

As the generation of tumour stroma is remarkably similar to the healing of wounds (Dvorak, 1986), platelet activation, coagulation of extravasated plasma fibrinogen and fibrinolysis are important endpoints of tumour–host cell interactions in growing tumours. Evidence for platelet activation in cancer patients is based on the assessment of circulating levels of β-thromboglobulin, a platelet α-granule component. Levels of β-thromboglobulin were significantly elevated, e.g. in prostatic cancer (Yazaki et al, 1987), in breast cancer (Ferriere et al, 1985) and in small-cell lung cancer (Milroy et al, 1988), compared with those of healthy individuals. VEGF appears to be released by activated platelets together with β-thromboglobulin, suggesting that VEGF is located in the α-granules of platelets (Möhle et al, 1997; Wartiovaara et al, 1998). Clotting of platelet-rich plasma (PRP) prepared from the blood of

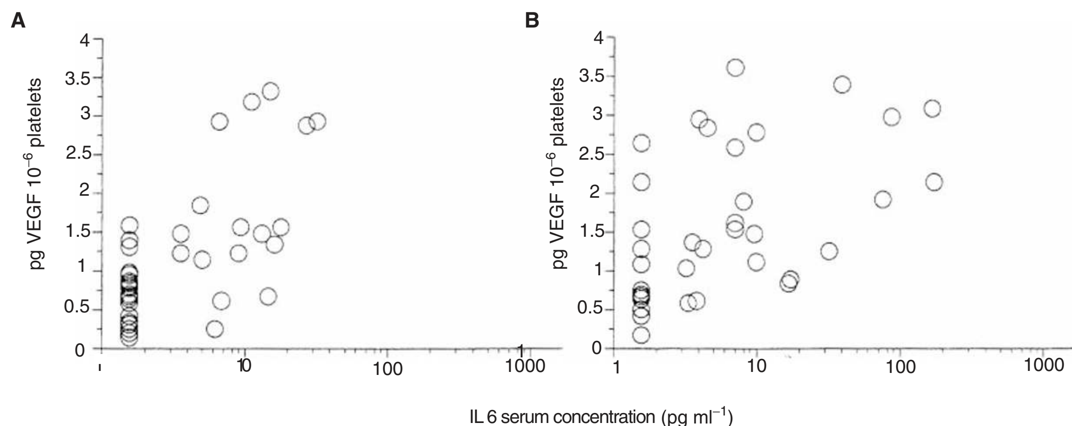

Figure 2 Theoretical VEGF content of platelets versus IL-6 serum levels for samples with a platelet number lower than the median (A; n = 40) and for samples with a platelet number higher than the median (**B**; n = 36). Respective r and P-values are: 0.67/<10^{-4} and 0.39/0.017

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eight healthy individuals with thrombin resulted in higher VEGF levels than when platelet-free plasma was used (Banks et al, 1998). The VEGF levels obtained after the clotting of the PRP correlated with the respective serum VEGF levels. The theoretical VEGF load of platelets, i.e. the VEGF concentration found after activation of the platelets calculated in terms of the number of platelets present, was highly comparable in PRP and serum. These observations support the hypothesis that platelets are an important source of circulating VEGF. In 27 patients with advanced breast cancer undergoing chemotherapy, a correlation (r = 0.8; P < 0.01) between serum VEGF levels and platelet counts was reported by Verheul et al (1997). In the group of eight healthy individuals (Banks et al, 1998), serum VEGF just failed to achieve a significant correlation with platelet number (r = 0.62; P = 0.1). In our population of 50 patients, corresponding to 80 blood samples, with various types of advanced cancer, a highly significant correlation between platelet number and serum VEGF concentration was found ($r = 0.61$; $P < 10^{-4}$). These results are in accordance with the reduced survival rates of cancer patients with preoperative thrombocytosis, e.g. in lung cancer (Møller Pedersen et al, 1996). We have indeed shown that high serum VEGF, and thus high platelet number, indicates fast growth kinetics in several tumour types (Dirix et al, 1996; Dirix et al, 1997). It is conceivable that platelets provide a means to concentrate the effect of secreted growth factors, e.g. VEGF, onto sites of perturbed integrity of the vascular wall, as present during wound healing and tumour growth. This scavenger function of platelets also conforms to the lack of apparent angiogenesis observed outside of the tumour in patients with high amounts of circulating VEGF protein. Although clear evidence for the endocytic uptake of VEGF by platelets is absent, the incorporation of other circulating proteins into platelet granules and into megakaryocytes has been extensively studied (Handagama et al, 1987; Harrison et al, 1989).

Since plasma VEGF levels are very low (Banks et al, 1998), and probably as a consequence of inefficient inhibition of platelet activation during sample handling, platelets can be regarded as the transporters of the active and stable fraction of circulating VEGF. The in vitro HUVEC proliferation assay results indeed suggest that the concentration of VEGF measured in the serum reflects the biological activity of VEGF. Given the considerable variability of the theoretical VEGF load of platelets (range: 0.16–3.64 pg VEGF 10^{-6} platelets), serum VEGF does not seem to be a simple reflection of the number of circulating platelets. As a practical conclusion, we therefore suggest using serum VEGF and not plasma VEGF as suggested by Banks et al (1998), as a measure of circulating VEGF. Since other circulating blood cells may also contain VEGF, measuring VEGF in whole blood might even reflect the active VEGF concentration more accurately (P Salven, personal communication).

We measured the serum IL-6 levels in the cohort of 50 patients with advanced cancer, (1) because IL-6 serum levels correlate with platelet number (Nakano et al, 1998) and intravenous administration of recombinant human IL-6 results in elevation of the platelet number (Clarke et al, 1996); (2) because IL-6 production is increased in tumour tissue (Rak et al, 1994; Degeorges et al, 1996) and during wound healing (Mateo et al, 1994); and (3) because transient tumour-stimulating effects have been observed during IL-6 treatment of cancer patients (Ravoet et al, 1994) and high IL-6 levels are associated with adverse prognosis in several tumour types and benign angiogenic diseases (Blay et al, 1992; Bross et al, 1996).

Serum levels of IL-6 correlate with platelet number and with serum VEGF concentration, but not with serum bFGF concentration. This result can be explained by the thrombopoietic action of IL-6 and the storage of VEGF within platelets. The calculated VEGF content of the platelets is larger when serum IL-6 levels are higher. This result implies an additional effect of IL-6 on VEGF production. Transient expression of IL-6 mRNA was shown in endothelial cells during the physiologic angiogenic processes associated with the development of ovarian follicles and with the embryonic implantation in the maternal decidua (Motro et al, 1990). Treatment of various cell lines, e.g. derived from an epidermoid human carcinoma and a glioma cell line, with IL-6 resulted in the induction of VEGF mRNA (Cohen et al, 1996). The effect of IL-6 on VEGF expression is mediated by specific DNA motifs located on the putative promotor region of VEGF as well as by specific elements in the 5′-untranslated region. Hypoxia has been shown to induce IL-6 mRNA and IL-6 protein in cultured human endothelial cells (Yan et al, 1995). IL-6 can thus be regarded as an indirect angiogenic factor produced by stromal cells and by tumour cells.

Although in benign prostate hyperplasia (Degeorges et al, 1996) and in early-stage radial melanoma growth phase (Rak et al, 1994), stroma-derived IL-6 might function as a growth inhibitor of tumour cells, at least in more advanced cancer, IL-6 might stimulate tumour growth by increasing the amount of active VEGF, stored in the platelets. The stimulation of platelet production by tumour-derived IL-6 favours the endocrine effect of angiogenic factors and facilitates the development of secondary tumours in distant tissues, exemplifying the parasitic nature of cancer.

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