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Clinical Significance of the Determination of Angiogenic Factors

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INTRODUCTION

ANGIOGENIC ACTIVITY is known to depend upon a balance between positive and negative angiogenesis regulators [1]. The alteration of this balance by mechanisms, such as the induction of vascular endothelial growth factor (VEGF), expression of TP53 or *RAS* mutations, *SRC* overexpression, and downregulation of thrombospondin-1 expression by *TP53* mutation, can switch the angiogenic phenotype and facilitate the growth of solid tumours [2–5]. Currently, measurement of intratumoral microvessel density (IMD) by immunocytochemistry appears to be the most reliable method of measuring angiogenic activity [6]. However, the determination of levels of each endothelial growth regulator in tumour tissues and in serum or urine can provide more information on the angiogenic characteristics of each tumour and might be an alternative method for evaluating angiogenic activity. For example, the level of intratumoral VEGF varies greatly between tumours and can be a valuable prognostic factor [7]. The endogenous angiogenesis inhibitor, angiostatin, which can cause tumour regression in human tumour xenografts models, has been detected in serum [8]. Since the biological properties of each endothelial growth factors is different, it will be of particular importance to know the role of each factor in human tumours.

Therefore, we have focused on the determination of angiogenic factors, particularly positive endothelial growth regulators including VEGF, platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and metalloproteinases (MMPs), in breast cancer patients. In this article, we review our experience and the most relevant data in the literature on the clinical significance of the determination of these endothelial growth regulators in human tumours.

EXPRESSION OF POSITIVE ENDOTHELIAL GROWTH REGULATORS AND CORRELATION WITH MICROVESSEL DENSITY

The most relevant biological properties of the angiogenesis regulators are illustrated in Figure 1. Among many new positive endothelial growth regulators, these factors seem to play central roles in angiogenesis in various types of solid tumours [9].

VEGF and bFGF are both involved in the process of new vessel formation. They are capable of inducing several types of proteases, such as plasminogen activators (PAs) and MMPs. VEGF, particularly $VEGF_{121}$ and $VEGF_{165}$ isoforms, with no or low heparin-binding activity, are secreted by tumour cells, and stimulate growth of endothelial cells where VEGF receptors are selectively expressed [10]. RT-PCR analysis has confirmed the expression of $VEGF_{121}$ and $VEGF_{165}$ isoforms in human cultured breast cancer cells and in primary breast tumour tissues [11]. Using immunocytochemical analysis, VEGF is mainly localised in the cytoplasm of tumour cells [7].

PD-ECGF/TP is an angiogenic enzyme engaged in nuclear acid metabolism [12–14]. TP has no mitogenic effect in microvascular endothelial cells, but it stimulates endothelial chemotaxis. Furthermore, TP potently induces the chemokinetics and histamine release in mast cells which are known to accumulate at the site of angiogenesis *in situ*. This suggests that TP also acts as a permeability factor through histamine release in a paracrine manner [15]. TP localises in the cytoplasm of tumour cells and in some stromal cells, including macrophages and lymphocytes, in tumour tissues as shown by immunocytochemical analysis [16]. A dominant stromal staining is particularly evident in gastro-intestinal tumours.

The mechanism through which TP induces angiogenesis is not well understood, but recently it has been found that 2-deoxy-D-ribose, a degradation product of thymidine caused by TP, has angiogenic activity both *in vitro* and *in vivo* [17].

A series of studies, evaluating the association between the expression of positive endothelial regulators with IMD in primary breast cancer found that VEGF and TP expression are positively associated with the increment of IMD, evaluated by counting vessel 'hot spots' using immunostaining with antifactor VIII-related antigen (RA) antibody (Table 1) [18]. The VEGF antibodies used were designed to recognise the common region of the amino acid sequence of all four VEGF isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and

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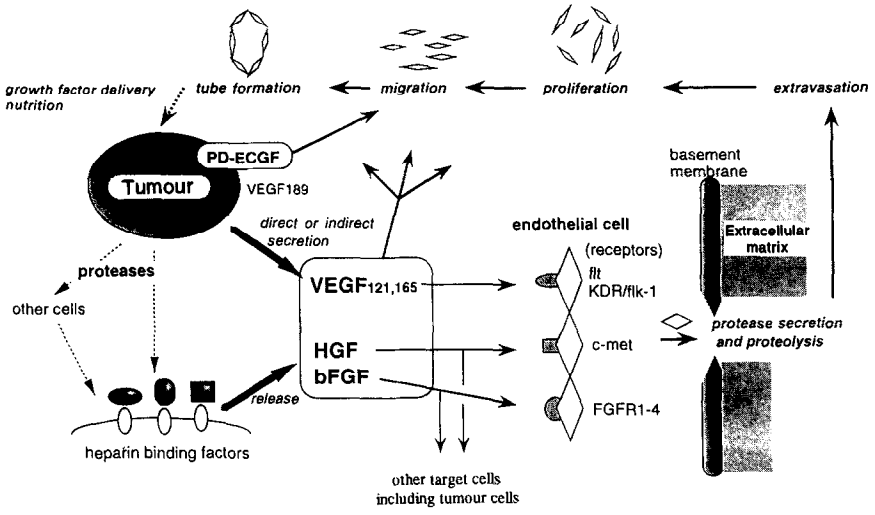

Figure 1. Schematic representation of the function of major positive angiogenic factors.

$VEGF_{206}$). The significant correlation between VEGF expression and has also been shown in other types of tumour including brain tumours, cervical neoplasias, lung cancer, stomach cancer and colon cancer (Table 2) [7, 9, 20–23]. Furthermore, the upregulation of the VEGF receptors, flt-1 and KDR, in tumour-associated endothelial cells was also observed in a variety of tumours including breast cancer, brain tumours, kidney tumours, bladder cancer, ovarian cancer and colon cancer [23–27]. Brown and associates clearly demonstrated an intense expression of VEGF mRNA in carcinoma cells and VEGF receptor mRNA in endothelial cells of small blood vessels adjacent to primary breast cancer [24]. Takahashi and colleagues reported an upregulation of both VEGF and KDR in highly vascularised tumour tissues in colon cancer [23]. These data suggest that VEGF is one of the most important growth factors involved in neovascularisation of human tumours.

The significant correlation between tumour TP expression and MID was also found in stomach and colon cancer (Ogata and Shirouzu, Kurume University Hospital, Japan, personal communication) using immunocytochemical analysis [28, 29]. In ovarian tumours, Reynolds showed that areas of high blood flow assessed using Doppler imaging, were significantly associated with increased expression of TP [30]. Fox and associates did not find a significant correlation between tumour TP expression and vascularity in primary breast cancer, but they suggested the role of TP occurs early in tumour angiogenesis through remodelling the existing vasculature, which is consistent with the chemotactic but non-mitogenic properties of TP. Moreover, TP expression in tumour cells was higher in low grade, small tumours, and endothelial cell TP expression was most prominent at the tumour periphery [31].

TP is frequently co-expressed with VEGF as found in our study of 256 primary breast tumours. Tumours co-expressing VEGF and TP presented with greater vascularity compared with those with a positive phenotype for either VEGF or TP, or negative for both. This suggests a co-operative function for TP and VEGF in neovascularisation of breast cancer. *In vitro* VEGF and TP synergistically influence endothelial growth under hypoxic conditions (Ishizuka, Roche Institute, Japan). However in bladder cancer, a differential expression of TP and VEGF between superficial and invasive tumours has been documented [32]. These results indicate a diverse role for these factors dependent on the organ or tissue.

We did not find a correlation between bFGF concentration determined by EIA in total tumour tissue extracts and IMD in our study [33], even though bFGF is the most potent mitogen *in vitro* of the many endothelial growth factors [34]. Since bFGF is normally bound to heparin and heparan sulphate proteoglycans in the extracellular matrix

Table 1. Expression of positive endothelial regulators and the relationship with the increment of IMD in human breast cancer tissues (Komagome study) [7, 16, 18, 33]

Positive endothelial regulators	Number of examined cases	Positivity	Correlation with IMD
VEGF-ICA	256	50.8%	$P < 0.001$*
VEGF-EIA	135	-	$P < 0.01$**
PD-ECGF/TP-ICA	256	52.7%	$P < 0.05$*
HGF-EIA	135	-	NS
bFGF-EIA	135	-	NS
MMP-2	122	40.1%	NS
MMP-9	122	62.2%	$P < 0.05$*

*χ^2-test; **t-test; NS, not significant.

Table 2. Prognostic significance of endothelial growth factor

Growth factor	Tumour type	Method	Number of cases	Correlation with IMD	Prognostic value	Reference
bFGF	Kidney	ICA	62	Unknown	Yes	[40]
bFGF	Breast	ICA	79	No	Yes (stromal staining)	[41]
VEGF	Breast	ICA	230	Yes	Yes	[11]
VEGF	Stomach	ICA	129	Yes	Yes	[22]
VEGF	Colon	ICA	300	Yes	Yes	Ogata and Shirouzu (personal communication)
VEGF	Colon	ICA	52	Yes	Yes	[23]
VEGF	Lung	ICA	204	Yes	No	[21]
PD-ECGF/TP	Colon	ICA	91	Yes	Yes (Stage III)	[29]
PD-ECGF/TP	Breast	ICA	240	No	No	[31]
PD-ECGF/TP	Stomach	ICA	120	Yes	Yes	[28]
HGF	Breast	EIA	258	Unknown	Yes	[46]
Midkine	Bladder	RNase protection	47	Unknown	Yes	[47]

(ECM) particularly in basement membranes, around vessels and stromal cells [35], the measurement of protease activities which can activate bFGF rather than the quantitation of tumour bFGF expression, might be useful for assessing the implications of FGF in vessel formation *in vivo*. No correlation was found between tumour HGF concentrations and IMD in breast cancer, possibly because HGF is normally stored in a bound form in the ECM or on the cell surface and needs protease activation.

Recently, Hildenbrand and associates found that intratumoral uPA and PAI-1 levels quantitated by EIA, were associated with IMD in 42 primary breast cancer tissues [36]. They also showed the importance of macrophage aggregation for microvessel formation. uPA expression by macrophages is known to lead to the release of the matrix-bound heparan sulphate proteoglycan, bFGF, and transforming growth factor (TGF)-β. In addition, uPA can cleave and activate HGF from the matrix- or cell surface-bound inactivated form [37]. We measured MMP activities in primary breast tumour tissues by zymography using SDS-PAGE containing gelatin (1%), and compared these levels with IMD. MMP-9 (92 KD gelatinase B) expression, assessed by the quantitation of the unstained zone corresponding to the enzymatic digestion by image-analyser, was significantly associated with IMD (Table 3). MMP-9 was more frequently expressed in tumours with 100 or more counts/mm^2 IMD than those with less than 100 counts/mm^2 IMD. Moreover, tumours expressing both MMP-9 and VEGF were those most vascularised (Table 3). No direct correlation between MMP-9 and VEGF was observed. It is possible that heparin-binding endothelial growth factors (for example, bFGF and HGF) when activated by proteases, including uPA and MMP-9, act co-operatively with VEGF *in situ*. bFGF and VEGF are known to act synergistically on the growth of microcapillary endothelial cells [38]. In glioma cells, bFGF is capable of inducing VEGF. The upregulation of VEGF by bFGF, particularly under hypoxic conditions, was also observed in vascular smooth muscle cells [39]. Since bFGF and VEGF can stimulate protease induction, a cyclic cascade might be present *in situ* involving secretion of endothelial growth factors by tumour cells, protease induction, activation of matrix-bound growth factors and upregulation of growth factor production in tumour and stromal cells. The co-expression of more endothelial regulators seems to be a common finding in active neovascularisation.

THE PROGNOSTIC IMPORTANCE OF ENDOTHELIAL GROWTH FACTORS

Nanus and associates first reported the importance of tumour bFGF expression as a prognostic indicator in renal tumours [40]. Subsequently, the prognostic value of other endothelial regulators have been documented (Table 2). Visscher and colleagues found that bFGF staining in stromal cells was significantly associated with aggressive, clinical behaviour of breast cancer [41]. In addition, they showed a tendency for co-expression of stromal bFGF and uPA in peritumoral host cells. We have reported the prognostic significance of VEGF expression, determined by immunostaining in 230 primary breast cancer patients [7]. Takahashi and associates also showed that the expression of VEGF and KDR, was notably higher in metastatic tumours than in non-metastatic tumours in colon cancer [23]. In addition, in Dukes' C colon cancer, VEGF status seems to be an independent prognostic indicator (Ogata, Kurume University Hospital, Japan). Maeda and associates reported that expression of VEGF, assessed by immunocytochemistry, was an independent, poor prognostic indicator in 95 gastric cancer patients who underwent curative surgery [28]. Conversely, Mattern and associates failed to demonstrate a prognostic value for VEGF expression in non-small cell

Table 3. Correlation between MMP-9/VEGF expression and IMD

IMD (counts/mm^2)	MMP-9/VEGF (n = %) −/− (n = 14)	+/− or −/+ (n = 55)	n (90) +/+ (n = 53)
<100	11 (79)	34 (62)	12 (23)
⩾100	3 (21)	21 (38)	41 (77)

$\chi^2 = 23.1$, $P < 0.01$.

lung cancer, although the mean survival time tended to be shorter in patients with VEGF positive tumours than in those with VEGF negative tumours [21]. Recently, we developed a VEGF-EIA method and examined intratumoral VEGF levels in 135 primary breast cancer patients [33]. The VEGF concentration was significantly related to IMD. Quantitative analysis of VEGF will be an important tool for assessing angiogenic activity. In a collaborative study with Gasparini's group, cytosolic VEGF concentrations appear to be of prognostic value in a series of 260 node-negative breast cancer patients (G. Gasparini, St. Bortolo Hospital, Vicenza, Italy).

The prognostic significance of TP has been assessed in stomach and colon cancers [28, 29]. In stomach cancer, liver metastasis was more frequent in patients with TP positive tumours than in those with negative tumours. However, no significant prognostic value of TP was detected in primary breast cancer patients. Our group also failed to find a prognostic value of TP expression in breast cancer (M. Toi, Tokyo Metropolitan Komagome Hospital, Japan). Since TP is responsible for thymidine metabolism, several types of anticancer drugs, such as methotrexate and 5-FU derivatives, which are widely used in adjuvant treatments, might cause a bias in the analysis of breast cancer patients [42–45]. In recurrent breast cancer patients, TP seemed to be a useful marker for predicting responsiveness to 5′-deoxy-5-fluorouridine treatment (M. Toi, Tokyo Metropolitan Komagome Hospital, Japan).

In addition, Yamashita and associates reported a prognostic value of HGF levels in primary tumours of breast cancer patients [46]. Moreover, O'Brien and colleagues postulated the importance of midkine, a basic heparin-binding growth factor secreted by a variety of cell types including tumour and endothelial cells, in human bladder cancer [47]. They noted, using an RNase protection assay, that overexpression of midkine predicted the early relapse and rapid progression in patients with invasive bladder cancer.

The prognostic significance of several types of proteolytic enzymes has been widely investigated. In particular, in primary breast cancer, large studies performed by Foekens and associates have demonstrated that cathepsin D and uPA/PAI expression are independent prognostic factors [48, 49].

DETERMINATION OF POSITIVE ANGIOGENESIS REGULATORS IN SERUM AND ITS CLINICAL RELEVANCE

Several angiogenic growth factors are detectable in the sera of cancer patients. Fujimoto and associates found elevated levels of bFGF in sera of renal cancer patients [50]. Since bFGF levels were elevated in the renal vein and serum bFGF disappeared after the resection of the affected kidney [51], the elevation of serum bFGF is considered to be caused by the tumour. In 105 cervical cancer patients, the serum bFGF level was reported to be aberrantly increased in 65.7% of cases (cut-off: 15 pg/ml) [52]. In addition, Duensing and associates showed a positive association between the aberrant increase of serum bFGF levels and pulmonary metastases in renal cancer patients [53]. Folkman's group observed increased urine levels of bFGF related to tumour progression in a wide spectrum of cancer patients [54].

Kondo and associates found serum VEGF in tumour-bearing mice and cancer patients, using an EIA method capable of detecting very low levels of VEGFs [55]. Using this EIA system, we measured serum VEGF levels in a variety of cancer patients: in breast cancer, serum VEGF levels were aberrantly increased in approximately 10% of patients with primary tumours and 40% with recurrent cancer (cut-off: 180 pg/ml) [56]. In primary tumours, aberrant VEGF serum levels were significantly associated with high IMD and VEGF expression in tumour cells, suggesting that circulating VEGF is produced by the tumour. The elevated serum VEGF levels decreased after surgical removal of the primary tumour. In a Western blot analysis, using the semi-purified sera from a recurrent breast cancer patient, the $VEGF_{165}$ isoform, commonly expressed in a variety of tumour cells, was detected. Recently, Dirix and associates repeatedly measured serum VEGF and bFGF levels in untreated colorectal cancer patients, and interestingly, they found that the patients with a tumour volume doubling time of less than 6 months showed higher bFGF and VEGF

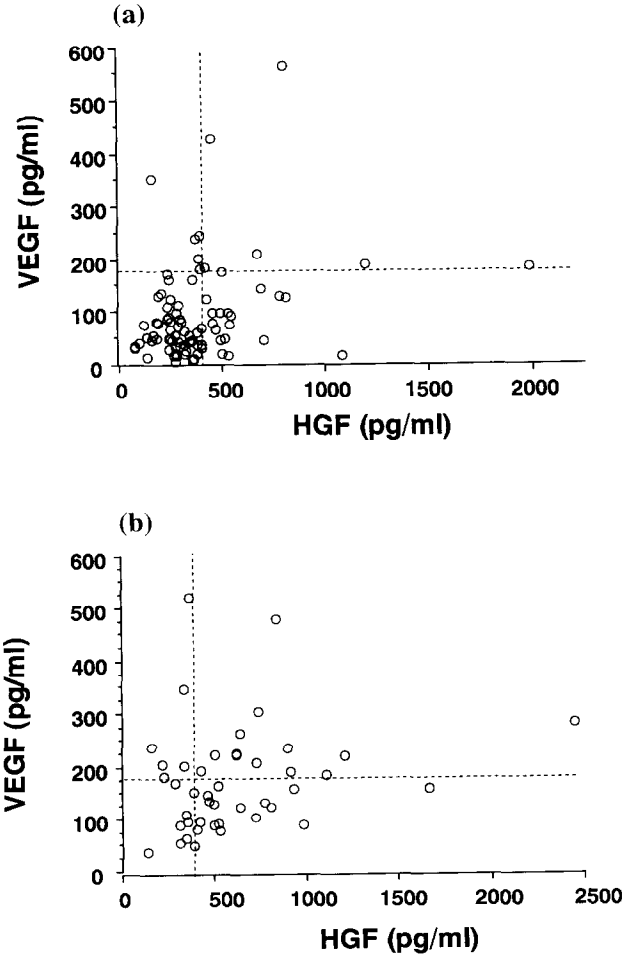

Figure 2. VEGF and HGF levels in the sera of breast cancer patients. The samples were taken prior to anticancer treatment. (a) Primary breast cancer patients. (b) Recurrent breast cancer patients. Of the recurrent breast cancer patients, 83% showed either VEGF or HGF aberrant elevation in the sera. No significant correlation between VEGF and HGF levels was demonstrated.

Table 4. Aberrant serum level, of VEGF, HGF, CEA and CA15-3 in recurrent breast cancer patients [56, 57]

Site of recurrence	Number of cases ($n = 46$)	VEGF elevated (%) ($n = 20$)	HGF elevated (%) ($n = 31$)	CEA elevated (%) ($n = 19$)	CA15-3 elevated (%) ($n = 26$)
Soft tissue	13	5 (38)	7 (54)	2 (15)	5 (38)
Bone	14	6 (43)	8 (57)	9 (64)	11 (79)
Lung	9	4 (44)	6 (67)	2 (22)	6 (67)
Liver	10	5 (50)	10 (100)	6 (60)	4 (40)

Cut-off: VEGF, >180 pg/ml; HGF, >400 pg/ml; CEA, >6.2 ng/ml; CA15-3, >27 IU/ml.

serum levels than the patients with more indolent tumours independent of the number of sites involved and the extent of the metastatic disease (L. Dirix, University Hospital Antwerp, Belgium).

Serum levels of HGF, which is not only a scatter factor for epithelial cells but also a potent growth factor for endothelial cells, are closely associated with the progression of disease [57]. Taniguchi and associates found that increased serum levels of HGF were detectable in approximately a third of patients with primary breast cancer. In patients with recurrent breast cancer, the detection rate of high serum levels of HGF increased up to 70% (cut-off: 400 pg/ml). Elevated levels of HGF in primary tumours was significantly related to high-grade lymphatic invasion. All patients with liver metastases exhibited an aberrant increase in HGF levels. HGF is likely to stimulate both tumour cell growth and invasion, as well as neovascularisation, particularly in tumours expressing c-met.

Aberrant expression of either VEGF or HGF in the sera was detected in 83% of recurrent breast cancer patients (Figure 2) [56, 57]. No direct association was found between VEGF and HGF levels. Because the elevated levels of VEGF and HGF concentrations were estimated to be high enough to elicit its biological functions for the endothelium, these circulating growth factors might function in an endocrine manner. In fact, Eagles and associates found HGF activity in the pleural effusion fluid of cancer patient.

Other growth factors or cytokines, including TGF-α and IL-8 are also elevated in the serum of cancer patients. TGF-α concentrations in gastro-intestinal cancer patients were reported to range from 119 to 760 pg/ml with a mean value of 269 pg/ml using EIA, whereas their serum levels ranged from 127 to 207 pg/ml with a mean value of 147 pg/ml [58]. An aberrant increase in serum TGF-α levels was also found in breast cancer patients [59]. Furthermore, proteases, such as MMP-9, pro-cathepsin D and tissue factor, have recently been shown to be increased in the sera of cancer patients [60–62]. Recently, Folkman emphasised the biological importance of negative angiogenesis regulators, such as angiostatin for the inhibition of primary tumours and metastasis [63].

Altered levels of serum endothelial growth factors might also be useful as tumour markers. For example, the increase in serum HGF levels was more frequent than that of some conventional tumour markers, including CEA and CA15-3 (Table 4) [56, 57].

CONCLUSION

Although many questions remain to be resolved on the regulation of circulating endothelial factors, including their binding with serum proteins, their metabolism and the balance between positive and negative regulators, the determination of these factors is potentially important for the assessment of angiogenic activity. Finally monitoring the circulating levels of endothelial regulators might be of particular value as biological targets for angio-inhibitors or antimetastatic drugs that are currently undergoing clinical trials.

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