Vascular Endothelial Growth Factor (VEGF) in Breast Cancer: Comparison of Plasma, Serum, and Tissue VEGF and Microvessel Density and Effects of Tamoxifen


ABSTRACT

The assessment of angiogenesis in breast cancer is of importance as a key indicator of survival and response to therapy. Circulating vascular endothelial growth factor (VEGF) measurements may provide a less subjective analysis than microvessel density (MVD) or immunohistochemical analysis of VEGF expression; however, most studies have used serum, which is now known to largely reflect platelet-derived VEGF concentrations. This study examined for the first time both plasma (VEGF_p) and serum (VEGF_s) VEGF concentrations in 201 blood samples from pre- and postmenopausal healthy controls and from patients with benign breast disease, localized breast cancer, breast cancer in remission, or metastatic breast cancer and related these to other clinicopathological markers. VEGF_p, but not VEGF_s, concentrations of patients with localized disease were significantly elevated compared with normal controls (P = 0.016). Patients with metastatic disease had higher VEGF_p and VEGF_s levels than normal controls (P < 0.001, P = 0.044 respectively), and higher VEGF_p, but not VEGF_s, than patients with benign disease (P = 0.009) and patients with localized disease (P = 0.004). However, the highest VEGF_p and VEGF_s concentrations were seen in patients in remission compared with normal controls (P < 0.001 and P = 0.008, respectively). VEGF_p concentrations in patients in remission were also higher than in patients with benign disease (P = 0.01) or patients with localized disease (P = 0.005). Tamoxifen treatment was significantly associated with higher circulating and platelet-derived VEGF levels. Circulating VEGF did not correlate with any clinicopathological factor, including MVD or VEGF expression. VEGF expression was significantly correlated with estrogen receptor status and inversely correlated with tumor grade. MVD correlated with tumor size. Tamoxifen-induced increases in VEGF may be important in clinical prognosis or associated pathologies.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from the existing vascular network, is essential for continued tumor growth and metastasis (1). One of the most potent and specific angiogenic factors is VEGF, also known as vascular permeability factor and vasculotropin (reviewed in Ref. 2). Evidence for the pivotal role of this cytokine in tumor angiogenesis includes the observations of increased expression in tumor cells of numerous human cancers together with up-regulation of the receptors on the associated endothelial cells and the inhibitory effect of anti-VEGF antibodies on tumor growth in vivo (reviewed in Ref. 2).

Since the pivotal findings in breast cancer of a correlation between tumor angiogenesis, as assessed by microvessel count and density, and metastasis (3), many studies have confirmed the clinical value of this parameter (4), and IMD is now established as one of the standard prognostic factors for predicting metastasis and relapse-free or overall survival. Longer-term survival is also predicted by IMD as an independent prognostic factor (5), and in addition, IMD has been found to add prognostic information to ER status in predicting response to tamoxifen treatment (6). Higher VEGF mRNA levels have been found in invasive breast carcinoma or DCIS, compared with benign or normal breast tissue (7–10), and in association with higher vessel counts (8, 10–13). Assessment of VEGF expression by immunohistochemistry or immunoassay of tissue extracts has shown significant correlations with microvessel counts or density (10, 14–16). In node-negative breast cancer patients, VEGF is a strong independent predictor of relapse-free (17, 18) and overall (18, 19) survival.

Clearly, measurement of circulating soluble markers of angiogenesis would be of considerable benefit over more subjective approaches such as immunohistochemical assessments, or immunoassays which involve laborious tissue extraction procedures. Since the original finding of elevated VEGF concentrations of in patients with cancer (20), many studies have reported similar findings in patients with breast cancer and many other types of cancer, with higher levels often found in metastatic disease than in localized disease or in progressive disease during treatment (21–24). Correlations with prognosis have also been reported for several cancers, e.g., ovarian cancer (25–27). However, the interpretation of such studies has been complicated by the demonstration that much of the VEGF measured in serum samples is released from platelets upon activation after venipuncture (28–30), with mRNA for VEGF being found in all blood cell types examined, including megakaryocytes, platelets, lymphocytes, CD34+ cells, granulocytes, and monocytes (31–34), and VEGF protein being shown to be released by neutrophils (35) and platelets (28–30, 33, 34) after activation in vitro. To measure true basal circulating levels of VEGF, rapidly processed citrated plasma samples are the only suitable material (28); they may, therefore, better reflect any circulating VEGF released by the tumor. However, this has yet to be investigated, and the biological significance of platelet- or leukocyte-derived VEGF has yet to be clarified.

This study examined for the first time both VEGF_p and VEGF_s in blood samples from pre- and postmenopausal healthy controls and patients with benign breast disease, localized breast cancer, breast cancer in remission, or metastatic breast cancer to establish the clinical validity of the different measurements. These results were related to the immunohistochemical expression of VEGF by the tumor, as determined by a new antibody specifically validated for use with paraffin-embedded material (36), and to the other established clinic-
pathological prognostic markers, namely MVD, tumor size and grade, lymph node status, and ER and PR status. In particular, the impact of therapy on circulating VEGF concentrations was investigated.

MATERIALS AND METHODS

Patients. The study included 138 patients with benign breast disease, localized breast cancer (prior to surgery), metastatic breast cancer, or in remission from breast cancer, together with 63 healthy controls (Table 1). Six patients with localized disease, 30 in remission, and 7 with metastatic disease were receiving tamoxifen at the time of sampling. Other hormone-based medication included aromide (one with localized disease, one in remission, and two with metastatic disease), Megace (one metastatic), and Danazol (one with localized disease). Two patients with localized disease, eight in remission, and five with metastatic disease were receiving chemotherapy. Nine of the 62 patients with localized disease had DCIS alone; the clinicopathological details of the remaining 53 patients in this group shown in Table 2. Patients with metastatic disease included 1 at presentation, 16 at relapse, and 5 during treatment for relapse. The duration of remission for the patients in the remission group ranged from <1 to 123 months. In seven healthy females, changes in circulating VEGF concentrations during the menstrual cycle were examined with blood samples taken on a minimum of four occasions during one cycle and concomitant samples being assayed for circulating estradiol, progesterone, luteinizing hormone, and follicle-stimulating hormone. The study had been approved by the local ethics committee, and informed consent was obtained.

Blood Sampling. Venous blood was collected in tubes containing either trisodium citrate [final concentration, 0.31% (w/v)] or no anticoagulant. Within

<table>
<thead>
<tr>
<th>Category</th>
<th>n (range)</th>
<th>Menopausal status</th>
<th>Peri</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign breast disease</td>
<td>12 (47–63)</td>
<td>6 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized breast cancer (presurgery)</td>
<td>62 (56–85)</td>
<td>4 14 44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remission</td>
<td>42 (38–85)</td>
<td>3 7 32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic disease</td>
<td>22 (32–82)</td>
<td>0 7 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy female controls</td>
<td>63 (37–72)</td>
<td>0 49 14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Serum and plasma samples were assayed for VEGF with the Remission group, 10 of the metastatic group, 9 of the benign group, and 20 of the control group because of a lack of hematological data for many samples.

Histopathology and Immunohistochemistry. Classification of tumors was according to the International Union against Cancer TNM system (37), with grade determined by the modified Bloom-Richardson criteria according to Elston and Ellis (38). Immunohistochemical staining for CD31, ER, PR, and VEGF was performed using a standard streptavidin-biotin-peroxidase detection system with the following primary antibodies (tissue culture supernatants): for ER, clone 1D5 (DAKO, Cambridge, United Kingdom) diluted 1:50; for PR, clone PgR 636 (DAKO) diluted 1:50; for CD31, clone J C70 (DAKO) diluted 1:30; and for VEGF, clone VEG1 (36, 39) used undiluted. Tissue sections for ER, PR, and VEGF immunolabeling were subject to antigen

where PCV is the packed cell volume, and the units for VEGF measurement were in pg/ml of serum or plasma and the platelet number was × 10³/μl of blood. These data were calculated for only 49 of the local disease group, 23 of the remission group, 10 of the metastatic group, 9 of the benign group, and 20 of the control group because of a lack of hematological data for many samples.

### Table 1: Characteristics of the 201 patients and healthy controls included in the study

<table>
<thead>
<tr>
<th>Category</th>
<th>n (range)</th>
<th>Menopausal status</th>
<th>Peri</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign breast disease</td>
<td>12 (47–63)</td>
<td>6 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized breast cancer (presurgery)</td>
<td>62 (56–85)</td>
<td>4 14 44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remission</td>
<td>42 (38–85)</td>
<td>3 7 32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic disease</td>
<td>22 (32–82)</td>
<td>0 7 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy female controls</td>
<td>63 (37–72)</td>
<td>0 49 14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Tumor characteristics of invasive breast cancer cases (n = 53) in the group of patients examined with locoregional disease prior to surgery

<table>
<thead>
<tr>
<th>Histology</th>
<th>Mean</th>
<th>Range</th>
<th>ND</th>
<th>Consistency</th>
<th>ER status</th>
<th>PR status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal (n = 47)</td>
<td>5.8</td>
<td>2.6–8.6</td>
<td>10</td>
<td>2</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Lobular (n = 2)</td>
<td>5.5</td>
<td>5.0–6.0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tubular (n = 2)</td>
<td>5.3</td>
<td>4.6–6.0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mucinous (n = 1)</td>
<td>8.3</td>
<td>6.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Medullary (n = 1)</td>
<td>11.3</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grade</td>
<td>(n = 9)</td>
<td>5.8</td>
<td>3.3–8.3</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>II (n = 22)</td>
<td>5.7</td>
<td>2.6–8.6</td>
<td>5</td>
<td>0</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>III (n = 19)</td>
<td>6.3</td>
<td>3.0–11.3</td>
<td>1</td>
<td>11</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Unknown (n = 3)</td>
<td>3</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumor size (UICC/TNM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1 (n = 32)</td>
<td>5.5</td>
<td>2.6–8.6</td>
<td>4</td>
<td>4</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>pT2 (n = 17)</td>
<td>6.8</td>
<td>3.6–11.3</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>pT3–4 (n = 2)</td>
<td>7.0</td>
<td>7.0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown (n = 2)</td>
<td>2</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Node status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (n = 32)</td>
<td>6.2</td>
<td>3.3–11.3</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Positive (n = 21)</td>
<td>5.5</td>
<td>2.6–8.6</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (n = 17)</td>
<td>6.4</td>
<td>3.0–11.3</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Positive (n = 33)</td>
<td>5.8</td>
<td>2.6–8.6</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>ND (n = 3)</td>
<td>6.0</td>
<td>6.0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PR status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (n = 29)</td>
<td>6.2</td>
<td>3.0–11.3</td>
<td>5</td>
<td>9</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Positive (n = 21)</td>
<td>5.6</td>
<td>2.6–8.6</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>ND (n = 3)</td>
<td>6.0</td>
<td>6.0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**ND, not done; UICC, International Union against Cancer.**

VEGF (pg)/10⁶ platelets = \[(VEGF_r - VEGF_f) \times (1 - PCV)\] platelet number
retrieval by immersion in boiling citrate buffer (pH 6.0) in a microwave three times for 5 min each, twice for 5 min each, and twice for 5 min each, respectively, before being either left to cool in the buffer for 20 min (ER and PR) or cooled on ice for 20 min (VEGF). For CD31 immunolabeling, antigen retrieval was carried out by immersing sections in boiling citrate buffer (pH 6.0) for 1 min in a domestic pressure cooker before cooling in water. A positive control was included in all runs, and for each case omission of the primary antibody was used as a negative control.

At least 10% of tumor cell nuclei were required to be positive for the tumor to be accepted as ER- or PR-positive, with cytoplasmic staining being disregarded. Staining intensity was variable, and positive staining of adjacent normal parenchymal elements was used as an internal control. VEGF staining was assessed semiquantitatively on a scale from 0 (negative) to 4 (strongly positive). IMD in invasive tumors was assessed after immunostaining for CD31 according to the recent international consensus (40), using a 25-point Chalkley graticule at ×200 magnification (field area, 0.67 mm²). The three most vascular fields, or “hotspots,” were initially identified at low power, and the maximum number of points on the graticule was counted for each area. Areas of sclerosis or dense inflammation were avoided. The mean Chalkley counts for the three areas were used in the statistical analysis. For cases of DCIS, “stromal” vascularity was assessed using a Chalkley graticule, and “periductal” vascularity was assessed by a semiquantitative, subjective grading system (40).

**Statistical Analysis.** All statistical analyses were performed using SPSS for Windows (Version 8.0; SPSS, Chicago). The primary analysis was the comparison of the five patient groups and the circulating VEGF variables. The Kruskal-Wallis test was used to compare the five groups overall, and for two-group comparisons we used the Mann-Whitney test. (Corrections for multiple significance tests were made using the Bonferroni correction.) For subgroup analyses and to investigate the relationships between the circulating VEGF variables and other variables, Spearman’s correlation, $\chi^2$, Kruskal-Wallis, ANOVA, Mann-Whitney, and Student’s $t$ tests were used as appropriate. $P < 0.05$ was considered statistically significant.

**RESULTS**

A limited evaluation of the VEGF immunoassay found VEGF recoveries of 85.1% (SD, 9.7%) for samples from patients with breast cancer ($n = 10$) compared with 86.3% (SD, 13.0%) for samples from healthy controls ($n = 10$) to which 130 pg/ml VEGF had been added. In general, intra-assay CVs were <10%, although they increased to 20% or more at lower concentrations of VEGF. Similarly, the inter-assay CVs were variable depending on the sample VEGF concentration, with values of 28% for a 18.2 pg/ml sample ($n = 15$) and 4.4% for a 186.0 pg/ml sample ($n = 10$).

**Fig. 1.** VEGF$_P$ (a), VEGF$_S$ (b), VEGF$_P$ minus VEGF$_S$ (c), and theoretical platelet VEGF concentration (d) in normal healthy controls (Controls), patients with benign breast disease (Benign), or breast cancer patients with localized breast cancer (Local disease), in remission (Remission), or with metastatic disease (Metastatic). The dashed line indicates the limit of detection of the immunoassay, the dotted line indicates the upper limit of normal. The bars and numbers illustrate the median for each group.
Significantly higher VEGF concentrations were found in the serum samples (VEGFₜ) compared with the corresponding citrated plasma samples (VEGFₚ; \( P < 0.0001 \)). When we used the 95th percentile as the cutoff, the upper limits of normal values for VEGF in the female control group were 62.5 pg/ml plasma for plasma, 590.2 pg/ml for serum, 546.9 pg/ml for the difference between serum and plasma (VEGFₐₜ), and 1.20 pg/10⁶ platelets; there were no significant differences when compared with a male control group, but the numbers were small (\( n = 14 \), data not shown). There were significant differences in age between the groups (\( P < 0.001 \)), but no significant effects of age or menopausal status were found on VEGF parameters. Analysis of the menstrual cycle data for seven healthy controls showed no definite trend for VEGFₜ, although within an individual cycle, variation was apparent with most values falling within 25% of the mean cycle value. No cyclical variation was detected in VEGFₚ because of the low and often undetectable circulating concentrations present.

The circulating VEGF concentrations for each study group are shown in Fig. 1. Significant differences between groups were seen in VEGFₚ (\( P < 0.001 \)) and VEGFₜ (\( P = 0.048 \)), but not in VEGFₐₜ or platelet VEGF concentrations. Further analysis revealed that VEGFₚ concentrations of patients with localized disease were significantly elevated compared with normal controls (\( P = 0.016 \)); patients with metastatic disease had higher levels than normal controls (\( P < 0.001 \)), patients with benign disease (\( P = 0.009 \)), and patients with localized disease (\( P = 0.004 \)). However, the highest concentrations were seen in patients in remission compared with normal controls (\( P < 0.001 \)), patients with benign disease (\( P = 0.01 \)), or patients with localized disease (\( P = 0.005 \)). VEGFₜ concentrations were significantly higher in patients in remission or those with metastatic disease compared with normal controls (\( P = 0.008 \) and 0.044, respectively). VEGFₐₜ was significantly higher only in the remission group compared with the control group (\( P = 0.023 \)).

When the data from all of the groups were considered, VEGFₚ
correlated strongly with VEGF_S ($\rho = 0.682; P < 0.001$), VEGF_diff ($\rho = 0.601; P < 0.001$), and platelet VEGF concentration ($\rho = 0.530; P < 0.001$). Not surprisingly, given the derivation and the relative proportions, VEGF_S correlated very strongly with VEGF_diff ($\rho = 0.992; P < 0.001$), but in addition, it correlated with platelet VEGF concentration ($\rho = 0.923; P < 0.001$). For the hematological variables, a significant but weak correlation was found between platelet count and VEGF_S ($\rho = 0.172; P = 0.05$) and VEGF_diff ($\rho = 0.204; P = 0.021$), and a negative correlation was found between VEGF_p and hemoglobin ($\rho = -0.227; P = 0.01$). When the groups were examined individually, correlations similar to those above were found for the VEGF parameters, but no significant correlations were seen between the VEGF and hematological variables, with the exception of a significant correlation between white cell count and VEGF_p in patients with metastatic disease ($P < 0.047; \rho = 0.489$).

Further analysis of the group of patients in remission revealed a significant effect of tamoxifen therapy on measured VEGF (Fig. 2). Significantly higher VEGF levels were seen in those patients in remission receiving tamoxifen ($P = 0.004$), as were VEGF_S, VEGF_diff, and platelet VEGF concentrations ($P = 0.01, 0.016$, and 0.012, respectively). No significant differences were seen in estradiol levels when subdivided on the basis of tamoxifen therapy. Multivariate analysis failed to reveal any further influencing variables. The median time since diagnosis tended to be greater in patients not receiving tamoxifen (60 months; range, 1–85 months) compared with those receiving tamoxifen (25 months; range, 2–108 months), but the difference did not reach statistical significance. Although the numbers of patients were small (14 non-tamoxifen versus 7 tamoxifen), similar significant effects for VEGF_p ($P = 0.004$) were seen in the group of metastatic patients, but the effects on other measured VEGF parameters failed to reach statistical significance ($P = 0.064, 0.172$, and 0.200, respectively).

The plasma and serum VEGF concentrations of patients in remission or metastatic groups not receiving tamoxifen were not significantly different from the normal female control group or the patients with localized breast cancer.

The histological types, grades, and other clinicopathological variables examined together with the immunolabeling results in the group of patients with localized invasive breast cancer are summarized in Table 2. Grade and immunohistological variables were unavailable for two patients because neoadjuvant therapy made assessment unreliable. The majority of patients had ductal carcinoma of no special type. CD31 immunolabeling gave a highly specific and intense staining of vascular endothelium both within and outside the areas of tumor. Stromal cells were negative, and epithelial cytoplasmic positivity was seen in only one case. Areas of intense inflammation were strongly CD31-positive and were avoided when selecting areas for assessment. VEGF immunohistochemical staining was heterogeneous across different tumors and within tumors, with areas of very intense and very pale staining. There was staining of normal breast tissue, especially of apocrine epithelium, but no stromal staining was seen. Labeling of intratumoral macrophages was also apparent in one case. Two patterns of increased vascularity were observed in the cases of pure DCIS: stromal and periductal. All cases demonstrated the former, whereas the typical “necklace” distribution of the latter was observed in only one case, which was an unusual case of apocrine DCIS exhibiting the most intense VEGF positivity of any sample.

In patients with localized invasive cancer, no significant relationships were found between the circulating VEGF variables and the recognized immunohistochemical or immunopathological prognostic indicators examined, with the exception of a significant correlation between VEGF_diff and ER status ($P = 0.325; P = 0.023$). Interestingly, an association between platelets and grade was found ($P = -0.359; P = 0.017$) and between VEGF immunostaining and white cell count ($P = 0.458; P = 0.006$), with that between VEGF immunostaining and platelet number just failing to reach statistical significance ($P = 0.331; P = 0.052$). The relationships between VEGF immunostaining or MVD and other recognized clinicopathological variables are shown in Table 3.

Of the six DCIS tumors for whom vascularity data were available, five had stromal vascular patterns of MVD staining, with one having both this and the necklaces of periductal vessels discussed above. The three most vascular (Chalkley assessment and semiquantitative) were all ER- and PR-negative, whereas the remaining three were positive. Similarly, the VEGF staining was more pronounced in the three with the highest MVD assessment.

### Table 3: Correlations between clinicopathological variables and VEGF immunostaining or microvessel density (Chalkley count) in patients with invasive breast cancer with locoregional disease prior to surgery

<table>
<thead>
<tr>
<th>Grade</th>
<th>Stage</th>
<th>T&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N</th>
<th>ER</th>
<th>PR</th>
<th>Chalkley count</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.326</td>
<td>0.452</td>
<td>0.733</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P = 0.021</td>
<td>P = 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>-0.664</td>
<td>-0.314</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ER</td>
<td>-0.484</td>
<td>0.339</td>
<td>P = 0.026</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PR</td>
<td>-0.597</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Chalkley count</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P = 0.001</td>
<td>P = 0.026</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a T and N, tumor size and nodal status, respectively, according to the TNM classification system; NS, not significant.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

In breast cancer, IMD is now established as one of the standard prognostic factors for predicting metastasis and relapse-free or overall survival (4, 5). The assessment of angiogenesis is also of potential relevance in identifying those who may benefit from antiangiogenic therapies. IMD is assessed primarily by quantification of MVD, and the techniques are laborious, require experience, and tend to observer variability (40). The measurement of circulating concentrations of specific angiogenic factors such as VEGF may provide less subjective measurements.

Initially interpreted as reflecting an “overspill” of tumor-derived VEGF into the systemic circulation, VEGFₕ is now known to be largely platelet-derived (28–30). A more accurate basal circulating level, which should reflect normal physiological angiogenesis and any disease-related overspill, should therefore be determined with minimal platelet activation, using citrated plasma (28). No study has as yet made these comparisons directly, and we report for the first time that VEGFₚ is a more sensitive measurement than VEGFₕ in terms of being significantly higher in patients with localized disease compared with controls or patients with benign disease, unlike VEGFₕ. However, it is also apparent that this measurement would not be an accurate discriminator between the groups because of considerable overlap. The lack of a significant difference in VEGFₕ between patients with localized breast cancer preoperatively and normal healthy controls confirms previous studies (22, 24, 41). Only two previous studies measured VEGFₚ in cancer, with concentrations being inversely related to survival and response to chemotherapy in gastrointestinal cancer (42) and with significant differences in patients with prostatic cancer depending on disease state, with a sensitivity of 81% and specificity of 71% for metastatic disease (43). Because the CVs of immunoassays at the low VEGF concentrations more often found in plasma samples are relatively high, interpretations of changes or differences should take into account their relative magnitude. Although decreases in VEGFₕ postoperatively (22, 23) have been described, the extent to which these measurements relate to tumor-derived VEGF is unclear. An alternative explanation may be the contribution of other tumor-derived cytokines, such as interleukin-6 (44), which is known to induce VEGF expression (45); has been shown to correlate with platelet number, VEGFₕ, and platelet VEGF content (46); and thus may conceivably play a role in acting systematically to regulate platelet VEGF content. Changes in VEGFₕ during chemotherapy coincide with chemotherapy-induced thrombocytopenia and a subsequent rebound increase in platelet number rather than the sustained decrease in VEGFₚ that would be expected if it was tumor-derived (30). Similarly, the finding of lower VEGFₕ in breast cancer patients with disseminated cancer undergoing therapy compared with those untreated (21, 24) may reflect chemotherapy-induced effects on platelets, effects which may not only be on platelet number but also on platelet volume (47).

Several studies have now shown that VEGFₕ correlates with platelet number (30, 46, 48), although significant interindividual variation exists for estimates of platelet VEGF content (28, 46, 49). The platelet VEGF concentration has been reported to be significantly higher in patients with cancer compared with normals, with medians of 1.6 pg/10⁶ platelets (range, 0.3–2.6 pg/10⁶ platelets) and 0.5 pg/10⁶ platelets (0.1–1.6 pg/10⁶ platelets), respectively (49), but this was not confirmed in the present study, in which we found corresponding medians of 0.47 pg/10⁶ platelets (range, 0.13–1.86 pg/10⁶ platelets) in invasive breast cancer and 0.54 pg/10⁶ platelets (range, 0.08–1.21 pg/10⁶ platelets) in controls. The variability in estimates of platelet-derived VEGF may reflect the use of isolated platelets rather than theoretical derivations from plasma and serum levels, sample sizes, and possible confounding factors such as therapy or the omission of a correction for hematocrit in derived concentrations. For peripheral blood mononuclear cells, median VEGF concentrations of 10.6 pg/10⁶ cells in cancer patients and 0.9 pg/10⁶ cells in normals have been reported (49), values that clearly make their potential contribution to serum VEGF much smaller than that of platelets given the differential in numbers, but with more marked differences between diseased and normal states. The role of platelets in tumor biology is not clear, but cancer patients frequently have higher platelet counts and an increased platelet consumption compared with healthy individuals; in addition, thrombocytosis is a negative prognostic factor in some cancers, and antitumor effects and improvements in survival have been seen with anticoagulation therapy (50).

In the all-groups analysis but not in the subgroups, VEGFₚ was negatively correlated with hemoglobin. A significant negative correlation between VEGFₕ and hemoglobin has been reported previously in patients with a variety of localized cancers (51). Such correlations may reflect the disease state, with low hemoglobin often reflecting more advanced disease; alternatively, they may be related to hypoxia-induced VEGF expression, with the decrease in hemoglobin reflecting the hypoxia.

There is no consensus regarding VEGF concentrations during the menstrual cycle; studies have found variable correlations with hormones and have variably reported higher levels of VEGFₕ in the follicular phase (52), the luteal phase (53), the early proliferative (follicular) and secretory (luteal) phases (54), or no consistent changes (23, 41). Interestingly, the patient in our study with the highest circulating VEGF concentrations was receiving Danazol at the time of sampling. Significantly higher levels have been reported in postmenopausal compared with premenopausal women (41, 52), which we could not confirm, possibly because of sample sizes.

The inference, after subgroup analysis, that tamoxifen produces significant elevations in circulating VEGF in some patients may have prognostic implications. This was also reflected by an increase in the amount of theoretical platelet-derived VEGF, which indicates that tamoxifen is likely to be acting systemically in terms of its actions on VEGF production. To our knowledge, no previous studies have demonstrated this effect. The antitumor activity of tamoxifen is largely attributed to antiestrogenic properties, although it has mixed agonist/antagonist activity depending on the tissue. However, the effects of tamoxifen are not entirely explained by its action on the ER: some receptor-positive patients fail to respond. Other known potentially therapeutic actions include regulation of circulating concentrations of the breast epithelial mitogen insulin-like growth factor-I and insulin-like growth factor-binding protein 1 (55, 56). Hormonal regulation of VEGF has been demonstrated, with progesterone and progestins such as megestrol acetate stimulating VEGF production by some breast cancer cell lines in vitro (57) and estradiol being shown to stimulate VEGF production in several systems, including endometrial cultures and the uterus in vivo (58–61). Tamoxifen itself has been shown to induce VEGF expression in the breast cancer cell line MCF7 by a mechanism that is not thought to be mediated by the ER (62).

The induction of VEGF by tamoxifen is paradoxical but may be implicated in tamoxifen-associated endometrial hyperplasia, with the endometrium being a possible source of some of the increase in VEGF seen in this study. Tamoxifen has been shown to increase VEGF expression in uterine tissue (63) and, more recently, to induce production of the novel angiogenic factor adrenomedullin by endometrial macrophages via a nonestrogenic mechanism (64). In addition, tamoxifen exerts a direct antiproliferative activity on VEGF-stimulated endothelial cell growth, an effect not mediated by the ER (65). Angiogenesis has been reported to add information to ER status in predicting response to tamoxifen, with the patients having the best...
prognosis being characterized by low vascularity, ER positivity, few nodes involved, and small low-grade tumors (6). The determination of the magnitude of the VEGF increase while on tamoxifen may add further prognostic information both with regard to breast cancer and the incidence of endometrial hyperplasia (66).

In the present study, 80% of tumors were found to be VEGF-positive, which is in agreement with previous reports. However no significant correlation between VEGF staining intensity and Chalkley count was seen, unlike in earlier reports (10, 14–16), although one study found this relationship to be true only for invasive ductal carcinomas or pure DCIS, with VEGF being lower and negatively correlated with angiogenesis in invasive lobular carcinoma (10). Our findings may reflect the relatively small sample size, the relatively uniform patient populations examined in many earlier studies, antibody specificity, the predominant use of tissue homogenates for assessment of intratumoral VEGF in previous studies, or variability in the techniques used to assess immunohistochemical staining or angiogenesis. This may also reflect differing relative dependencies on other specific angiogenic factors such as thymidine phosphorylase. IMD has also been reported to correlate with menstrual status, age, and tumor grade (67), although this is unconfirmed (6). In the present study, a significant association with tumor size was found that may be expected given the dependence of tumor expansion on angiogenesis, confirming previous reports (67, 68). The association of intratumoral VEGF with other clinical prognostic factors is unresolved, with no correlation being found with age, tumor type or size, or hormone receptor status in several studies (14, 15, 18); however, correlations with tumor size (17, 19) and grade (19) and an inverse correlation with ER in node-negative breast cancer patients have also been reported (19). In contrast, we found intratumoral VEGF to be correlated with ER status, which may reflect an estrogenic regulation of VEGF in some patients. Similarly, VEGF	extsubscript{act} was correlated with ER status. Unexpectedly, VEGF was inversely correlated with tumor grade. The prognostic use of intratumoral VEGF assessment is still not clear (15, 16), but in node-negative breast cancer, several studies have found VEGF to be a strong independent predictor of relapse-free (17, 18) and overall (18, 19) survival. This is being examined prospectively in our study cohort at present. No association between circulating levels of VEGF and intratumoral VEGF assessed immunohistochemically was seen, confirming previous results (69), although an additional study has reported that the serum-positive rate is associated with MVD and intratumoral VEGF expression when determined by immunohoussay (23).

This study illustrates that VEGF	extsubscript{p} shows better discrimination than VEGF	extsubscript{s} in patients with breast cancer and highlights the tamoxifen-associated increase in VEGF concentrations occurring in some patients. The significance of this in relation to disease course or subsequent endometrial pathology, together with the relevance of platelet-tumor cell interactions in tumor angiogenesis and progression, warrants further prospective studies.

ACKNOWLEDGMENTS

We thank Lynne Hill, Christine Gascoigne, Julie Booth, Sue Buckle, and Amanda Brazier for assistance.

REFERENCES


Vascular Endothelial Growth Factor (VEGF) in Breast Cancer: Comparison of Plasma, Serum, and Tissue VEGF and Microvessel Density and Effects of Tamoxifen

Jacqueline Adams, Pauline J. Carder, Sarah Downey, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/60/11/2898

Cited articles  This article cites 63 articles, 18 of which you can access for free at: http://cancerres.aacrjournals.org/content/60/11/2898.full#ref-list-1

Citing articles  This article has been cited by 28 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/60/11/2898.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.