Expression of the Angiogenic Factors Vascular Endothelial Cell Growth Factor, Acidic and Basic Fibroblast Growth Factor, Tumor Growth Factor β-1, Platelet-derived Endothelial Cell Growth Factor, Placenta Growth Factor, and Pleiotrophin in Human Primary Breast Cancer and Its Relation to Angiogenesis

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ABSTRACT

Angiogenesis is a significant prognostic factor in breast cancer, but the factors that control angiogenesis in vivo are not well defined. Multiple angiogenic polypeptides are known, and we have determined the expression of seven of these in primary human breast cancers; the relationship of expression to estrogen receptor and vascular density was also examined. Vascular endothelial growth factor (VEGF) and its four isoforms (121, 165, 189, and 206 amino acids), transforming growth factor (TGF)-β1, pleiotrophin, acidic and basic fibroblast growth factor (FGF), placental growth factor, and thymidine phosphorylase (platelet-derived endothelial cell growth factor) were quantitated by RNase protection analysis. β-FGF was also measured by ELISA. The estrogen receptor (ER), epidermal growth factor receptor, and vascular density were analyzed in 64 primary breast cancers. All tumors expressed at least six different vascular growth factors. VEGF was most abundant, and the transcript for the 121-amino acid form predominated. Other angiogenic factors expressed at high levels were thymidine phosphorylase and TGF-β1. Expression of most of the angiogenic factors did not correlate with that of ER or vascular density. However, thymidine phosphorylase did, with a correlation coefficient of 0.3 (P = 0.03). There were significant associations of pleiotrophin with acidic FGF expression (P = 0.001) and TGF-β with platelet-derived endothelial cell growth factor expression (P = 0.001). Thus, angiogenesis may involve a coordinate regulation of some vascular growth factors. High VEGF expression correlated with poor prognosis in univariate analysis (P = 0.03), as did ER and epidermal growth factor receptor expression. Basic FGF was also assessed by ELISA and was more highly expressed in tumors than normal breast tissues (median, 346 μg/ml cytosol; range, 54–1323 versus median, 149; range, 32–509; P = 0.01). Implications for therapy are that broad spectrum agents that block features common to these factors may be useful (e.g., antagonism of heparin-binding activity agents), because so many angiogenic factors are expressed. Inhibiting endothelial migration or agents directly toxic to endothelium would be of value in a combined approach to therapy.

INTRODUCTION

Angiogenesis is an essential step in tumor growth and metastasis (1). Angiogenesis in tumors, however, is quite different from that seen in normal tissues, with leaky vessels, aberrant blood flow, and areas of necrosis, as well as increased vascularity (2). Recent studies in breast cancer and a range of other tumor types have shown that quantification of angiogenesis can be used as an independent prognostic factor (3–5). Angiogenesis has been quantified after staining breast cancer and a range of other tumor types have shown that seven of these in primary human breast cancers; the relationship

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2 To whom requests for reprints should be addressed.

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incubated in triplicate overnight at 4°C on microtiter plates coated with a murine monoclonal antibody against human bFGF. Unbound proteins were washed off, and an enzyme-linked polyclonal antibody specific for bFGF was added to "sandwich" the bFGF immobilized during the first incubation. A substrate solution for horseradish peroxidase was added, and the color developed in proportion to the amount of antibody-bound bFGF. The absorbance of the color was read at 450 nm.

A standard curve, consisting of known amounts of bFGF, was carried through the procedure, and the concentrations of bFGF in the unknown samples were determined from this standard curve. Concentrations of bFGF were expressed as picograms per milligram cytosol protein.

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections. Sections were predigested with 1.25 mg of protease type XXIV (Sigma Chemical Co., Poole, United Kingdom) per 100 ml PBS for 20 min at 37°C before application of the primary anti-CD31 antibody.

**Assessment of Microvessel Density**

VCs were determined without knowledge of patient outcome. The three most vascular areas where the highest number of discreet microvessels stained were chosen by two observers over a conference microscope. A microvessel was defined as any immunoreactive endothelial cell(s) that was separate from adjacent microvessels. Vessels within the sclerotic body of the tumor were not included. These maximal areas of neovascularization were identified by scanning at low power (×40 and ×100). VCs were then estimated by both observers using a 25-point Chalkley eyepiece graticule at ×250. (The graticule covered an area of 0.159 mm² at this magnification.) The graticule was rotated in the eyepiece to where the maximum number of graticule dots overlaid immunohistochemically identified vessels or their lumens. We have shown previously that this method of vascular assessment correlates strongly with field counts in invasive breast carcinomas (n = 31; r = 0.79; paired t test; P = 0.00005; Ref. 17). VCs for individual tumors were then produced using the mean of the three graticule counts.

**Isolation of RNA**

Total RNA was prepared by the method of Chomczynski and Sacchi (18) or by the guanidinium isothiocyanate lysis and cesium chloride gradient method (19). For the RNase protection assays, radiolabeled riboprobes were synthesized with [α-32P]CTP (Amersham) from linearized plasmid DNA using the in vitro transcription method (19).

**Construction of Plasmids to Generate Probes for RNase Protection Analysis**

aFGF and bFGF. A 293-bp HindIII/PstI fragment of the human aFGF coding region from plasmid pc3-5 was cloned into the HindIII/PstI site of pBluescript SK⁺. After linearization with BamHII, a 293-bp antisense probe was generated with T7 RNA polymerase. For bFGF, a 214-bp EcoRI/BamHII fragment of human bFGF cDNA in pHL1-7 was cloned into the EcoRI/BamHII site of pBluescript SK⁺. The resulting plasmid was linearized with EcoRV, and an antisense probe was generated with T3 RNA polymerase. Plasmids pc3-5 and pHL1-7 were gifts from Drs. Judith Abraham and John Fiddes (California Biotechnology, Mountain View, CA).

PDECGF (Thymidine Phosphorylase). Plasmid pc5 incorporating the full-length cDNA of PDECGF was digested with Ncol. The 5’ overhangs were end filled using DNA polymerase 1 (Klenow fragment), and the plasmid was then digested with BamHII. The 241-bp fragment generated corresponding to 817–1058 bp of the coding region of PDECGF was cloned into the EcoRI/BamHII sites of pBluescript SK⁺. The resultant construct was linearized with HindIII before generation of antisense transcripts with T3 RNA polymerase.

VEGF. Two probes were used; the first was designed to protect the full length of the smallest isoform (VEGF121, yielding a 444-base band, with a lower band of 345 representing the remaining isofoms). This 520-base probe was generated by linearizing the full-length cDNA for VEGF121, cloned into pBluescript SK⁺ with EcoRV and transcribing with T7 RNA polymerase. To determine the isoforms contributing to this second smaller band, a new construct was designed to protect as its largest fragment, VEGF165, with the remaining isoforms forming two bands lower on the gel. The cDNA coding for the mature protein was cloned into pBluescript KS⁺ in the XbaI and EcoRI sites, and the probe was generated using NotI and T3 polymerase. The largest protected fragment was 567 bases, with the intermediate fragment being 345 from both the VEGF121 and VEGF165 isoforms, and a third band of 150 bases representing VEGF165 alone.

Pleiotrophin. A 240-bp DNA fragment corresponding to bases 558–798 of the coding region of pleiotrophin was amplified from a partial cDNA clone (gift from Dr. Peter Milner, Jewish Hospital, St. Louis, MO) by the PCR. Restriction sites were incorporated into the PCR primers, and the fragment obtained was cloned into the EcoRI/XbaI sites of pBluescript KS⁺. The construct was linearized with XbaI before generation of antisense transcripts with T3 RNA polymerase.

**RESULTS**

**Patient Characteristics.** There were 64 patients in the study. The clinicopathological features of their tumors are shown in Table 1.

**Nuclease Protection Assays for Angiogenic Factors.** For each angiogenic factor, there was approximately a 2-log range of expression. Results were standardized from one gel to another for any one angiogenic factor with a control RNA common to each. Results for VEGF and TGF-β are shown in Fig. 1 for the same cases. Because of the sensitivity of RNase protection assays, in nearly every case there was a signal detectable, and for all factors there was a log-normal distribution (Table 1). In every tumor, at least six of the seven factors analyzed were detectable.

**Analysis of bFGF by ELISA.** bFGF protein was detected in every tumor using this assay. Levels were significantly higher (median, 346; range, 54–1323 pg/mg cytosol) than in normal tissue controls from reduction mammaplasties and also higher than nonmalignant tissue from mastectomy specimen sections (median, 149; range, 32–509 pg/mg cytosol; P < 0.01). Sections from these were analyzed to exclude direct tumor involvement. bFGF ELISA results were compared with the nuclease protection results, and there was a highly significant correlation (Fig. 2: pairwise correlation test; coefficient 0.58; P < 0.001).

**VEGF Isoforms.** Three major products were detected by RNase protection assay, in order of decreasing abundance: 121<165<189. The predominant form was the 121-amino acid variant, which comprised
Median (range) values are RNAS nuclelease protection assays standardized by glyceraldehyde-3-phosphate dehydrogenase.

<table>
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<th>Histology</th>
<th>n</th>
<th>VEGF</th>
<th>TGF-β</th>
<th>aFGF</th>
<th>bFGF</th>
<th>PDECGF</th>
<th>PTN</th>
<th>PLGF</th>
<th>bFGF ELISA (pg/mg)</th>
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<td>16 (2, 436)</td>
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<td>36</td>
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<td>21 (1, 305)</td>
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<td>≥6</td>
<td>41</td>
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<td>Overall</td>
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<td>4 (1, 22)</td>
<td>5 (0, 48)</td>
<td>32 (3, 231)</td>
<td>14.5 (0, 436)</td>
<td>4.5 (1, 37)</td>
<td>347 (55, 1324)</td>
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</table>

50–90% of total VEGF mRNA detected (Fig. 1). The second lower band represented a hybridization product common to all of the remaining isoforms (i.e., VEGFs 165, 189, and 206). By using a riboprobe to the full-length 189 isoform, it was possible to determine which of the remaining isoforms was the most abundant (i.e., VEGF 165).

Coexpression Patterns of Vascular Growth Factors. One aim of the study was to examine coexpression of angiogenic factors. It was found that there was a highly significant correlation of pleiotrophin expression with aFGF (Fig. 3a) and also of TGF-β with PDECGF (Fig. 3b; pairwise correlation; P = 0.014 and 0.004, respectively). This was also analyzed by Spearman rank correlation coefficient, which showed a correlation of 0.73 (P < 0.001), and correlation coefficient 0.49 (P < 0.001), respectively.

Correlation of Nuclease Protection Assays with ER and Other Pathological Variables. Since many growth factors are estrogen regulated in breast cancer cell lines (e.g., TGF-β1 and TGF-α), we
EXPRESSIOI\N OF ANGIOGENIC FACTORS

The predominant expression of the mRNA for the 121-amino acid form in breast cancer contrasts with gliomas (that predominantly express the 165 amino acid form; Ref. 25) and suggests that the diffusible VEGF121 may play a more significant role in breast cancer angiogenesis.

Although this was not the major purpose of the study, patients with mRNA higher than the median for VEGF had a poorer survival. VEGF was the only angiogenic growth factor associated with poor relapse-free survival. Larger numbers will be needed to confirm this. The potential role of VEGF protein, as well as RNA assay, in prognosis and ultimately selection for antiangiogenic therapy should be assessed further. Toi et al. (26) has shown that assessment of VEGF by immunohistochemistry is associated with poor prognosis.

bFGF has been shown to synergize markedly with VEGF in vitro capillary growth (27), and in every case we studied, bFGF protein was also detectable. Some groups have reported that bFGF is expressed in

looked for correlation of ER and EGFr with expression of angiogenic factors. There was no correlation of ER or EGFr with any of the angiogenic factors. Lymph node metastasis and tumor size did not correlate either. Representative data are shown for mRNA of bFGF mRNA and VEGF (Fig. 2).

TGF-β levels were higher in tumors smaller than 2 cm (median, 7; range, 3–22) compared with larger tumors (median, 5; range, 1–28; P = 0.05; Mann-Whitney test).

Vessel Counts and Angiogenic Factors. Use of the Chalkley counting method showed a 3-fold range in counts (3–9 per 250× field). We showed previously that using the median of 6 provided a split of prognostic importance in a larger series (22). However, using a cut-point of <6 and ≥6 showed no correlation of vessel counts with angiogenic factor expression (Fig. 4a). Similarly, using a level that defined the upper one-third with highest vessel counts (≥7) showed no correlation. Using vessel counts as a continuous variable showed that PDECGF correlated with vessel counts (pairwise comparison; correlation coefficient 0.3; P = 0.03). TGF-β1 also showed a significant association with a correlation coefficient of 0.35 (P = 0.018).

Prognosis and VEGF Expression. Relapse-free survival was analyzed using the median angiogenic factor value in univariate analysis. VEGF values above the median were associated with high relapse rate (P = 0.03; Fig. 5). ER expression >10 fmol/mg cytosol was associated with better prognosis (P = 0.0008), and high EGFr was associated with poor prognosis (P = 0.01).

DISCUSSION

This study shows that primary breast cancers express multiple angiogenic factors. The factors belong to several different growth factor families, some specific for endothelium (e.g., VEGF), others having pleiotrophic effects (e.g., TGF-β1).

The RNase protection technique can only show which factors are expressed, rather than the location and cell types producing them. However, because it is a profile of the total mass of tumor, it does give an assessment that is not so subject to sampling bias.

Factors most highly expressed at the RNA level were VEGF and PDECGF. VEGF causes increased vascular permeability (23), as well as angiogenesis, and may be one of the most important mediators of tumor angiogenesis. VEGF splice variants have different properties (e.g., the 165- and 189-amino acid forms bind to the cell surface and heparins; Ref. 24). The predominant expression of the mRNA for the 121-amino acid form in breast cancer contrasts with gliomas (that predominantly express the 165 amino acid form; Ref. 25) and suggests that the diffusible VEGF121 may play a more significant role in breast cancer angiogenesis.

Fig. 3. Coexpression of angiogenic factors. a, aFGF compared with pleiotrophin RNA expression. b, TGF-β1 expression compared with PDECGF.
Luqmani et al. (32) showed lower bFGF mRNA expression in breast tumors than in normal breast tissues. We compared expression as measured by protein assays and found higher expression. There was a good correlation in our assays between mRNA and protein, suggesting that regulation of fibroblast growth factor at a transcriptional level may be one mechanism of control. However, protease production is markedly increased in breast tumors (e.g., urokinase; Ref. 31), and this may increase the extractability of bFGF from stroma. Thus, our results suggest that bFGF is one of the most important growth factors in primary breast cancer, based on differential expression of protein.

Pleiotrophin has been analyzed previously in primary breast cancer by Northern blotting and nuclease protection (11). Our results confirm the initial findings in a larger series. In the former study, tumors were classified as positive or negative, but the present study showed expression in all cases. Expression was log-normally distributed, and if the very low expressers with <5% of the highest expression level are considered negative, the results are comparable.

Placenta growth factor is another member of the VEGF family and was recently shown to be angiogenic (33). Levels in breast tumors are several logs lower than expression in placenta, but most tumors did express placenta growth factor. TGF-β1 was ubiquitously expressed and could have an indirect role in tumor angiogenesis, via attraction of inflammatory cells (34, 35). However, transfection experiments have shown in vivo stimulation of angiogenesis in xenografts (36). aFGF was expressed and has recently been shown to be a growth factor for capillary blood vessels, but not large-vessel endothelium, in three-dimensional cultures (9).

Multiple factors were expressed, and there was evidence of a coordinated expression of TGF-β1 with PDECGF, and aFGF with pleiotrophin. A switch to an angiogenic phenotype has been postulated (37). This study would suggest that the switch does not coordinately regulate multiple factors. One factor may be rate limiting and synergize with others (e.g., VEGF). Alternatively, release of bFGF from the stroma could be rate limiting. As a result of increased angiogenesis, other cell types might have enhanced access to the tumor (macrophages) with independently regulated growth factors. Toi et al. (26) recently showed an association of VEGF with PDECGF expression using an immunohistochemistry approach, which allows sampling of focal areas.

**TGF-β1 Expression Correlation with PDECGF Expression.**
PDECGF is known to be induced by several cytokines, including tumor necrosis factor-α, interleukin 1, and IFN-γ. It is possible that there is also an effect of TGF-β1 on PDECGF expression or induction...
of an inflammatory cell infiltrate producing these factors. Additionally, we have recently shown a significant association of PDECGF expression with small tumor size in a study of 240 patients by immunohistochemistry (22). The association of TGF-β1 with small tumors is of borderline significance at (P = 0.07), and there may be coexpression because of the association with tumor size.

Angiogenesis counts are also independent of ER status, as reported previously by us and others (3, 4); therefore, angiogenesis appears to be regulated by nonendocrine pathways. This may be why it is an independent prognostic factor in several studies.

Nevertheless, because in the normal premenopausal woman there is cyclical development of angiogenesis in the endometrium and ovary, it was possible that there would be endocrine regulation of angiogenesis in the breast. There was no correlation with ER, yet other estrogen-regulated genes, such as PS2, and the progesterone receptor can be shown to be associated with ER expression in tumor hormone genotypes. It is possible that a more detailed study of premenopausal breast cancer patients at different phases of the menstrual cycle would detect this.

Vessel counts correlated with relapse and survival, but the only angiogenic factors correlating with vessel counts were PDECGF and TGF-β1. This may reflect the different techniques used. Vascular microscopy, whereas the measurement of RNA or protein is an assessment in the breast. There was no correlation with ER, yet other breast cancer patients at different phases of the menstrual cycle would express with small tumor size in a study of 240 patients by

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REFERENCES


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