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[1]

Michele Relf, Susan LeJeune, Prudence A. E. Scott, Stephen Fox, Kenneth Smith, Russell Leek, Amir Moghaddam, Ruth Whitehouse, Roy Bicknell, and Adrian L. Harris[2]

Molecular Angiogenesis Group, Molecular Oncology Laboratories, Imperial Cancer Research Fund, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, England

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 endothelial cells with antibodies to factor VIII-associated antigen (4) or to the cell adhesion molecule PECAM (platelet endothelial cell

adhesion molecule, CD31; Ref. 3). The higher the vascular density of the tumor, the worse the prognosis.

We have recently shown that the endothelium in breast cancers has a mitotic index 50-fold greater than that in nonmalignant tissues, and this proliferation is mainly at the periphery of the tumor (6). However, there was no relationship of the labeling index of the endothelium to that of the tumor. This suggests that different growth factors may be regulating the tumor growth, compared with the endothelial growth.

Many angiogenic factors have been described in the last seven years (reviewed in Ref. 7). However, which factors are expressed in human breast cancer, the regulation of their expression, and their relationship to estrogen regulation of growth or to quantitative vascular density have not been described.

VEGF[3] exists as several splice variants, yielding proteins of 121, 165, 189, and 206 amino acids, respectively (8). The transcript corresponding to the 206-amino acid form has only been detected in a fetal liver cell cDNA library (8). aFGF and bFGF have been well characterized as angiogenic factors (9). Pleiotrophin has been reported to be angiogenic (10) and is highly expressed in a subset (60%) of breast cancers (11). Placental growth factor is another member of the VEGF family. TGF-β1 inhibits endothelial cell growth *in vitro* but stimulates angiogenesis *in vivo*, probably through induction of an inflammatory angiogenic infiltrate (12). PDECGF is thymidine phosphorylase and was initially purified as the major angiogenic activity in platelets (13, 14). We have recently demonstrated that it is strongly angiogenic *in vivo* (15), possibly through modulation of nucleotide metabolism.

Therefore, we quantitated expression of the above factors, which represent a variety of mechanisms of angiogenesis, by RNase protection assays and compared the expression with vascular density, ER expression, and other pathological variables as a basis for rational future therapeutic antiangiogenic approaches to breast cancer treatment.

MATERIALS AND METHODS

Extraction of Tumor Membranes and Cytosols

Tumor membranes and cytosols were prepared as described previously (16).

bFGF Immunoassay

Concentrations of bFGF in human breast cancer cytosolic extracts were quantified using a "Quantikine" human bFGF immunoassay (R&D Systems, Inc., Minneapolis, MN). Cytosols, prepared as described previously, were stored at −80°C before measurement of bFGF levels. Diluted cytosols were

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 above 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 12.5 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 ($\times 40$ and $\times 100$). VCs were then estimated by both observers using a 25-point Chalkley eyepiece graticule at $\times 250$. (The graticule covered an area of 0.159 mm^2 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-^{32}P]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 *Hind*III/*Pst*I fragment of the human aFGF coding region from plasmid pJC3–5 was cloned into the *Hind*III/*Pst*I site of pBluescript SK$^+$. After linearization with *Bam*HI, a 293-bp antisense probe was generated with T7 RNA polymerase. For bFGF, a 214-bp *Eco*RI/*Bam*HI fragment of human bFGF cDNA in pHFL1–7 was cloned into the *Eco*RI/*Bam*HI site of pBluescript SK$^+$. The resulting plasmid was linearized with *Eco*RV, and an antisense probe was generated with T3 RNA polymerase. Plasmids pJC3–5 and pHFL1–7 were gifts from Drs. Judith Abraham and John Fiddes (California Biotechnology, Mountain View, CA).

PDECGF (Thymidine Phosphorylase). Plasmid pPL5 incorporating the full-length cDNA of PDECGF was digested with *Nco*I. The 5' overhangs were end filled using DNA polymerase I (Klenow fragment), and the plasmid was then digested with *Bam*HI. The 241-bp fragment generated corresponding to 817–1058 bp of the coding region of PDECGF was cloned into the *Eco*RV/*Bam*HI sites of pBluescript SK$^+$. The resultant construct was linearized with *Hind*III 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 (VEGF$_{121}$ yielding a 444-base band, with a lower band of 345 representing the remaining isoforms). This 520-base probe was generated by linearizing the full-length cDNA for VEGF$_{121}$ cloned into pBluescript SK$^-$ with *Eco*RV 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, VEGF$_{189}$, 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 *Xba*I and *Eco*RI sites, and the probe was generated using *Not*I and T3 polymerase. The largest protected fragment was 567 bases, with the intermediate fragment being 345 from both the VEGF$_{121}$ and VEGF$_{165}$ isoforms, and a third band of 150 bases representing VEGF$_{165}$ 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 *Eco*RI/*Xba*I sites of pBluescript KS$^+$. The construct was linearized with *Xba*I before generation of antisense transcripts with T3 RNA polymerase.

TGF-β1. Constructs were as described previously (20).

Placenta Growth Factor. A 220-bp fragment of placenta growth factor PCR amplified from the plasmid pUC18-p23 (a gift from Dr. G. Persico, Naples University, Naples, Italy) was cloned into pBluescript KS$^+$. The plasmid was linearized with *Bam*HI, and a 270-bp fragment was generated with T3 RNA polymerase.

RNase Protection Analysis

Antisense probes were hybridized to 10 μg of total cellular RNA, and free unhybridized probe and RNA were removed by digestion with RNase T1 and RNase A. Protected fragments were analyzed by electrophoresis in 6% polyacrylamide/urea sequencing gels followed by autoradiography. In each hybridization reaction, an antisense transcript (20) corresponding to human glyceraldehyde-3-phosphate dehydrogenase was included as an internal control, and tRNA was included as a negative control. mRNA abundance was quantitated by scanning laser densitometry (Bioimage densitometer, Millipore, Bedford, MA), and signals were normalized to the glyceraldehyde-3-phosphate dehydrogenase signal. Placental mRNA was used as a positive control for comparative purposes.

EGFr and ER Assays

ER content was determined using an ELISA technique (Abbott Laboratories, Chicago, IL). Tumors were considered positive when cytoplasmic ER levels exceeded 10 fmol/mg cytosolic protein. EGFr was measured using ligand binding of ^{125}I-labeled epidermal growth factor to tumor membranes (21). Concentrations >20 fmol/mg membrane protein were considered positive as reported previously (3).

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 mammoplasties 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

Table 1 *Expression of angiogenic growth factors by patient and tumor variables*

Median (range) values are RNase nuclease protection assays standardized by glyceraldehyde-3-phosphate dehydrogenase.

	n	VEGF	TGF-β	aFGF	bFGF	PDECGF	PTN	PLGF	bFGF ELISA (pg/mg)
Histology									
Other	13	9 (1, 152)	6 (1, 28)	3.5 (1, 10)	8 (2, 30)	20 (9, 119)	9 (5, 89)	7 (9, 119)	372 (168, 895)
Ductal	51	6 (0, 41)	6 (1, 23)	4 (1, 22)	4 (0, 48)	33 (3, 231)	18 (0, 436)	4.5 (3, 231)	340 (55, 1324)
Age (years)									
≤50	21	4 (1, 33)	5.5 (1, 23)	5.5 (1, 22)	3 (0, 30)	23 (3, 158)	9.5 (1, 436)	3 (1, 37)	306 (55, 951)
>50	43	8 (0, 152)	6 (1, 28)	4 (1, 16)	6.5 (0, 48)	33.5 (5, 231)	18 (0, 305)	5 (1, 188)	385 (64, 1324)
Grade									
I/II	42	6 (0, 152)	6 (1, 28)	4 (1, 16)	5 (0, 46)	32 (5, 231)	18 (1, 436)	5 (1, 37)	382 (55, 1324)
III	22	8 (1, 41)	4.5 (1, 14)	2 (1, 22)	3.5 (0, 48)	32 (3, 158)	7 (0, 305)	4 (1, 188)	259 (156, 779)
Size (cm)									
≤2	22	8 (0, 152)	7 (3, 22)	6 (1, 16)	6 (0, 46)	40 (7, 231)	21 (3, 436)	4.5 (2, 18)	316 (130, 1324)
>2	42	6 (1, 41)	5 (1, 28)	2.5 (1, 22)	3 (0, 48)	20 (3, 184)	12 (0, 364)	4.5 (1, 188)	369 (55, 1230)
Nodes									
Neg	35	6 (0, 41)	6 (1, 21)	4 (1, 16)	7 (0, 48)	32 (3, 231)	14 (0, 364)	4 (1, 36)	430 (147, 1324)
Pos	29	8 (1, 38)	5.5 (1, 28)	3.5 (1, 22)	4 (0, 26)	30 (5, 119)	16 (2, 436)	5.5 (1, 188)	259 (55, 895)
ER (fmol/mg)									
<10	19	5 (1, 41)	5 (1, 14)	2 (1, 12)	3 (0, 46)	20 (3, 184)	10 (0, 436)	4 (1, 188)	253 (130, 1324)
≥10	45	7 (0, 152)	6 (1, 28)	4 (1, 22)	5.5 (0, 48)	32 (5, 231)	18 (1, 364)	5 (1, 37)	353 (55, 1230)
EGFr (fmol/mg)									
<20	28	6 (0, 38)	6 (1, 28)	4.5 (1, 15)	4.5 (0, 48)	26 (3, 231)	13 (1, 305)	5 (1, 37)	328 (55, 895)
≥20	36	8 (1, 152)	5.5 (1, 20)	3 (1, 22)	5 (0, 46)	32.5 (3, 158)	21.5 (0, 436)	4 (1, 188)	419 (130, 1324)
Vessel count (Chalkley)									
<6	23	8 (1, 19)	6 (1, 14)	4 (1, 10)	7 (0, 48)	22 (5, 75)	21 (1, 305)	4.5 (1, 37)	347 (64, 1324)
≥6	41	6 (0, 152)	6 (1, 28)	4 (1, 22)	4 (0, 26)	33 (3, 231)	12 (0, 436)	4.5 (1, 188)	350 (55, 1230)
Overall	64	6.5 (0, 152)	6 (1, 28)	4 (1, 22)	5 (0, 48)	32 (3, 231)	14.5 (0, 436)	4.5 (1, 188)	347 (55, 1324)

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
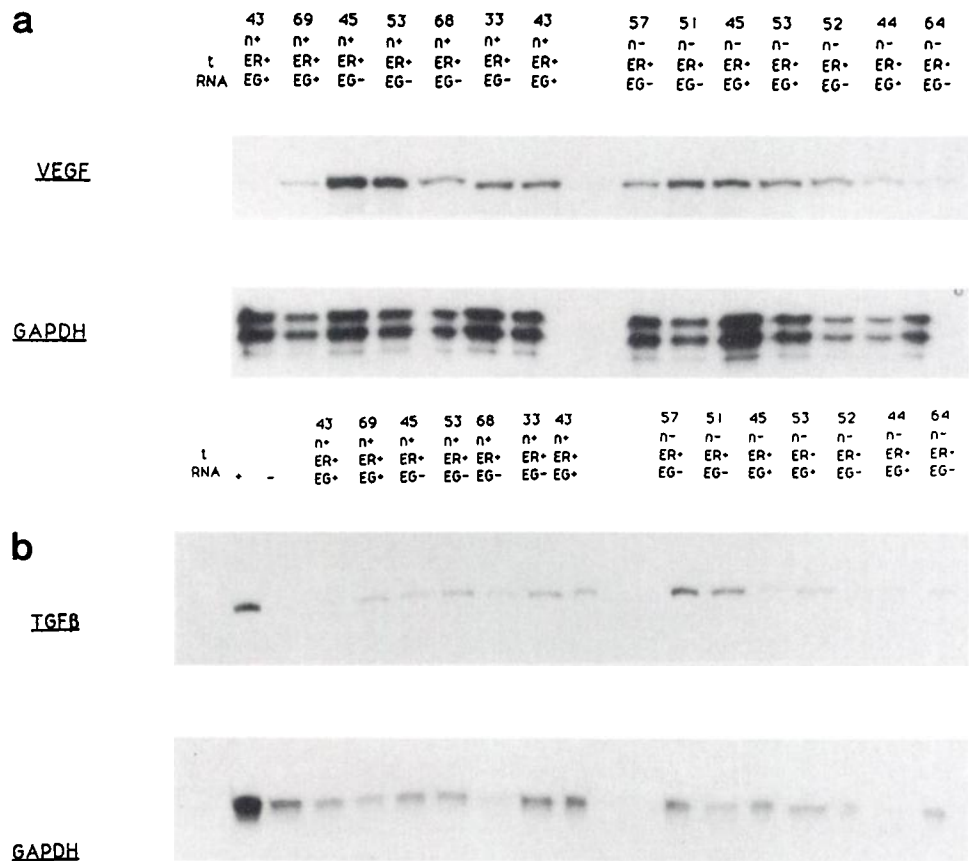

Fig. 1. Nuclease protection assays for vascular growth factors. *a*, nuclease protection assay for VEGF. Samples are segregated by ER, EGFr, and node status. *b*, nuclease protection assays for TGF-β.
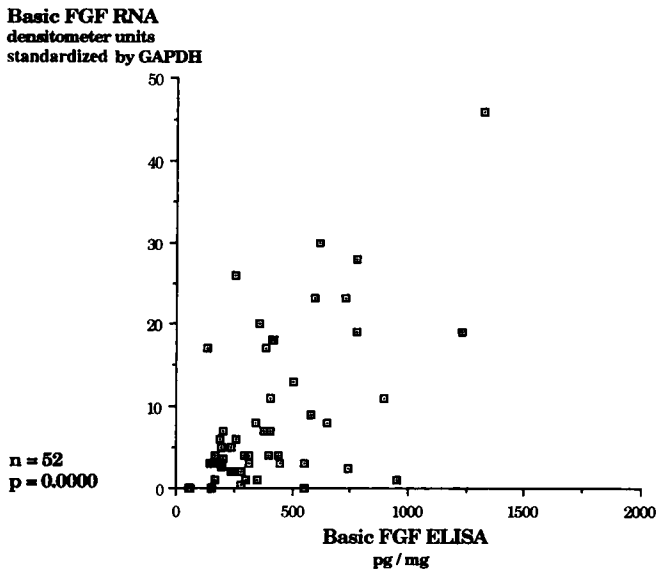
Basic FGF RNA
densitometer units
standardized by GAPDH

Fig. 2. bFGF ELISA compared with bFGF RNA expression.

(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 $VEGF_{121}$ 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
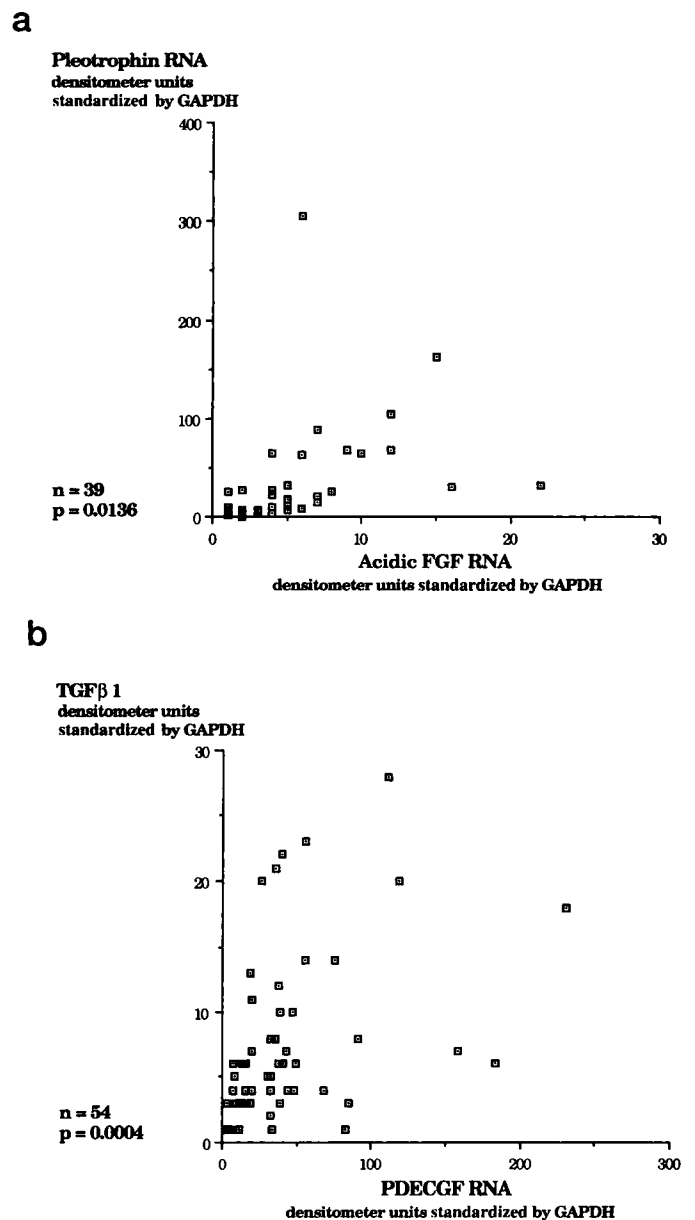
looked for correlation of ER and EGFr with expression of angiogenic factors. There was no correlation of ER or EGFr with any of the angiogenic factors. Lymph node metastasis and tumor size did not correlate either. Representative data are shown for mRNA of bFGF mRNA and VEGF (Fig. 4a).

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. 4b). 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

a

Pleotrophin RNA
densitometer units
standardized by GAPDH

b

TGFβ 1
densitometer units
standardized by GAPDH

Fig. 3. Coexpression of angiogenic factors. a, aFGF compared with pleiotrophin RNA expression. b, TGF-β1 expression compared with PDECGF.

a

bFGF RNA
VEGF RNA

b

bFGF RNA
VEGF RNA
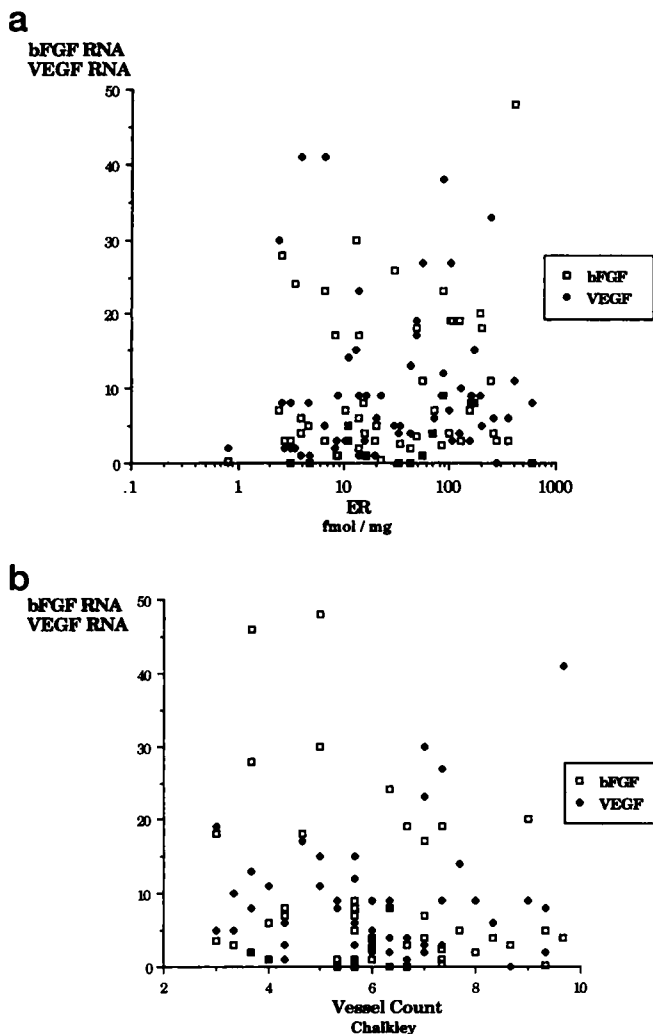

Fig. 4. bFGF and VEGF expression in breast cancers. a, bFGF and VEGF expression compared with ER expression. b, bFGF and VEGF expression compared with vessel counts (Chalkley counts).

residual normal tissue rather than in the tumors directly (28, 29). However, this does appear to depend both on antibodies used and the technique. Furthermore, stromal proteases may still release bFGF and stimulate capillary growth at the interface of normal and invading tissue (30, 31).

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

Relapse Free Survival by VEGF status

Fig. 5. VEGF mRNA expression and relapse-free survival.

967
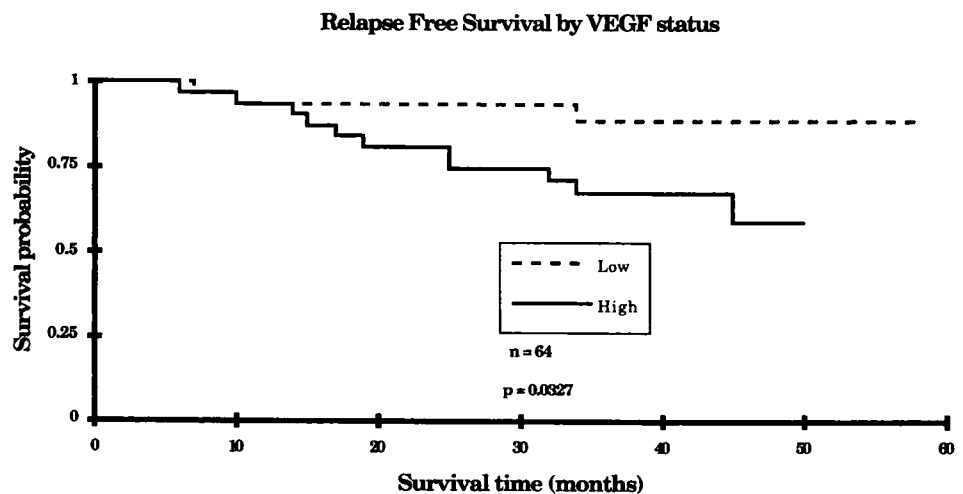
of an inflammatory cell infiltrate producing these factors. Additionally, we have recently shown a significant association of PDECGF expression with small tumor size in a study of 240 patients by immunochemistry (22). The association of TGF-β1 with small tumors is of borderline significance at (P = 0.07), 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 homogenates. 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 counting depends on selecting the most vascular field detected microscopically, whereas the measurement of RNA or protein is an average of a larger mass of tumor. Nevertheless, it is important to demonstrate which angiogenic factors are present within the tumor mass, because production of these factors by stromal cells, macrophages, and other nonmalignant populations may be relevant to tumor angiogenesis. Histological examination of angiogenic factor expression around blood vessels may help to resolve this further.

This study was not able to address the issue of differential expression in tumor tissue versus normal tissues for most of the angiogenic factors, apart from bFGF, because of low amounts of tissue near tumor available for assays.

There are several implications of our findings for antiangiogenic therapy. Agents designed to specifically block one vascular growth factor from binding to its receptor may not be effective, unless that factor is the most important in vivo. A recent example is interference with VEGF receptor function by gene therapy (38). It is unlikely that complete inhibition would be possible because of the multiple factors involved. Thus, broad spectrum agents that could inhibit features in common between these vascular growth factors may be an important approach. Because the majority of the factors described here are heparin binding, drugs that block this function should be assessed further. These include pentosan (39) and suramin analogues (40–42). Finally, agents that block endothelial growth and migration, downstream events from initial growth factor receptor interactions, may also be a particularly important approach. These agents include AGM 1470 (43–45) or recombinant toxins and vascular targeting (46). A combined approach should also be evaluated.

ACKNOWLEDGMENTS

REFERENCES

1. Folkman, J. What is the evidence that tumors are angiogenesis dependent? J. Natl. Cancer Inst., 82: 4–6, 1990.
2. Denekamp, J. Inadequate vasculature in solid tumours: consequences for cancer research strategies. Br. J. Radiol., 24: 111–117, 1992.
3. Horak, E. R., Leek, R., Klenk, N., LeJeune, S., Smith, K., Stuart, N., Greenall, M., Stepniewska, K., and Harris, A. L. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. Lancet, 340: 1120–1124, 1992.
4. Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. A., Moore, H., Meli, S., and Gasparini, G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J. Natl. Cancer Inst., 84: 1875–1887, 1992.
5. Dickinson, A. J., Fox, S. B., Persad, R. A., Hollyer, J., Sibley, G. N. A., and Harris, A. L. Quantification of angiogenesis as an independent predictor of prognosis in invasive bladder carcinomas. Br. J. Urol., 74: 762–766, 1994.
6. Fox, S. B., Gatter, K. C., Bicknell, R., Going, J. J., Stanton, P., Cooke, T. G., and Harris, A. L. Relationship of endothelial-cell proliferation to tumor vascularity in human breast-cancer. Cancer Res., 53: 4161–4163, 1993.
7. Scott, P. A. E., and Harris A. L. Current approaches to targeting cancer using antiangiogenesis therapies. Cancer Treat. Rev., 20: 393–412, 1994.
8. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing. Mol. Endocrinol., 5: 1806–1814, 1991.
9. Slavin, J. Fibroblast growth factors: at the heart of angiogenesis. Cell Biol. Int., 19: 431–444, 1995.
10. Fang, W., Hartmann, N., Chow, D. T., Riegel, A. T., and Wellstein, A. Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. J. Biol. Chem., 267: 25889–25897, 1992.
11. Riegel, A. T., and Wellstein, A. The potential role of the heparin-binding growth-factor pleiotrophin in breast-cancer. Breast Cancer Res. Treat., 31: 309–314, 1994.
12. Sunderkotter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R., and Sorg, C. Macrophages and angiogenesis. J. Leukocyte Biol., 55: 410–422, 1994.
13. Ishikawa, K., Miyazono, U., Hellman, H., Drexler, C., Wernstedt, K., Hagiwara, K., Usuki, F., Takaku, K., Risau, W., and Heldin, C. H. Identification of the angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. Nature (Lond.), 338: 557–562, 1989.
14. Sumizawa, T., Furukawa, T., Haraguchi, M., Yoshimura, A., Takeyasu, A., Ishizawa, M., Yamada, Y., and Akiyama, S. Thymidine phosphorylase activity associated with platelet-derived endothelial cell growth factor. J. Biochem. (Tokyo), 114: 9–14, 1993.
15. Moghaddam, A., Zhang, H. T., Fan, T. P., Hu, D. E., Lees, V. C., Turley, H., Fox, S. B., Gatter, K. C., Harris, A. L., and Bicknell, R. Thymidine phosphorylase is angiogenic and promotes tumor growth. Proc. Natl. Acad. Sci. USA, 92: 998–1002, 1995.
16. Sacks, P. M., Smith, K., Norman, A. P., Greenall, M., LeJeune, S., and Harris, A. L. Cathepsin D levels in primary breast cancers: relationship with epidermal growth factor receptor, oestrogen receptor and axillary node status. Eur. J. Cancer, 29A: 426–428, 1993.
17. Fox, S. B., Leek, R. D., Weekes, M. P., Whitehouse, R. M., Gatter, K. C., and Harris, A. L. Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count and computer image analysis. J. Pathol., 177: 275–283, 1995.
18. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156–159, 1987.
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. Current Protocols in Molecular Biology, pp. 4.7.1–4.7.6. John Wiley and Sons, 1994.
20. McCarthy, S. A., and Bicknell, R. Responses of pertussis toxin treated microvascular endothelial cells to transforming growth factor b. No evidence for G-protein involvement. J. Biol. Chem., 267: 21617–21622, 1992.
21. Nicholson, S., Sainsbury, J. R., Needham, G. K., Chambers, P., Farndon, J. R., and Harris, A. L. Quantitative assays of epidermal growth factor receptor in human breast cancer: cut-off points of clinical relevance. Int. J. Cancer, 42: 36–41, 1988.
22. Fox, S. B., Westwood, M., Moghaddam, A., Comley, M., Turley, H., Whitehouse, R. M., Bicknell, R., Galter, K. C., and Harris, A. L. The angiogenic factor platelet-derived endothelial cell growth factor thymidine phosphorylase is up-regulated in breast cancer epithelium and endothelium. Br. J. Cancer, 73: 275–280, 1996.
23. Connolly, D. T. Vascular permeability factor: a unique regulator of blood vessel function. J. Cell. Biochem., 47: 219–223, 1991.
24. Houck, K. A., Leung, D. W., Rowland, A. M., Winer, J., and Ferrara, N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J. Biol. Chem., 267: 26031–26037, 1992.
25. Berkman, R. A., Merrill, M. J., Reinhold, W. C., Monacci, W. T., Saxena, A., Clark, W. C., Robertson, J. T., Ali, I. U., and Oldfield, E. H. Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. J. Clin. Invest., 91: 153–159, 1993.
26. Toi, M., Hoshina, S., Takayanagi, T., and Tominaga, T. Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. Jpn. J. Cancer Res., 85: 1045–1049, 1994.
27. Pepper, M. S., Ferrara, N., Orci, L., and Montesano, R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem. Biophys. Res. Commun., 189: 824–831, 1992.
28. Janot, F. N. A., Morrison, R. S., Liu, T. J., Taylor, D. L., and Clayman, G. L. Expression of basic fibroblast growth factor in squamous cell carcinoma of the head and neck is associated with degree of histologic differentiation. Int. J. Cancer, 64: 117–123, 1995.
29. van der Laan, B. F., Freeman, J. L., and Asa, S. L. Expression of growth factors and growth factor receptors in normal and tumorous human thyroid tissues. Thyroid, 5: 67–73, 1995.
30. Vlodavsky, I., Fuks, Z., Ishai-Michaeli, R., Bashkin, P., Levi, E., Korner, G., Bar-Shavit, R., and Klagsrun, M. Extracellular matrix-resident basic fibroblast growth factor: implication for the control of angiogenesis. J. Cell. Biochem., 45: 167–179, 1991.

31. Brunner, N., Pyke, C., Hansen, C. H., Romer, J., Grondahl, H. J., and Dano, K. Urokinase plasminogen activator (uPA) and its type 1 inhibitor (PAI-1): regulators of proteolysis during cancer invasion and prognostic parameters in breast cancer. Cancer Treat. Res., 71: 299–309, 1994.

32. Luqmani, Y. A., Graham, M., and Coombes, R. C. Expression of basic fibroblast growth factor, FGFR1 and FGFR2, in normal and malignant human breast, and comparison with other normal tissues. Br. J. Cancer, 66: 273–280, 1992.

33. Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M. G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K-J., Yang Chou, J., and Persico, M. G. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PIGF), are transcribed from a single gene of chromosome 14. Oncogene, 8: 925–931, 1993.

34. Phillips, G. D., Whitehead, R. A., Stone, A. M., Ruebel, M. W., Goodkin, M. L., and Knighton, D. R. Transforming growth factor beta (TGF-β) stimulation of angiogenesis: an electron microscopic study. J. Submicrosc. Cytol. Pathol., 25: 149–155, 1993.

35. Gruber, B. L., Marchese, M. J., and Kew, R. Angiogenic factors stimulate mast-cell migration. Blood, 86: 2488–2493, 1995.

36. Ueki, N., Nakazato, M., Ohkawa, T., Ikeda, T., Amuro, Y., Hada, T., and Higashino, K. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. Biochim. Biophys. Acta, 1137: 189–196, 1992.

37. Folkman, J., and Hanahan, D. Switch to the angiogenic phenotype during tumorigenesis. In: C. C. Harris (ed.), Multistage Carcinogenesis, pp. 339–347. Tokyo: Japan Scientific Societies Press, 1992.

38. Millauer, B., Shawver, L. K., Plate, K. H., Risau, W., and Ullrich, A. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. Nature (Lond.), 367: 576–579, 1994.

39. Zugmaier, G., Lippman, M. E., and Wellstein, A. Inhibition by pentosan polysulphate (PPS) of heparin-binding growth factors released from tumour cells and blockage by PS of tumour growth in animals. J. Natl. Cancer Inst., 84: 1716–1724, 1992.

40. Pesenti, E., Sola, F., Mongelli, N., Grandi, M., and Spreafico, F. Suramin prevents neovascularisation and tumour growth through blocking of basic fibroblast growth factor activity. Br. J. Cancer, 66: 367–372, 1992.

41. Takano, S., Gately, S., Neville, M. E., Herblin, W. F., Gross, J. L., Engelhard, H., Perricone, M., Eidsvoog, K., and Brem, S. Suramin: an anticancer and angiosuppressive agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator. Cancer Res., 54: 2654–2660, 1994.

42. Braddock, P., Hu, D-E., Fan, T-P., Stratford, I., Harris, A., and Bicknell, R. A structure-activity analysis of antagonism of the growth factor and angiogenic activity of basic fibroblast growth factor by suramin and related polyanions. Br. J. Cancer, 69: 890–898, 1994.

43. Ingber, D., Fujita, D., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H., and Folkman, J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. 348: 555–557, 1990.

44. Kamei, S., Okada, H., Inoue, Y., Yoshioka, T., Ogawa, Y., and Toguchi, H. Antitumour effects of angiogenesis inhibitor TNP-4709 in rabbits bearing VX-2 carcinoma by arterial administration of microspheres and oil solution. J. Pharmacol. Exp. Ther., 264: 469–474, 1993.

45. Takamiya, Y., Friedlander, R. M., Brem, H., Malick, A., and Martuza, R. L. Inhibition of angiogenesis and growth of human nerve-sheath tumors by AGM 1470. J. Neurosurg., 78: 470–476, 1993.

46. Biro, S., Siegall, C., Fu, Y-M., Speir, E., Pastan, I., and Epstein, S. E. In vitro effects of a recombinant toxin targeted to the fibroblast growth factor receptor on rat vascular smooth muscle and endothelial cells. Circ. Res., 71: 640–645, 1992.