High Tumor Levels of Vascular Endothelial Growth Factor Predict Poor Response to Systemic Therapy in Advanced Breast Cancer[1]

John A. Foekens,[2] Harry A. Peters, Nicolai Grebenchtchikov, Maxime P. Look, Marion E. Meijer-van Gelder, Anneke Geurts-Moespot, Theo H. van der Kwast, C. G. J. (Fred) Sweep, and Jan G. M. Klijn

Division of Endocrine Oncology (Department of Medical Oncology), Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam [J. A. F., H. A. P., M. P. L., M. E. M. v. G., J. G. M. K.]; Department of Pathology, University Hospital Rotterdam, Rotterdam [T. H. v. d. K.]; and Department of Chemical Endocrinology, University Hospital Nijmegen, Nijmegen [N. G., A. G-M., C. G. J. S.], the Netherlands

ABSTRACT

Vascular endothelial growth factor (VEGF), a potent angiogenic factor, has been reported to be associated with a poor prognosis in primary breast cancer and in several other cancer types. In the present study, we have measured with ELISA the levels of VEGF in cytosolic extracts of 845 primary breast tumors of patients who developed a recurrence during follow-up. All of the patients received tamoxifen ($n = 618$) or cyclophosphamide, methotrexate, 5-fluorouracil (CMF) or 5-fluorouracil, Adriamycin, cyclophosphamide (FAC) chemotherapy ($n = 227$) as first-line systemic therapy after diagnosis of advanced disease. VEGF levels were not related to age or menopausal status but were negatively related to the cytosolic levels of estrogen receptor and progesterone receptor ($P < 0.0001$). In patients who relapsed within 1 year after primary surgery, tumor VEGF levels were higher than in patients who showed a longer disease-free interval ($P = 0.0005$). In patients with a first relapse in the viscera, VEGF levels were higher compared with those that relapsed to the bone or soft tissue ($P = 0.0004$). In univariate analysis for response to first-line tamoxifen therapy, patients with high or intermediate levels showed a poor rate of response, compared with patients with low tumor-VEGF levels ($P = 0.0001$). Similarly, in multivariate analysis for response to tamoxifen treatment, corrected for age, site of relapse, disease-free interval, and estrogen receptor and progesterone receptor status, VEGF status was an independent predictive factor ($P = 0.009$). In concordance, higher levels of VEGF were associated with a short progression-free survival and postrelapse overall survival (both, $P < 0.0001$). On first-line chemotherapy, the rate of response decreased with higher tumor levels of VEGF, both in univariate ($P = 0.003$) and in multivariate analysis ($P = 0.004$). Furthermore, higher VEGF levels were associated with a short progression-free survival ($P = 0.003$) and postrelapse overall survival ($P = 0.001$). In conclusion, the tumor VEGF level is an important independent marker that predicts a poor efficacy of both tamoxifen and chemotherapy in advanced breast cancer. Knowledge of the tumor level of VEGF might be helpful in selecting individual patients who may benefit from treatments with antiangiogenic agents combined with conventionally used drugs.

INTRODUCTION

Angiogenesis, required for tumor growth and metastasis (1, 2), is balanced by a variety of positive and negative regulators of microvessel growth (3). An unbalance of these regulators results in a switch to an angiogenic tumor phenotype (4–6). Quantification of MVD[3] in histological specimens of primary breast tumors and lymph-node metastases was shown to be related to a poor RFS and overall survival (7–10). VEGF, first described as vascular permeability factor (11), consists of several splice variants yielding proteins of 121, 145, 165, 189, and 206 amino acids (12, 13). In tissue, $VEGF_{165}$ is the predominant isoform, and $VEGF_{121}$ and $VEGF_{165}$ are secreted into the circulation (14). Within tumors the tumor cells are the main source of VEGF; however, tumor-associated stroma has also been shown to produce VEGF (15). VEGF behaves as a growth factor ligand that binds to specific tyrosine kinase receptors VEGFR-1 (flt) and VEGFR-2 (KDR/flk-1) on endothelial cells (16, 17).

In patients with breast cancer, serum and plasma VEGF levels have been found to be elevated in patients with larger tumors and with metastatic disease (18, 19). In human primary breast tumors, the immunocytochemically assessed VEGF showed a close correlation with MVD, and high expression levels were associated with a poor relapse-free survival (20). The levels of VEGF measured by ELISA in tumor cytosols correlated with microvessel count as well (21). However, in this small heterogeneous study including only 89 patients, the level of cytosolic VEGF was not correlated with RFS (21). On the other hand, several groups of investigators reported that an increased expression level of VEGF mRNA (22) or protein, as measured by ELISA in tumor cytosols (23–25), was associated with a poor prognosis in primary breast cancer patients. Similarly, in patients treated with adjuvant endocrine or chemotherapy, intratumoral MVD or a high level of VEGF in primary breast tumor cytosols were shown to be related to a poor prognosis (26–30). From these studies, however, no conclusions can be drawn regarding the association of systemic treatment with the level of VEGF or the extent of MVD because there were no randomized untreated control groups available.

Recently, functional estrogen response elements in the gene coding for VEGF have recently been reported (31, 32). There is evidence that steroid hormones can regulate VEGF production in human breast cancer cells. In human breast cancer cells *in vitro* (33, 34) and in 7,12-dimethylbenzanthracene-induced rat mammary tumors *in vivo* (35), VEGF mRNA and/or protein production was found to be stimulated by estrogens and progestins. The antiestrogen ICI 182.780 inhibited the estradiol-stimulated VEGF production of the MCF-7 breast cancer cells, whereas tamoxifen did not. Tamoxifen, when used alone, even stimulated VEGF production by a mechanism thought to be independent of ER (34). Currently no published data on the relationship between the tumor level of VEGF and the efficacy of response to systemic endocrine therapy, nor to chemotherapy, in patients with advanced breast cancer are available. In the present study, we aimed to assess in a relatively large series of patients whether the tumor level of cytosolic VEGF might be predictive for the efficacy of tamoxifen and/or chemotherapy in advanced breast cancer patients.

Received 2/1/01; accepted 5/7/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Grant DDHK 2000-2256 of the Dutch Cancer Society, Amsterdam, the Netherlands.

[2] To whom requests for reprints should be addressed, at Josephine Nefkens Institute, Room BE426, P. O. Box 1738, 3000 DR, Rotterdam, the Netherlands. Phone: 31-10-4088-369; Fax: 31-10-4088-377; E-mail: Foekens@bidh.azr.nl.

[3] The abbreviations used are: MVD, microvessel density; RFS, relapse-free survival; PFS, progression-free survival; PR-OS, postrelapse overall survival; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; ER, estrogen receptor; PgR, progesterone receptor; DFI, disease-free interval; CR, complete response; PR, partial response; SDis, stable disease; PD, progressive disease; CMF, cyclophosphamide, methotrexate, 5-fluorouracil; FAC, 5-fluorouracil, Adriamycin, cyclophosphamide; OR, odds ratio; RHR, relative hazard rate; CI, confidence interval; EORTC, European Organization for Research and Treatment of Cancer; CV, coefficient of variation.

MATERIALS AND METHODS

Patients and Treatment. Our study design was approved by the medical ethical committee of the Erasmus University Rotterdam, the Netherlands. A series of 845 patients with primary operable breast cancer who underwent resection of their primary tumor between 1978 and 1995, and who developed a recurrence that was treated with first-line tamoxifen (618 patients) or chemotherapy (227 patients), were selected. At the time of surgery for their primary tumor, the median age of the tamoxifen-treated patients was 59 years (range 26–90 years), and the chemotherapy-treated patients was 47 years (range 24–79 years). The differentiation grade of the tumor was based on histological and cellular characteristics, as stated in the reports of the regional pathologists, and it is not based on a central pathological review of all of the tumor samples and, thus, reflects daily practice. The length of PFS was defined as the time from the start of treatment of advanced disease until the start of next treatment because of PD or until the time of intercurrent death. All of the patients were assessed by standard Union International Contre Cancer criteria as having CR and PR. Patients with no change for more than 6 months (SDis) have a PR-OS similar to patients with PR (36, 37). Therefore, for overall response, objective response (CR + PR) and SDis were combined.

First-Line Tamoxifen Treatment. All of the patients received tamoxifen (40 mg daily) as first-line endocrine therapy after diagnosis of advanced disease. None of the patients had received neoadjuvant therapy, and none of the patients were exposed to hormonal treatment at an earlier stage (hormonaïve). Adjuvant polychemotherapy was given to 117 patients (CMF in 76 patients, FAC in 41 patients). At start of tamoxifen treatment, 137 (22%) patients were premenopausal and 481 (78%) patients were postmenopausal. Of the patients, 523 (85%) had a an ER-positive (≥10 fmol/mg of protein) tumor, whereas 83 (13%) had an ER-negative tumor and 12 (2%) an unknown receptor status. The median follow-up of the patients still alive after surgery is 93 months (range, 5–167 months) and after start of tamoxifen treatment is 39 months (range, 4–135 months). One hundred twenty-one patients are still alive, whereas 497 (80%) died. On tamoxifen therapy given for advanced disease, tumor progression occurred in 575 patients (93%) during follow-up. Of these patients, 401 were subsequently treated with one or more additional hormonal agents (mostly high-dose progestins), and, thus far, 330 patients received systemic chemotherapy (mainly, CMF, or with Adriamycin instead of methotrexate, FAC).

First-Line Chemotherapy. All of the patients received polychemotherapy as first-line treatment (CMF in 111 and FAC in 116 patients) after diagnosis of advanced disease. None of these patients had received neoadjuvant therapy. Adjuvant chemotherapy was given to 44 patients (CMF in 31 patients, FAC in 13 patients) and adjuvant hormonal therapy was given to 44 patients as well, either alone (42 patients) or in combination with CMF (2 patients). At start of chemotherapy, 123 patients were premenopausal (54%) and 104 patients were postmenopausal (46%). Of these patients, 123 (54%) had an ER-negative tumor, whereas 101 (44%) had an ER-positive tumor and 3 (1%) an unknown receptor status. The median follow-up of the patients still alive after surgery is 75 months (range, 13–118 months) and after start of chemotherapy is 18 months (range, 4–79 months). Thirty-three patients are still alive, and 194 died (85%). On chemotherapy, tumor progression occurred in 215 patients (95%) during follow-up. Of these patients, 142 were eventually treated with endocrine therapy, 106 (tamoxifen in 63 patients, progestins in 41 patients, others in 2 patients) immediately after progression on first-line CMF or FAC and 36 after 1 to 3 additional chemotherapy regimens.

Tumors and Assays. Tumor tissues were stored in liquid nitrogen and pulverized in the frozen state with a microdismembrator as recommended by the EORTC for processing of breast tumor tissue for cytosolic ER and PgR determinations (38). The resulting tissue powder was suspended in EORTC receptor buffer [10 mM K_2HPO_4, containing 1.5 mM dipotassium EDTA, 3 mM NaN_3, 10 mM monothioglycerol, and 10% v/v glycerol (pH 7.4)]. The suspension was centrifuged for 30 min at 100,000 × *g* at 4°C to obtain the supernatant fraction (cytosol). ER and PgR levels were determined by ligand-binding assay or enzyme immunoassay, as described previously (39).

VEGF levels were determined in breast tumor cytosols with an ELISA developed by the EORTC Receptor and Biomarker Group. The assay specifically measures $VEGF_{165}$ and $VEGF_{121}$, the main isoforms of VEGF. The details of the assay procedure, including those of the specificity and performance, have been described elsewhere (40). To increase the sensitivity, modifications involved the detecting procedures in which the horseradish peroxidase-labeled goat antirabbit detecting antibody was replaced for monoclonal antirabbit alkaline phosphatase-conjugated antibody (A-2556; Sigma Chemical Co., St. Louis, MO). Incubation with the detecting antibody, 1:8000 diluted in PBS, containing 1% w/v BSA and 0.1% v/v Tween 20, was performed for 2 h at ambient temperature. Subsequent incubation with 100 μl of substrate solution, 0.1 mg/ml 4-methylumbelliferyl phosphate (free acid; Molecular Probes Inc, Eugene, OR) in alkaline phosphatase buffer [0.1 M Tris-HCl, 0.1 M NaCl, 10 mM $MgCl_2$ (pH 9.5)] and was performed for 1 h at ambient temperature. The reaction was stopped with 150 μl of 0.15 M glycine (pH 10.5), and fluorescence was measured with a fluorometric plate reader (Ascent FL Labsystems, Breda, The Netherlands). To enable the assessment of the between-assay variations (% CV), in each of 32 assay-runs an aliquot of a pooled breast cancer cytosol sample was analyzed. The between-assay CV was 12.6% and the within-assay CV of samples measured in duplicate was 5.8% at a level of 0.88 ng/ml.

Statistics. The strength of the associations of VEGF with ER and PgR were tested with Spearman rank correlation (r_S). The associations of VEGF (used as continuous variable) with other variables (used as grouping variables) was tested with the nonparametric Wilcoxon rank-sum test or the Kruskal-Wallis test, followed by a Wilcoxon-type test for trend across ordered groups if appropriate. In uni- and multivariate analysis, the relation with response-to-therapy was examined with logistic regression analysis. Multivariate analysis was performed with variables eliminated in a step-down fashion. ORs were calculated and presented with their 95% CIs. Variables with a $P < 0.1$ were retained in the final multivariate models for response to tamoxifen and chemotherapy. The likelihood ratio test in regression models was used to test for differences and for interactions. Isotonic regression analysis (41) was applied to define cutpoints for VEGF after it had been established that, in a test for trend using log-transformed VEGF values, high VEGF levels were significantly associated with a poor rate of response or a shorter PFS on tamoxifen therapy ($P = 0.002$ and $P = 0.001$, respectively), and chemotherapy ($P = 0.003$ and $P = 0.05$, respectively). With isotonic regression analysis, the hazard rate for failure is estimated as a function of the VEGF value under the assumption of a monotone-decreasing failure rate (no response or progression) with increasing VEGF levels. The cutpoints chosen to classify tumors as VEGF-low, intermediate and -high, were 0.22 and 1.73 ng/mg of protein, respectively, in analysis of response and survival on tamoxifen treatment. The same cutpoints were adapted in the analysis of response and survival on chemotherapy because there were no reasons to assume that they might be different from those defined for the patients who were treated with tamoxifen. Cox univariate regression analysis was used in the analysis of PFS and PR-OS. The assumption of proportional hazards was verified graphically. RHRs were calculated and presented with their 95% CIs. Survival curves were generated using the method of Kaplan and Meier (42) and the log-rank test for trend was used to examine survival data. All of the *P*s are two-sided and relate to all of the available data during the total period of follow-up.

RESULTS

Levels and Associations. The median level of VEGF determined in 845 cytosols was 0.22 ng/mg of protein (range, 0–542 ng/mg protein). Table 1 shows their median levels and quartiles in subgroups of tumors and their relationships with patient and tumor characteristics. The tumor levels of VEGF were not related to menopausal status or with age (Spearman correlation, $r_s = 0.05$) at the time of primary surgery. If the primary tumor had high levels of VEGF, the first metastases more often developed in the viscera and bone, and less frequently in soft tissues ($P = 0.0004$). Patients who had a DFI of less than 1 year had higher VEGF levels in the primary tumor than those with a DFI of ≥1 year ($P = 0.0005$). VEGF levels were higher in hormone receptor-negative tumors compared with receptor-positive tumors ($r_s = -0.14$ for ER, and $r_s = -0.19$ for PgR, respectively; for both $P < 0.0001$). Tumor VEGF levels were not significantly correlated with nodal status ($P = 0.09$) or with primary tumor size ($P = 0.51$) or grade ($P = 0.20$).

Table 1 *Relationships of VEGF with patient and tumor characteristics*

Characteristic	Frequency[a]	VEGF median value (quartiles)[b]	*P*
All patients	845	0.22 (0.01, 0.82)	
Menopausal status[c]			
Premenopausal	349	0.20 (0, 0.71)	
Postmenopausal	496	0.24 (0.01, 0.98)	0.08[d]
First site of relapse[e]			
Soft tissue	125	0.13 (0, 0.59)	
Bone	335	0.18 (0, 0.61)	
Viscera	385	0.30 (0.04, 1.13)	0.0004[f]
DFI			
<1 yr	249	0.33 (0.04, 1.31)	
≥1 yr	596	0.20 (0, 0.72)	0.0005[d]
ER status[g]			
Negative	206	0.45 (0.06, 1.59)	
Positive	624	0.18 (0, 64)	<0.0001[g]
PgR status[h]			
Negative	283	0.36 (0.07, 1.40)	
Positive	538	0.17 (0, 0.59)	<0.0001[h]

[a] Because of missing values, numbers do not always add up to 845.
[b] All of the values are in ng/mg of protein (25th and 75th percentiles).
[c] At time of primary surgery.
[d] *P* for Wilcoxon rank-sum test.
[e] In case of multiple sites, the site with the worst prognosis was considered dominant.
[f] *P* for Wilcoxon-type test for trend.
[g] *P* for Spearman rank correlation.
[h] Cutoff points used for ER and PgR: 10 fmol/mg of protein.

Univariate Analysis for Response to Tamoxifen Therapy. Of the 618 patients who received tamoxifen as first-line treatment for advanced disease, 351 (57%) responded (29 CR, 83 PR, 239 SDis). The median duration of response in these responders was 16.1 months.

Table 2 shows that postmenopausal and older patients had a higher rate of response to tamoxifen treatment than premenopausal and younger patients. Furthermore, patients who first relapsed to the viscera showed a worse rate of response (51% response) compared with patients of whom the soft tissue or the bone was the first site of relapse (60 and 61% response, respectively). In patients with a DFI of <1 year (40% response; OR set at 1) the fraction of responding patients was smaller than in patients with a DFI of ≥1 year (63% response; OR, 2.49). The application of adjuvant chemotherapy was not related to the rate of response to tamoxifen treatment in advanced disease. Patients with ER-positive or PgR-positive tumors had a higher response rate (OR, 3.40 and 2.10, respectively) than patients with ER-negative or PgR-negative tumors (OR, 1). Compared with the 320 patients with low levels of VEGF (<0.22 ng/mg protein) in the tumor cytosols [64% response (22% CR + PR and 42% SDis; OR, 1)], the 220 patients with intermediate VEGF levels (≥0.22 and <1.73 ng/mg protein) and the 78 patients with high VEGF levels (≥1.73 ng/mg protein), showed a worse rate of response [intermediate, 52% response (16% CR + PR and 36% SDis; OR, 0.61); high, 40% response (9% CR + PR and 31% SDis; OR, 0.37); $P = 0.0001$]. Lymph-node status, or size and grade of the primary tumor, which are strong prognostic factors in patients with primary breast cancer, were not significantly related to the rate of response to tamoxifen treatment in patients with advanced disease. These factors were, therefore, not further considered in the present study.

In Kaplan-Meier analysis of the 618 tamoxifen-treated patients, those with intermediate and high VEGF levels showed a shorter PFS ($P < 0.0001$; Fig. 1*A*) and PR-OS ($P < 0.0001$; Fig. 1*B*) compared with patients with low VEGF levels. After 3 years, more than twice as many patients were alive in cases in which the tumor had low VEGF

Table 2 *Univariate and multivariate analysis for response to first-line tamoxifen therapy in patients with advanced breast cancer*

			Univariate analysis			Multivariate analysis[c]				
	Frequency[a]	Response rate (%)	*P*	OR[b]	(95% CI)[b]	*P*	OR[b]	(95% CI)[b]	Duration of response (mo)[d]	Survival (mo)[e]
All patients	618	57							16.1	26.2
Menopausal status[f]										
Premenopausal	137	47		1					16.3	25.9
Postmenopausal	481	60	0.007	1.69	(1.15–2.47)				16.1	26.2
Age (yr)[f]										
≤40	40	43		1			1		13.7	19.0
41–55	175	51		1.43	(0.72–2.87)		1.43	(0.68–2.99)	14.2	26.9
56–70	237	58		1.85	(0.94–3.65)		2.11	(1.02–4.34)	15.3	27.0
>70	166	64	0.02	2.45	(1.22–4.96)	0.008	2.78	(1.31–5.89)	18.5	26.1
First site of relapse										
Soft tissue	92	60		1			1		16.3	31.4
Bone	287	61		1.05	(0.65–1.70)		0.80	(0.47–1.36)	17.3	30.4
Viscera	239	51	0.05	0.69	(0.42–1.12)	0.09	0.58	(0.34–1.00)	14.5	17.0
DFI										
<1 yr	168	40		1			1		11.9	18.5
≥1 yr	450	63	<0.0001	2.49	(1.73–3.58)	<0.0001	2.30	(1.55–3.42)	16.8	31.1
Adjuvant therapy										
No	484	56		1					16.3	26.0
Yes	134	59	0.57	1.12	(0.76–1.65)				14.8	27.0
ER status[g]										
Negative	83	31		1			1		12.7	13.6
Positive	523	61	<0.0001	3.40	(2.07–5.58)	0.009	2.14	(1.20–3.83)	16.4	29.4
PgR status[g]										
Negative	155	43		1			1		12.5	17.0
Positive	442	62	<0.0001	2.10	(1.45–3.05)	0.09	1.47	(0.95–2.28)	17.4	31.4
VEGF levels[h]										
Low	320	64		1			1		18.4	32.6
Intermediate	220	52		0.61	(0.43–0.87)		0.69	(0.47–1.00)	14.7	22.2
High	78	40	0.0001	0.37	(0.22–0.61)	0.009[i]	0.45	(0.26–0.78)	12.2	15.9

[a] Because of missing values, numbers do not always add up to 618.
[b] OR (95% CI).
[c] The final multivariate model with all of the factors known included 597 patients.
[d] Median time until progression (mo) in responding patients.
[e] PR-OS (mo) after start of first-line tamoxifen treatment of all 618 patients.
[f] At time of start of first-line tamoxifen treatment.
[g] Cutpoints: 10 fmol/mg protein.
[h] Low: <0.22 ng/mg protein; intermediate: ≥0.22 and <1.73 ng/mg protein; high: ≥1.73 ng/mg protein.
[i] The increment in χ^2 is 9.52.

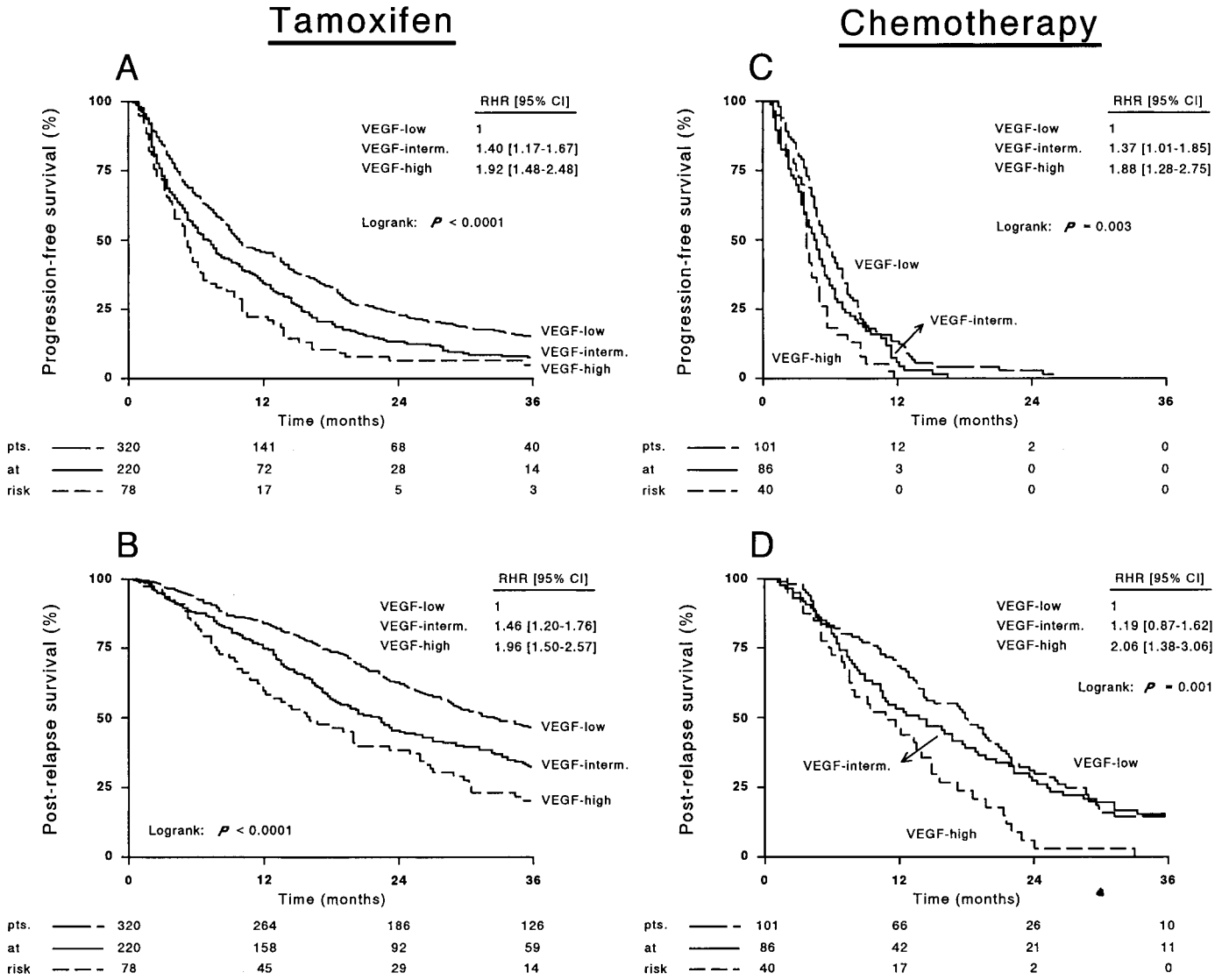

Fig. 1. PFS (*A, C*) and PR-OS (*B, D*) after the start of tamoxifen treatment (*A, B*) or chemotherapy (*C, D*) as a function of the level of VEGF: *low*, low values; *interm.*, intermediate values; *high*, high values. For cutpoints, see Table 2, Footnote *h*. The number of patients below the *X*-axis represents the number at risk in the low, intermediate, and high VEGF groups, at the indicated time points.

levels (46% alive) compared with those with high VEGF levels (20% alive). The median PFS decreased from 9.9 months for those with low VEGF levels, via 7.0 months for those with intermediate VEGF levels, to 5.1 months for those with high levels of VEGF in the tumor cytosols. Similarly, the PR-OS decreased from 32.6 months, via 22.2 months, to 15.9 months with VEGF levels increasing from low, via intermediate, to high, respectively. The median duration of response in the 351 patients responding to tamoxifen (Table 2) decreased from 18.4 months for patients with low (RHR, set at 1), via 14.7 months for those with intermediate (RHR, 1.35; 95% CI, 1.06–1.71) to 12.2 months for those with high tumor levels of VEGF (RHR, 1.86; 95% CI, 1.86–2.77; $P = 0.002$). The median PR-OS in the 351 responding patients decreased from 42.5 via 36.2 to 28.7 months for tumors with low (RHR, 1), intermediate (RHR, 1.51; 95% CI, 1.16–1.98) and high levels of VEGF (RHR, 1.94; 95% CI, 1.26–2.98), respectively ($P < 0.001$).

Univariate Analysis for Response to Chemotherapy. Of the 227 patients treated with first-line chemotherapy, 120 (53%) responded (16 CR, 67 PR, 37 SDis). The proportion of response was higher for the 116 patients who received FAC (63% response; 8 CR, 45 PR, 20 SDis) than for the 111 patients who received CMF (42% response; 8 CR, 22 PR, 17 SDis; $P = 0.002$). The median duration of response in the 120 responding patients was 7.4 months; this was not different between the patients who received FAC (7.6 months) or CMF (7.1 months).

Table 3 shows that on first-line chemotherapy, the premenopausal patients responded more favorably (61% response) than the postmenopausal patients (43% response). In patients with a DFI of <1 year, the rate of response (44% response; OR, 1) was lower compared with patients with a DFI of ≥ 1 year (58% response; OR, 1.69), although not significant ($P = 0.06$). The first site of relapse, the application of former adjuvant systemic therapy, and the ER or PgR status, were not related to the rate of response to first-line chemotherapy. Higher levels of VEGF in the tumor cytosols predicted a poor outcome on chemotherapy ($P = 0.003$). Of the 101 patients with low VEGF levels, 64% (43% CR + PR, 22% SDis; OR, 1) responded. This compares with 48% responders (37% CR + PR, 10% SDis; OR, 0.50) in the 86 patients with intermediate VEGF levels, and to 35% responders (20% CR + PR, 15% SDis; OR, 0.30) in the 40 patients with high VEGF levels, respectively (Table 3). Lymph-node status, or

Table 3 *Univariate and multivariate analysis for response to first-line chemotherapy in patients with advanced breast cancer*

	Frequency[a]	Response rate (%)	Univariate analysis			Multivariate analysis[c]			Duration of response (mo)[d]	Survival (mo)[e]
			P	OR[b]	(95% CI)[b]	*P*	OR[b]	(95% CI)[b]		
All patients	227	53							7.4	14.3
Menopausal status[f]										
Premenopausal	123	61		1			1		7.0	18.1
Postmenopausal	104	43	0.008	0.49	(0.29–0.83)	0.01	0.49	(0.28–0.85)	8.5	11.7
Age (yr)[f]										
≤40	45	58		1					6.4	15.7
41–55	111	54		0.86	(0.43–1.73)				7.1	17.6
56–70	63	51		0.75	(0.35–1.63)				8.5	13.7
>70	8	25	0.36	0.24	(0.04–1.34)				4.5	4.7
First site of relapse										
Soft tissue	33	52		1					5.4	17.6
Bone	48	54		1.11	(0.46–2.70)				7.4	19.9
Viscera	146	53	0.97	1.05	(0.49–2.24)				7.6	13.1
DFI										
<1 yr	81	44		1			1		7.6	12.4
≥1 yr	146	58	0.06	1.69	(0.98–2.93)	0.08	1.67	(0.94–2.96)	7.4	16.7
Adjuvant therapy										
No	141	53		1					7.1	16.7
Yes	86	52	0.90	0.97	(0.56–1.65)				7.6	13.6
ER status[g]										
Negative	123	49		1					6.5	11.7
Positive	101	58	0.15	1.48	(0.87–2.51)				8.5	19.3
PgR status[g]										
Negative	128	48		1					6.6	11.3
Positive	96	59	0.10	1.56	(0.91–2.66)				7.6	20.0
VEGF levels[h]										
Low	101	64		1			1		7.6	17.8
Intermediate	86	48		0.50	(0.28–0.91)		0.48	(0.26–0.87)	7.1	13.7
High	40	35	0.003	0.30	(0.14–0.64)	0.004[i]	0.31	(0.14–0.68)	6.6	10.7

[a] Because of missing values, numbers do not always add up to 227.
[b] OR (95% CI).
[c] The final multivariate model included all 227 patients.
[d] Median time until progression (mo) in responding patients.
[e] PR-OS (mo) after start of first-line chemotherapy in all of the 227 patients.
[f] At time of start of chemotherapy.
[g] Cutpoints: 10 fmol/mg protein.
[h] Low: <0.22 ng/mg protein; intermediate: ≥0.22 and <1.73 ng/mg protein; high: ≥1.73 ng/mg protein.
[i] The increment in χ^2 is 11.0.

size and grade of the primary tumor, were not significantly related to the rate of response to chemotherapy in patients with advanced disease, and were not further considered in the present study.

In Kaplan-Meier analysis of the 227 patients who were treated with chemotherapy, compared with tumors with low VEGF levels, those with intermediate and high levels showed a shorter PFS ($P = 0.003$; Fig. 1*C*) and PR-OS ($P = 0.001$; Fig. 1*D*). The median PFS and PR-OS of all of the 227 patients decreased from 5.6 and 17.8 months for those with low tumor VEGF levels, via 4.6 and 13.7 months for those with intermediate VEGF levels, to 3.8 and 10.7 months for those with high VEGF levels, respectively. The decrease in the median duration of response on chemotherapy as a function of the VEGF level in the 120 responding patients was not significantly affected. It decreased from 7.6 months for those with low, via 7.1 months for those with intermediate, to 6.6 months for those with high tumor VEGF levels (Table 3; $P = 0.29$). In PR-OS analysis of these responding patients, compared with patients with low VEGF tumor levels (RHR, 1), those with intermediate (RHR, 1.20; 95% CI, 0.78–1.86) and high levels (RHR, 2.73; 95% CI, 1.49–5.00) showed a poor survival ($P = 0.004$). The median survival time in patients with high VEGF levels was only 13.4 months, compared with 20.7 and 21.8 months for those with intermediate and low levels, respectively.

Multivariate Analysis for Response to Tamoxifen or Chemotherapy. The independent relationship of VEGF levels with the rate of response to systemic treatment in advanced breast cancer was studied using multivariate logistic regression analysis. In both the analysis of response to tamoxifen treatment (Table 2) and the analysis of response to chemotherapy (Table 3), corrected for the classical variables, increasing levels of VEGF were significantly related to a poor outcome of treatment ($P = 0.009$ and $P = 0.004$, respectively).

In addition to VEGF added as a categorical variable, young age, a short DFI, and ER-negativity independently predicted a poor rate of response to tamoxifen treatment as well. The contributions of the first site of relapse and PgR to the multivariate model were not statistically significant (both, $P = 0.09$; Table 2). The marginal contribution of PgR was attributable to the inclusion of ER in the model. In a separate multivariate analysis in which VEGF was added to the model as a log-transformed continuous variable instead of a categorical variable, the contribution of VEGF was statistically significant as well ($P < 0.05$). Furthermore, when ER and PgR were both included as log-transformed continuous variables in the model (ER, $P = 0.004$; PgR, $P = 0.01$), the contribution of VEGF as a categorical variable was statistically significant ($P = 0.03$). In this latter model, compared with tumors with low VEGF levels (OR, 1), those with intermediate and high levels had ORs and 95% CIs of 0.71 (0.48–1.04) and 0.49 (0.28–0.87), respectively. There were no statistically significant interactions between VEGF and ER or PgR in the analysis of response to tamoxifen treatment, neither when analyzed as continuous variables, nor when analyzed as categorical variables.

In the multivariate analysis for response to chemotherapy, in addition to VEGF added as a categorical variable ($P = 0.004$), only menopausal status was a significant predictor of a poor rate of response ($P = 0.01$), whereas the contribution of a short DFI was only of borderline significance ($P = 0.08$; Table 3). In a separate multivariate analysis in which VEGF was included as a log-transformed continuous variable, its contribution was statistically significant as well (OR, 0.86; 95% CI, 0.77–0.95; $P = 0.004$). When the type of chemotherapy (FAC or CMF) was additionally included as a covariate in the model, the estimates of VEGF were not affected (OR, 0.86; 95% CI, 0.77–0.96; $P = 0.006$). This suggests that the relationship of

VEGF to the rate of response to chemotherapy did not depend on the presence of the anthracyclin in the polychemotherapy regimen given. There were no statistically significant interactions between categorically added ER (or PgR) and VEGF with respect to response to chemotherapy. However, when analyzed as log-transformed continuous variables in the multivariate analysis for response to chemotherapy, there appeared to be a significant first-order interaction between VEGF and ER ($P = 0.01$), but not between VEGF and PgR ($P = 0.14$).

Response to Treatment in ER Subgroups. Because we observed a statistically significant interaction of VEGF and ER with response to chemotherapy, we performed exploratory analyses for the rate of response in subgoups of ER-positive and ER-negative patients as a function of VEGF status. The predictive value of VEGF for a poor response to chemotherapy was confined to the subgroup of 123 ER-negative patients, *i.e.,* intermediate and high levels of VEGF were associated with a lower fraction of responding patients ($P = 0.026$). Compared with the 44 tumors with low VEGF levels (64% response; OR, 1), the ORs and 95% CIs for the 51 tumors with intermediate levels (45% response) was 0.46 (0.21–1.07), and for the 28 tumors with high levels (32% response) was 0.27 (0.10–0.74), respectively. In the 101 ER-positive patients, the decrease in the fraction of responders as a function of the level of VEGF (64, 53, and 45% response for those with low, intermediate, and high VEGF levels, respectively) was not statistically significant ($P = 0.37$). In the analysis of the rate of response to tamoxifen treatment as a function of the level of VEGF, the association of VEGF with the fraction of responders was confined to the subgroup of 523 ER-positive patients. Of 285 patients with ER-positive and VEGF-low tumors, 192 (67%) responded favorably (OR, 1). This compares with 101 (56% response) of 180 tumors with intermediate VEGF levels (OR, 0.62; 95% CI, 0.42–0.91) and to 25 (43% response) of 58 tumors with high VEGF levels (OR, 0.37; 95% CI, 0.21–0.65; $P < 0.001$). In 83 ER-negative patients, the response rates were 31% for those with low, 34% with intermediate, and 26% with high VEGF levels, respectively ($P = 0.83$).

DISCUSSION

Angiogenesis is a necessity for tumors to grow at the primary and metastatic sites. Therefore, many new therapies aimed at the inhibition of angiogenesis, *e.g.,* the use of natural inhibitors or drugs that block VEGF action and VEGFR-associated tyrosine kinase activation, are currently under investigation (reviewed in Refs. 3 and 43). Combinations of antiangiogenic drugs with conventional hormonal or chemotherapeutic agents are attractive treatment options to explore (44). For the selection of patients who may benefit from these combined treatment modalities, knowledge of the tumor phenotype with respect to the expression of potential target proteins, or pathways, is essential. In preclinical breast cancer models, angiogenesis and/or VEGF production may be regulated by hormones (26, 33–35, 45, 46) or chemotherapeutic agents (47, 48). Furthermore, in human breast tumors, a reduction in MVD was observed after treatment of patients with neoadjuvant chemoendocrine therapy (49). Moreover, antiestrogens, including tamoxifen, have been shown to inhibit VEGF-stimulated endothelial cell proliferation by a process not mediated by the ER (50). Because VEGF is considered essential for tumor growth, and because the VEGF-induced VEGFR tyrosine kinase activity could be targeted in various ways, we have investigated in the present study whether tumor VEGF levels are related to the efficacy of response to tamoxifen and chemotherapy in advanced-breast-cancer patients.

The present finding that patients with a short DFI had significantly higher tumor levels of VEGF as compared with those with a longer DFI, is consistent with the results of earlier reports in which high tumor levels of VEGF were found to be related to a poor prognosis in primary breast cancer (20, 22–25). We observed in our study with 845 recurrences that the tumors that had metastasized to viscera as first site of relapse had higher levels of VEGF as compared with those that had metastasized to soft tissues or bone. These results are in accordance with those recently reported by Linderholm *et al.* (30) in a study involving 362 node-positive patients of whom 130 showed a recurrence during follow-up. However, although not comparable to the results of VEGF measurements as performed by us and Linderholm *et al.* (30), in an earlier study of Gasparini *et al.*, including 254 node-negative patients of whom 46 relapsed (51), no relationship between MVD and first site of relapse was observed. There is no consensus in the literature with respect to the association of VEGF with ER and/or PgR. In the present study, we found significant but weak negative correlations between the levels of VEGF and ER or PgR, in analogy to some studies (28, 30) but in contrast to others (22, 23, 25). A positive relationship between VEGF and ER expression has been reported as well (19). It should be emphasized that, in this latter study, VEGF and ER were assessed by immunohistochemistry, whereas in the previous studies, tumor extracts were analyzed (22, 23, 25, 28, 30). The reasons for the discrepant findings may be the different methodologies used to assess VEGF and hormone receptor levels and the different patient populations included in the various studies (node-negative, node-positive, unselected breast cancer patients, and primary and advanced breast cancer patients). These weak negative correlations (or absence of correlations) between VEGF and ER and PgR in the primary breast tumors is surprising in view of the evidence that VEGF production in breast cancer cells is stimulated by estrogens and progestins *in vitro* and/or *in vivo* (33–35). One plausible explanation for this apparent discrepancy is that, in the extracts of homogenized breast tumor tissues, additional VEGF is present that is produced by noncancer cells such as fibroblasts (15, 52, 53) and macrophages (54). In this respect, up-regulation of VEGF in mammary fibroblasts in response to hypoxia, a major inducer of VEGF in tumors (55), has been reported (56). A further explanation for the observed lack of a positive relation between VEGF and ER and PgR could be a constitutive expression of high levels of VEGF by ER-negative breast cancer cells (57), whereas its expression is under the control of estrogen in the better differentiated ER-positive breast cancer cells. Moreover, *VEGF* gene expression is regulated by many cytokines or growth factors (58), with expression levels that vary widely between ER-positive and ER-negative breast cancer cells (59).

In univariate analysis of the efficacy of response on first-line tamoxifen treatment in patients with advanced breast cancer, a high level of tumor VEGF was significantly related to a poor outcome. In multivariate analysis for response, this relationship remained significant, even when corrected for classical predictive factors for response, including hormone receptor status. Similarly, the duration of response and the length of PFS and PR-OS were significantly reduced in patients with high tumor levels of VEGF. In our exploratory analysis, the predictive value of VEGF for the outcome on tamoxifen treatment appeared to be confined to patients with ER-positive tumors. The mechanisms by which high VEGF levels, or high angiogenesis, in ER-positive tumors are associated with a poor outcome on tamoxifen treatment can only be speculated on. Possible mechanisms that have been put forward by Gasparini *et al.* (27), involve the production of growth factors by stroma and vessels that stimulate the tumor cells directly, such that the inhibitory effect of tamoxifen on tumor growth is bypassed by paracrine tumor growth stimulatory pathways. Furthermore, it was argued that stromal cells, such as macrophages, produce growth factors that stimulate both the tumor and the vessels, resulting in high angiogenesis with hormone resistance (27). A further

possibility is that, under tamoxifen pressure, the tumor cells as well produce growth factors that potentially stimulate, directly or indirectly, angiogenesis. In this respect, tamoxifen has been shown to increase tumor growth factor β1 expression by breast tumor cells *in vitro* (60) as well as stromal fibroblasts *in vivo* (61). Tumor growth factor β1 in its turn is capable of increasing VEGF production by breast cancer cells (57) and breast tumor-associated macrophages (62). Moreover, VEGF production increases to support the survival of endothelial cells under unfavorable conditions (63), such as hypoxia (64) and high cell density (65). Therefore, it is tempting to speculate that failure to respond to tamoxifen treatment results in part from a stress (tamoxifen?)-induced endothelial cell survival. Our present results on the relationship between VEGF and tamoxifen resistance in clinically advanced breast cancer cannot directly be compared with those of others because published data are lacking. There are two studies available showing an adverse relationship between the primary tumor level of VEGF and the length of RFS and OS after adjuvant tamoxifen therapy in ER-positive node-positive primary breast cancer patients (28, 30). Furthermore, for this same patient group there are two published studies showing an inverse association between MVD and prognosis after adjuvant tamoxifen treatment (26, 27). In all of these studies, the discriminatory power of VEGF or MVD were of similar size as has been reported for untreated node-negative breast cancer patients (8, 23–25). Therefore, from these studies, no conclusion on the efficacy of adjuvant tamoxifen treatment in relation to angiogenesis or VEGF expression can be made because of the lack of direct comparison with untreated control groups.

Similar to its association with a poor outcome on tamoxifen therapy, we found high tumor-VEGF levels to be associated with a poor rate of response and a short PFS and PR-OS, on chemotherapy given for advanced breast cancer. In our exploratory analysis, this relationship seemed to be confined to ER-negative tumors. Our results cannot be compared with those in the literature because this is the first study on tumor-VEGF levels and the efficacy of chemotherapy in advanced breast cancer patients. There is, however, one study on the (lack of a) relationship between MVD and the efficacy of doxorubicin monotherapy in patients with locally advanced breast cancer (66), and there are a few studies (partly conflicting with respect to PFS and PR-OS) available overall suggesting an adverse relation between MVD (28, 29, 67) or VEGF (30) and the efficacy of adjuvant polychemotherapy in primary breast cancer. Similar to the studies exploring the relationship between MVD or VEGF with the efficacy of adjuvant tamoxifen treatment, these adjuvant chemotherapy studies are not conclusive as well because no untreated control groups could be included. The question remains why high tumor levels of VEGF are associated with a poor response to chemotherapy in patients with advanced breast cancer. One explanation could be that VEGF by inducing endothelial cell proliferation indirectly contributes to the drug-resistant phenotype of a tumor via the expression of drug-resistance-associated proteins such as glutathione *S*-transferase-π (68).

In conclusion, our exploratory analysis suggests that for patients with a high tumor-VEGF level, treatment with tamoxifen or chemotherapy alone may not prove to be beneficial to the patient with advanced breast cancer. It seems reasonable to postulate that tumors of this type may be responsive to angiogenesis inhibitors given alone or in combination with conventional anticancer treatments. In particular, patients with ER-positive tumors, combined with high levels of VEGF, might benefit from a combination of tamoxifen with an antiangiogenic treatment.

ACKNOWLEDGMENTS

We thank the community hospitals in the region, especially the surgeons and internists of the St. Clara Hospital, Ikazia Hospital, and St. Franciscus Gasthuis at Rotterdam, and the Ruwaard van Putten Hospital at Spijkenisse, for their assistance in collecting the patients' clinical follow-up data.

REFERENCES

1. Folkman, J. What is the evidence that tumors are angiogenesis dependent? J. Natl. Cancer Inst. (Bethesda), *82:* 4–6, 1990.
2. Folkman, J. Tumor angiogenesis: therapeutic implications. N. Engl. J. Med., *285:* 1182–1186, 1971.
3. Folkman, J. Clinical applications of research on angiogenesis. N. Engl. J. Med., *333:* 1757–1763, 1995.
4. Dameron, K. M., Volpert, O. V., Tainsky, M. A., and Bouck, N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science (Wash. DC), *265:* 1582–1584, 1994.
5. Carmeliet, P., and Jain, R. K. Angiogenesis in cancer and other diseases. Nature (Lond.), *407:* 249–257, 2000.
6. Fidler, I. J., and Ellis, L. M. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell, *79:* 185–188, 1994.
7. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. N. Engl. J. Med., *324:* 1–8, 1991.
8. Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N., Moore, D. H., Meli, S., and Gasparini, G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J. Natl. Cancer Inst. (Bethesda), *84:* 1875–1887, 1992.
9. 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 metastasis and survival in breast cancer. Lancet (N. Am. Ed.), *340:* 1120–1124, 1992.
10. Guidi, A. J., Berry, D. A., Broadwater, G., Perloff, M., Norton, L., Barcos, M. P., and Hayes, D. F. Association of angiogenesis in lymph node metastasis with outcome of breast cancer. J. Natl. Cancer Inst. (Bethesda), *92:* 486–492, 2000.
11. Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science (Wash. D C), *219:* 983–985, 1983.
12. 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.
13. Veikkola, T., Karkkainen, M., Claesson-Welsh, L., and Alitalo, K. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res., *60:* 202–212, 2000.
14. Neufeld, G., Cohen, T., Gengrinovitch, P., and Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J., *13:* 9–22, 1999.
15. Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E. C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R. K., and Seed, B. Tumor induction of VEGF promoter activity in stromal cells. Cell, *94:* 715–725, 1998.
16. De Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science (Wash. D C), *255:* 989–991, 1992.
17. Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Böhlen, P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem. Biophys. Res. Commun., *187:* 1579–1586, 1992.
18. Yamamoto, Y., Toi, M., Kondo, S., Matsumoto, T., Suzuki, H., Kitamura, M., Tsuruta, K., Taniguchi, T., Okamoto, A., Mori, T., Yoshida, M., Ikeda, T., and Tominaga, T. Concentrations of vascular endothelial growth factor in the sera of normal controls and cancer patients. Clin. Cancer Res., *2:* 821–826, 1996.
19. Adams, J., Carder, P. J., Downey, S., Forbes, M. A., MacLennan, K., Allgar, V., Kaufman, S., Hallam, S., Bicknell, R., Walker, J. J., Cairnduff, F., Selby, P. J., Perren, T. J., Lansdown, M., and Banks, R. E. Vascular endothelial growth factor (VEGF) in breast cancer: comparison of plasma, serum, and tissue VEGF and microvessel density and effects of tamoxifen. Cancer Res., *60:* 2898–2905, 2000.
20. Toi, M., Inada, K., Suzuki, H., and Tominaga, T. Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. Breast Cancer Res. Treat., *36:* 193–204, 1995.
21. Obermair, A., Kucera, E., Mayerhofer, K., Speiser, P., Seifert, M., Czerwenka, K., Kaider, A., Leodolter, S., Kainz, C., and Zeillinger, R. Vascular endothelial growth factor (VEGF) in human breast cancer: correlation with disease-free survival. Int. J. Cancer, *74:* 455–458, 1997.
22. Relf, M., LeJeune, S., Scott, P. A., Fox, S., Smith, K., Leek, R., Moghaddam, A., Whitehouse, R., Bicknell, R., and Harris, A. L. Expression of the angiogeneic 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. Cancer Res., *57:* 963–969, 1997.
23. Gasparini, G., Toi, M., Gion, M., Verderio, P., Dittadi, R., Hanatani, M., Matsubara, I., Vinante, O., Bonoldi, E., Boracchi, P., Gatti, C., Suzuki, H., and Tominaga, T. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J. Natl. Cancer Inst. (Bethesda), *89:* 139–147, 1997.

24. Linderholm, B., Tavelin, B., Grankvist, K., and Henriksson, R. Vascular endothelial growth factor is of high prognostic value in node-negative breast carcinoma. J. Clin. Oncol., *16:* 3121–3128, 1998.
25. Eppenberger, U., Kueng, W., Schlaeppi, J. M., Roesel, J. L., Benz, C., Mueller, H., Matter, A., Zuber, M., Luescher, K., Litschgi, M., Schmitt, M., Foekens, J. A., and Eppenberger-Castori, S. Markers of tumor angiogenesis and proteolysis independently define high- and low-risk subsets of node-negative breast cancer patients. J. Clin. Oncol., *16:* 3129–3136, 1998.
26. Macauley, V. M., Fox, S. B., Zhang, H., Whitehouse, R. M., Leek, R. D., Gatter, K. C., Bicknell, R., and Harris, A. L. Breast cancer angiogenesis and tamoxifen resistance. Endocr. Rel. Cancer, *2:* 97–103, 1995.
27. Gasparini, G., Fox, S. B., Verderio, P., Bonoldi, E., Bevilacqua, P., Boracchi, P., Dante, S., Marubini, E., and Harris, A. L. Determination of angiogenesis adds information to estrogen receptor status in predicting the efficacy of adjuvant tamoxifen in node-positive breast cancer patients. Clin. Cancer Res., *2:* 1191–1198, 1996.
28. Gasparini, G., Toi, M., Miceli, R., Vermeulen, P. B., Dittadi, R., Biganzoli, E., Morabito, A., Fanelli, M., Gatti, C., Suzuki, H., Tominaga, T., Dirix, L. Y., and Gion, M. Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy or hormone therapy. Cancer J. Sci. Am., *5:* 101–111, 1999.
29. Viens, P., Jacquemier, J., Bardou, V. J., Bertucci, F., Penault-Llorca, F., Puig, B., Gravis, G., Oziel-Taieb, S., Resbeut, M., Houvenaeghel, G., Camerlo, J., Birbaum, D., Hassoun, J., and Maraninchi, D. Association of angiogenesis and poor prognosis in node-positive patients receiving anthracycline-based adjuvant chemotherapy. Breast Cancer Res. Treat., *54:* 205–212, 1999.
30. Linderholm, B., Grankvist, K., Wilking, N., Johansson, M., Tavelin, B., and Henriksson, R. Correlation of vascular endothelial growth factor content with recurrences, survival, and first relapse site in primary node-positive breast carcinoma after adjuvant treatment. J. Clin. Oncol., *18:* 1423–1431, 2000.
31. Mueller, M. D., Vigne, J-L., Minchenko, A., Lebovic, D. I., Leitman, D. C., and Taylor, R. N. Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors α and β. Proc. Natl. Acad. Sci. USA, *97:* 10972–10977, 2000.
32. Hyder, S. M., Nawaz, Z., Chiappetta, D., and Stancel, M. Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res., *60:* 3183–3190, 2000.
33. Hyder, S. M., Murthy, L., and Stancel, G. M. Progestin regulation of vascular endothelial growth factor in human breast cancer cells. Cancer Res., *58:* 392–395, 1998.
34. Ruohola, J. K., Valve, E. M., Karkkainen, M. J., Joukov, V., Alitalo, K., and Harkonen, P. L. Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Mol. Cell. Endocrinol., *149:* 29–40, 1999.
35. Nakamura, J., Savinov, A., Lu, Q., and Brodie, A. Estrogen regulates vascular endothelial growth/permeability factor expression in 7,12-dimethyl-benz(*a*)anthracene-induced rat mammary tumors. Endocrinology, *137:* 5589–5596, 1996.
36. Ravdin, P. M., Green, S., Dorr, T. M., McGuire, W. L., Fabian, C., Pugh, R. P., Carter, R. D., Rivkin, S. E., Borst, J. R., Belt, R. J., Metch, B., and Osborne, C. K. Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: results of a prospective Southwest Oncology Group study. J. Clin. Oncol., *10:* 1284–1291, 1992.
37. Foekens, J. A., Portengen, H., Look, M. P., van Putten, W. L. J., Thirion, B., Bontenbal, M., and Klijn, J. G. M. Relationship of PS2 with response to tamoxifen therapy in patients with recurrent breast cancer. Br. J. Cancer, *70:* 1217–1223, 1994.
38. EORTC Breast Cancer Cooperative Group. Revision of the standards for the assessment of hormone receptors in human breast cancer; report of the second E. O. R. T. C. Workshop, held on 16–17 March, 1979, in the Netherlands Cancer Institute. Eur. J. Cancer, *16:* 1513–1515, 1980.
39. Foekens, J. A., Portengen, H., van Putten, W. L. J., Peters, H., Krijnen, H. L. J. M., Alexieva-Figusch, J., and Klijn, J. G. M. Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols. Cancer Res., *49:* 5823–5828, 1989.
40. Span, P. N., Grebenchtchikov, N., Geurts-Moespot, J., Westphal, J. R., Lucassen, A. M. J., and Sweep, C. G. J. EORTC Receptor and Biomarker Study Group Report: a sandwich enzyme-linked immunosorbent assay for vascular endothelial growth factor in blood and tumor tissue extracts. Int. J. Biol. Markers, *15:* 184–191, 2000.
41. Barlow, R. E., Bartelomew, D. J., Bremmer, J. M., and Brunck, H. D. Statistical Interference under Order Restrictions. London: John Wiley & Sons, 1972.
42. Kaplan, E. L., and Meier, P. Non-parametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.
43. Ferrara, N., and Alitalo, K. Clinical applications of angiogenic growth factors. Nat. Med., *5:* 1359–1364, 1999.
44. Gasparini, G., and Harris, A. L. Clinical importance of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool. J. Clin. Oncol., *13:* 765–782, 1995.
45. Shweiki, D., Itin, A., Neufeld, G., Gitay-Goren, H., and Keshet, E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J. Clin. Investig., *91:* 2235–2243, 1993.
46. Kristensen, C. A., Hamberg, L. M., Hunter, G. J., Roberge, S., Kierstead, D., Wolf, G. L., and Jain, R. K. Changes in vascularization in human breast cancer xenografts responding to antiestrogen therapy. Neoplasia, *1:* 518–525, 1999.
47. Lau, D. H., Xue, L., Young, L. J., Burke, P. A., and Cheung, A. T. Paclitaxel (Taxol): an inhibitor of angiogenesis in a highly vascularized transgenic breast cancer. Cancer Biother. Radiopharm., *14:* 31–36, 1999.
48. Browder, T., Butterfield, C. E., Kräling, B. M., Shi, B., Marshall, B., O'Reilly, M. S., and Folkman, J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res., *60:* 1878–1886, 2000.
49. Makris, A., Powles, T. J., Kakolyris, S., Dowsett, M., Ashley, S. E., and Harris, A. L. Reduction in angiogenesis after neoadjuvant chemoendocrine therapy in patients with operable breast carcinoma. Cancer (Phila.), *85:* 1996–2000, 1999.
50. Gagliardi, A., Hennig, B., and Collins, D. C. Antiestrogens inhibit endothelial cell growth stimulated by angiogenic growth factors. Anticancer Res., *16:* 1101–1106, 1996.
51. Gasparini, G., Weidner, N., Bevilacqua, P., Maluta, S., Dalla Palma, P., Caffo, O., Barbareschi, M., Boracchi, P., Marubini, E., and Pozza, F. Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. J. Clin. Oncol., *12:* 454–466, 1994.
52. Senger, D. R., Van de Water, L. VEGF expression by epithelial and stromal cell compartments. Am. J. Pathol., *157:* 1–3, 2000.
53. Speirs, V., and Atkin, S. L. Production of VEGF and expression of the VEGF receptors Flt-1 and KDR in primary cultures of epithelial and stromal cells derived from breast tumours. Br. J. Cancer, *80:* 898–903, 1999.
54. Lewis, J. S., Landers, R. J., Underwood, J. C. E., Harris, A. L., and Lewis, C. E. Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. J. Pathol., *192:* 150–159, 2000.
55. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (Lond.), *359:* 843–845, 1992.
56. Hlatky, L., Tsionou, C., Hahnfeldt, P., and Coleman, C. N. Mammary fibroblasts may influence breast tumor angiogenesis via hypoxia-induced vascular endothelial growth factor up-regulation and protein expression. Cancer Res., *54:* 6083–6086, 1994.
57. Donovan, D., Harmey, J. H., Toomey, D., Osborne, D. H., Redmond, H. P., and Bouchier-Hayes, D. J. TGF β-1 regulation of VEGF production by breast cancer cells. Ann. Surg. Oncol., *4:* 621–627, 1997.
58. Ferrara, N. Vascular endothelial growth factor and the regulation of angiogenesis. Recent Prog. Horm. Res., *55:* 15–35, 2000.
59. Dickson, R. B., and Lippman, M. E. Growth factors in breast cancer. Endocr. Rev., *16:* 559–589, 1995.
60. Chen, H., Tritton, T. R., Kenny, N., Absher, M., and Chiu, J. F. Tamoxifen induces TGF-β 1 activity and apoptosis of human MCF-7 breast cancer cells *in vitro*. J. Cell Biochem., *61:* 9–17, 1996.
61. Butta, A., MacLennan, K., Flanders, K. C., Sacks, N. P., Smith, I., McKinna, A., Dowsett, M., Wakefield, L. M., Sporn, M. B., Baum, M., and Colletta, A. A. Induction of transforming growth factor β_1 in human breast cancer *in vivo* following tamoxifen treatment. Cancer Res., *52:* 4261–4264, 1992.
62. Harmey, J. H., Dimitriadis, E., Kay, E., Redmond, H. P., and Bouchier-Hayes, D. Regulation of macrophage production of vascular endothelial growth factor (VEGF) by hypoxia and transforming growth factor β-1. Ann. Surg. Oncol., *5:* 271–278, 1998.
63. Bruns, C. J., Liu, W., Davis, D. W., Shaheen, R. M., McConkey, D. J., Wilson, M. R., Bucana, C. D., Hicklin, D. J., and Ellis, L. M. Vascular endothelial growth factor is an *in vivo* survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. Cancer (Phila.), *89:* 488–499, 2000.
64. Shweiki, D., Neeman, M., Itin, A., and Keshet, E. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. Proc. Natl. Acad. Sci. USA, *92:* 768–772, 1995.
65. Koura, A. N., Liu, W., Kitadai, Y., Singh, R. K., Radinsky, R., and Ellis, L. M. Regulation of vascular endothelial growth factor expression in human carcinoma cells by cell density. Cancer Res., *56:* 3891–3894, 1996.
66. Paulsen, T., Aas, T., Børresen, A-. L., Verhaug, J. E., Lønning, P. E., and Akslen, L. A. Angiogenesis does not predict clinical response to doxorubicin monotherapy in patients with locally advanced breast cancer. Int. J. Cancer, *74:* 138–140, 1997.
67. Protopapa, E., Delides, G. S., and Revesz, L. Vascular density and the response of breast carcinomas to mastectomy and adjuvant chemotherapy. Eur. J. Cancer, *29A:* 1391–1393, 1993.
68. Terrier, P., Townsend, A. J., Coidre, J. M., Triche, T. J., and Cowan, K. H. An immunohistochemical study of π class glutathione *S*-transferase expression in normal human tissue. Am. J. Pathol., *137:* 845–853, 1990.