Prognostic Significance of Vascular Endothelial Growth Factor Protein in Node-Negative Breast Carcinoma

Giampietro Gasparini, Masakazu Toi, Massimo Gion, Paolo Verderio, Ruggero Dittadi, Mitsuya Hanatani, Isamu Matsubara, Orazio Vinante, Emanuela Bonoldi, Patrizia Boracchi, Carlo Gatti, Hideo Suzuki, Takeshi Tominaga*

Background: The clinical outcome is generally positive for patients with node-negative breast carcinoma (i.e., those who do not have detectable metastases in the lymph nodes) who have been treated with surgery or surgery plus radiation therapy. In about 30% of the patients, however, the disease recurs, and they are at risk of death. Determination of valid new prognostic indicators would improve the ability to identify patients at high risk of recurrence. Breast cancer can entail substantial development of new blood vessels within the tumor tissue, and it is known that the growth and metastasis of solid tumors are dependent on such angiogenesis. The conversion of tumor cells to an angiogenic phenotype may be preceded by a change in the balance of angiogenic growth factors and angiogenesis inhibitors. Purpose: This study was conducted to determine if the levels of vascular endothelial growth factor (VEGF) protein, a potent endothelial growth factor and mediator of vascular permeability and angiogenesis, measured in the primary tumors of women with node-negative breast cancer are associated with known prognostic factors and patient survival. Methods: By use of a selective enzymatic immunoassay, levels of VEGF protein were measured in cytosolic extracts of primary tumor tissue surgically obtained from 260 women with node-negative breast carcinoma who had been treated with surgery with or without radiation therapy but not with adjuvant therapy and who had been followed for a median time of 66 months. The relationships between VEGF concentrations and other prognostic dichotomous variables or clinical outcome were tested by the use of the Kolmogorov–Smirnov test and univariate and multivariate Cox analyses, respectively. The relationship between VEGF and hormone receptors (i.e., those for estrogen and progesterone) was examined by the use of Spearman’s correlation analyses. All P values resulted from the use of two-sided statistical tests. Results: Tumors from 247 (95%) of the 260 patients had detectable VEGF, ranging in concentration from 5.0 to 6523 pg/mg protein (median, 126.25 pg/mg protein). No statistically significant associations were found between VEGF and the other prognostic factors (e.g., age, menopausal status, histologic tumor type, tumor size, and hormone receptors) examined. Levels of VEGF were found to be prognostic for both relapse-free and overall survival in univariate and multivariate analyses (likelihood ratio tests; all four P values <.001). In the multivariate analysis, the first-order interaction term of VEGF and estrogen receptor was also prognostic for overall survival (likelihood ratio test; P = .05). Conclusions: The results show that cytosolic levels of VEGF in tumor tissue samples are indicative of prognosis for patients with node-negative breast carcinoma. [J Natl Cancer Inst 1997;89:139-47]

Angiogenesis is necessary for the growth and invasiveness of primary tumors (1) and is an integral part of the cascade of biologic events involved in tumor metastasis (2). The mechanisms by which neovascularization stimulates tumor progression are as follows: 1) delivery of nutrients and oxygen necessary for tumor cells to grow (perfusion effect) (3), 2) facilitation of penetration of tumor cells through the vessel wall and their transport to distant organs (metastatic effect) (4), and 3) secretion of some cytokines (i.e., interleukins 1-6 and 8) and growth factors (granulocyte colony-stimulating factor, angiogenic peptides, transforming growth factor-β1, and insulin-like growth factors) (J,2,5) from endothelial cells that directly stimulate tumor cells (paracrine effect) (5). The switch to the angiogenic phenotype may be due to the overexpression of a number of endothelial growth factors and/or to the reduced expression of endogenous angiogenesis inhibitors (1).

Vascular endothelial growth factor (VEGF; also known as vascular permeability factor) is a potent and widely distributed angiogenic peptide (6). This growth factor is a dimeric 34-42 kd glycosylated basic protein, with moderate affinity for heparin, encoded in four molecular isoforms (7). The two larger isoforms (VEGF189 and 206) remain cell associated, whereas the smaller isoforms (VEGF121 and 165) are secreted as soluble molecules (8). The latter isoforms induce their angiogenic effects by binding to the specific transmembrane tyrosine kinase receptors KDR/flk-1 and flt-1, also called VEGFR-1 and VEGFR-2, respectively, which are selectively expressed on vascular blood

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See “Notes” following “References.”
endothelial cells (6). VEGF expression increases in response to several stimuli, such as hypoxia (9); certain oncogene products, including mutant ras (10); and over-expression of transforming growth factor-α (11).

Immunohistochemical and in situ messenger RNA hybridization studies (12-15) have shown that VEGF and its receptors are overexpressed in several human tumors. In particular, VEGF expression is present in approximately half of invasive breast cancers, and it is significantly associated with high intratumoral microvessel density (16) and prognosis (17).

VEGFs are multifunctional molecules that have been implicated in vasculogenesis (18,19), endothelial cell proliferation and migration (20), vascular permeability (21), and stromal degradation through the activation of some proteolytic enzymes involved in tumor invasiveness and angiogenesis (22).

In vivo experimental studies (23,24) have shown that human tumor cell lines that have been transfected with VEGF exhibit enhanced biologic aggressiveness, vascularity, and metastatic ability. Conversely, VEGF-deficient embryonic stem cells exhibit reduced tumorigenesis in nude mice when compared with the appropriate control cells (19). Previous studies [reviewed in (25)] where angiogenesis was assessed measuring intratumoral microvessel density have shown that human breast cancer presents a heterogeneous and variable vascularization. Furthermore, several (26,27) but not all [reviewed in (28)] studies have demonstrated that the degree of vascularization is of prognostic value for patients with node-negative breast carcinoma.

In general, patients with node-negative breast carcinoma treated with adequate surgery (with or without radiation therapy) have a good prognosis. However, in approximately 30% of the patients the disease recurs, and they are at risk of death. The assessment of valid new prognostic indicators would improve the clinical management of patients with node-negative breast carcinoma by the identification of the subgroup of patients at high risk of recurrence (26).

We conducted this study to assess one of the possible angiogenic pathways responsible for neovascularization of node-negative breast carcinoma and its clinical significance. Therefore, we determined the concentrations of VEGF protein in the cytosol of primary tumors and examined their relationship with the known prognostic indicators and clinical behavior in a series of 260 women with node-negative breast carcinoma.

**Methods**

**Study Population**

In the study, we included a cohort of 260 consecutive patients with node-negative breast carcinoma who were referred to the Laboratory of Tumoral Markers of the Regional Hospital of Venice from collaborating institutions in Italy. In all patients, a verbal informed consent was obtained for therapy. Criteria of eligibility were as follows: histologic diagnosis of invasive breast cancer without axillary lymph node involvement (N0) (with at least axillary lymph node levels I and II being free of metastases), primary tumors Tx or T1 (any subcategory), T2a and T3a, no distant metastasis (M0), and no other previous or concomitant invasive cancer and sufficient pathologic frozen material for the determination of concentrations of VEGF and hormone receptors in cytosolic extracts.

We adopted the 1989 pathologic staging system of the American Joint Committee on Cancer (29). Briefly, eligible primary tumors were classified as follows: Tx = primary tumor cannot be assessed; T1 (any category admitted) = tumor 2 cm or less in greatest dimension; T2a = tumor greater than 2 cm but not greater than 5 cm in greatest dimension, with no fixation to pectoral fascia or muscle; and T3a = tumor greater than 5 cm in greatest dimension, with no fixation to pectoral fascia or muscle.

All of the patients had surgical therapy consisting of modified (Patey) radical mastectomy (125 patients) (30) or conservative surgery (quadrantectomy with axillary lymph node dissection) (135 patients) (31). The patients treated with the latter modality also received a 5-6-week course of radiation therapy on the residual breast within 6 weeks of surgery. Radiation therapy consisted of 50 Gy administered in two tangentially opposing fields by the use of high-energy equipment in addition to a boost of 10 Gy on the scar with orthovoltage equipment (31). Conservative surgery and radiation therapy were given as an alternative to mastectomy in those patients with primary tumors less than 3 cm in diameter. The patients with T1-T3a N0 M0 tumors have been enrolled in a trial of follow-up surveillance up to recurrence and, in accordance with institutional guidelines, none of these patients received adjuvant therapy.

**Follow-up**

Every 4 months a physical examination was performed in all study subjects at the participating institutions. Radiographic studies, including chest roentgenogram, mammography, and ultrasound examination of the liver, were carried out twice a year during the first 3 years and then yearly. Relapse-free and overall survival were calculated as the period from surgery to the date of first recurrence of breast cancer or death, respectively. Primary treatment failure was defined as the first documented evidence of new disease manifestation(s) in locoregional area(s), distant organs, or a combination of the above.

**Histopathologic Studies**

Surgical specimens were fixed in formalin, embedded in paraffin, cut into 4-μm-thick sections prior to histologic examination or were snap frozen in liquid nitrogen, stored at −70 °C, and later thawed on ice and analyzed for levels of the hormone receptors.

Tumor histologic types were classified by the use of conventional criteria (32), and all identifiable lymph nodes were histologically examined. For preparation of cytosolic extracts, the frozen tissue samples were pulverized by the use of a micro-disemembrator (Braun, Melsungen, Federal Republic of Germany) and were then homogenized with 10 vol of cold (i.e., 4 °C) 10 mM phosphate buffer (pH 7.4) containing 1.5 mM EDTA, 10 mM sodium molybdate, 3 mM NaF, and 10% glycerol. Low salt extract (cytosol) was obtained by centrifugation at 100 000g for 1 hour at 4 °C. The supernatant fraction was appropriately diluted according to the protein concentration and was used for the VEGF and specific hormone-receptor assays.

**Determination of VEGF Concentration**

The concentrations of VEGF were determined by the use of a colorimetric VEGF enzymatic immunoassay. This assay employed an antihuman VEGF polyclonal antibody (Toagosei Co. Ltd., lot No. 091895) prepared from the serum of rabbits that had been immunized with recombinant human VEGF121–glutathione S-transferase fusion protein. To make recombinant VEGF protein, complementary DNA encoding VEGF121 was prepared by the use of total RNA from human chronic myelogenous leukemia K562 cells, then a restriction fragment of 0.5-kd in size containing the coding sequence was obtained by digestion of the DNA with EcoRI and NcoI endonucleases and ligated into the appropriate site of the vector of the glutathione S-transferase fusion gene system (Pharmacia Biotech, Inc., Tokyo, Japan). Recombinant VEGF121–glutathione S-transferase fusion protein was isolated from a culture of Escherichia coli HB101 (Japan Collection of micro-organisms, Tokyo) that had been transfected with the construct. The anti-VEGF antibody is known to react with the VEGF121 and VEGF165 isoforms, and it has been suggested that it should be able to recognize the other two isoforms because of the shared amino acid sequences (33,34). Reverse transcription–polymerase chain reaction analysis has shown that VEGF121 and VEGF165 are expressed in human breast carcinomas (17). We have tested and confirmed the specificity of the anti-VEGF antibody in western blotting experiments (data not shown).

Ninety-six-well microtiter plates (Lab-systems, Helsinki, Finland) were coated with 10 ng/mL of the purified anti-VEGF antibody in 0.1 M NaCl and 0.25 M carbonate buffer (pH 9.0) and then blocked with 1% bovine serum albumin, 0.2 M carbonate buffer (pH 9.5), 0.1 M NaCl, and 0.1% NaN3. Aliquots (100 μL) of tumor cytosolic extract samples serially diluted with human recombinant

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VEGF$_{121}$ (standards) were added to the wells and incubated for 1 hour at 22 °C. After six washes in bovine serum albumin, 100 μL of peroxidase-conjugated Fab’ of the anti-VEGF antibody was added to each well and incubated for 1 hour at 22 °C. The enzyme reaction was carried out at 22 °C for 30 minutes with o-phenylenediamine (Sigma Chemical Co., St. Louis, MO) as a substrate. The 490-nm absorbance was measured by a microplate reader (Molecular Devices Corp., Sunnyvale, CA), and the cytosolic concentrations of VEGF were estimated from the standard curve determined from the serially diluted VEGF.

Aliquots of serially diluted recombinant human VEGF$_{121}$, ranging from 3800 to 0 pg/mL, were subjected to the VEGF enzymatic immunoassay to assess the analytic sensitivity of the assay. Five independent measurements were carried out for each concentration, and the mean of the readings was plotted as a standard curve. The regression formula ($Y = 3007 + 5.34 \times 10^{-4}X - 3.71 \times 10^{-8}X^2$ [r = 1.000]) (where $X$ = standard VEGF concentrations, $Y$ = absorbance at 490 nm by plate reader, and r = Spearman’s correlation coefficient between X and Y) was determined. The minimal detectable dose was defined as the value of ±2 standard deviation (SD) above the zero standard; this limit value was found to be 5.0 pg/mg protein.

### Determination of Hormone Receptor Levels

Estrogen receptor and progesterone receptor levels were measured by the dextran-coated charcoal method as recommended by the European Organization for Research and Treatment of Cancer (35) by the use of a single-saturating dose technique with 4 mM concentration of tritium-labeled steroid hormone.

### Statistical Analyses

The linear relationship between VEGF (continuous variable) and the other continuous variables (hormone receptors) in their original scale of values was investigated by use of Spearman’s correlation analyses.

The distribution of VEGF within the modalities of each of the other variables was compared with the Kolmogorov–Smirnov test (36). For the sake of simplicity, only two modalities were adopted for each variable. This is a distribution-free test; the null hypothesis is the equality of the two distributions of VEGF, and the general alternative is that they are different. The Kolmogorov–Smirnov statistic is that value that measures the maximum difference between the two empirical distribution functions of VEGF. The corresponding asymptotic statistic (KS$_a$) is obtained by the product of the Kolmogorov–Smirnov statistic with the square root of the total number of subjects. For each value of KS$_a$, the probability of observing a larger value of the statistic test under the null hypothesis is provided.

The patterns of overall and relapse-free survival were estimated by means of the product-limit method (Kaplan–Meier). The role of each prognostic variable in the null hypothesis is provided. The probability of observing a larger value of the statistic test under the null hypothesis of the corresponding asymptotic statistic (KS$_a$) is provided by the product of the Kolmogorov–Smirnov statistic with the square root of the total number of subjects. For each value of KS$_a$, the probability of observing a larger value of the statistic test under the null hypothesis is provided.

The interactions that were considered to be clinically relevant were VEGF/estrogen receptor and VEGF/progesterone receptor. Because of the relatively low number of events, these interactions were first investigated in a bivariate fashion, resorting to a regression Cox model that considered the main effects and the interaction terms between them.

In the multivariate analysis, we included the variables that were statistically significantly prognostic in univariate analysis (at the 15% significance level) and the first-order interaction terms that were statistically significant in the bivariate analyses. A final, more parsimonious, model was then obtained by the use of a backward selection procedure in which only the variables reaching the more conventional significance level of 5% were retained. All of the $P$ values presented were obtained by two-sided tests.

### Results

Overall, we found that 257 breast cancers had measurable cytosolic levels of VEGF protein, including 10 tumors with concentrations below the detection limits of the method (5 pg/mg protein), ranging from 0.7 to 4.0 pg/mg protein.

Therefore, 247 (95%) of the 260 patients with node-negative breast carcinoma had detectable concentrations of VEGF protein in their tumors. In this series, a wide range of concentrations of VEGF in the cytosol, ranging from 5.0 to 6523 pg/mg protein (median value, 126.25 pg/mg protein), was observed. The intra-assay coefficient of variation was less than 9.7% through the entire range.

Table 1 shows the characteristics of the patients studied.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients (%)</th>
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<tbody>
<tr>
<td>Patients enrolled</td>
<td>260</td>
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<tr>
<td>Median age, y (range):</td>
<td>60 (34-85)</td>
</tr>
<tr>
<td>Menopausal status</td>
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<td>Premenopausal</td>
<td>72 (27.6)</td>
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<td>16 (6.1)</td>
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<td>Postmenopausal</td>
<td>172 (66.3)</td>
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<td>Ductal invasive</td>
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<td>Lobular invasive</td>
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<tr>
<td>Others</td>
<td>34 (13.4)</td>
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<td>Tumor size* and other measured parameters</td>
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<tr>
<td>T1</td>
<td>133 (51.0)</td>
</tr>
<tr>
<td>a</td>
<td>0</td>
</tr>
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<td>b</td>
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<td>c</td>
<td>128</td>
</tr>
<tr>
<td>T2a</td>
<td>96 (37.0)</td>
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<td>T3a</td>
<td>1 (5)</td>
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<td>T3x</td>
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<td>Median size, mm (range):</td>
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<td>Median value of estrogen receptor, fmol/mg protein (range):</td>
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<td>Median value of progesterone receptor, fmol/mg protein (range):</td>
<td>35 (0-1135)</td>
</tr>
<tr>
<td>Median value of vascular endothelial growth factor, pg/mg protein (range):</td>
<td>126.25 (0-6523)</td>
</tr>
</tbody>
</table>

Surgical therapy

- Radical mastectomy: 125 (48)
- Conservative + radiotherapy: 135 (52)

*Tumor size, see (29).
Using the Kolmogorov–Smirnov test, we found that VEGF distribution within the subcategories of the other dichotomous variables was not statistically significant: age (>55 versus ≤55 years: Ksa = .82; \( P = .52 \)), menopausal status (postmenopausal versus perimenopausal/premenopausal: Ksa = .70; \( P = .71 \)), histologic type (ductal versus lobular/other: Ksa = .63; \( P = .83 \)), and tumor size (T1-3a versus Tx: Ksa = .83; \( P = .49 \)).

The correlations between VEGF and hormone receptors were weak; the Spearman’s coefficients were −.0345 and −.1786 for estrogen receptor and progesterone receptor, respectively.

For both relapse-free and overall survival, residual analysis suggested a logarithmic–linear relationship between the loga-

<table>
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<th>Variable</th>
<th>Relapse-free survival</th>
<th>LRT</th>
<th>Overall survival</th>
<th>LRT</th>
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<tr>
<td></td>
<td>( \beta )</td>
<td>SE</td>
<td>( \chi^2 )</td>
<td>( P )</td>
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<td>.33</td>
<td>.57</td>
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<td>Other versus ductal</td>
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<td>.92</td>
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<tr>
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<tr>
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<td>.71</td>
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<td>Coefficient spline</td>
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<td>.258</td>
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<td>( b )</td>
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<td>.021</td>
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<td></td>
<td>( c )</td>
<td>( -0.475 )</td>
<td>.198</td>
<td>5.65</td>
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<td>&gt;7 versus 0-4</td>
<td>1.49</td>
<td>.58</td>
<td>6.64</td>
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\*\( \beta \) = beta estimate; SE = standard error; LRT = likelihood ratio test.

†Tumor size, see (29).

‡ER = estrogen receptor expressed as ln values.

§PgR = progesterone receptor expressed as ln values.

| VEGF = vascular endothelial growth factor. \( a \) = first component, \( b \) = second component, \( c \) = third component (see ‘Results’ section). Classes = the natural VEGF values corresponding to the logarithmic values are: 0-4 = 0-54 pg/mg protein; 4-5.5 = 55-244 pg/mg protein; 5.5-7 = 245-1096 pg/mg protein, and greater than 7 = greater than 1096 pg/mg protein.

To identify the appropriate regression model, we compared the likelihood ratio test obtained by using the model with four knots with that with three knots or by using the model with only the linear term, respectively. For relapse-free survival, the values of the likelihood ratio test for the three above-mentioned models were as follows: 33.2 (3 df), 26.2 (2 df), and 23.6 (1 df), respectively. For overall survival, the corresponding values for the above-mentioned models were: 20.3 (3 df), 15.9 (2 df), and 12.8 (1 df), respectively. The cubic spline approach showed that the most suitable model to describe the functional relationship between VEGF and the hazard is a spline with four knots and the linearity constraints on the tails. The equation describing the above-mentioned spline function has three terms (38), and the corresponding regression coefficients are referred to as ‘‘\( a \),’’ ‘‘\( b \),’’ and ‘‘\( c \)’’ in Tables 2 and 3. Since these regression coefficients are not directly interpretable, we provide the graphic representation of the spline function itself (Fig. 1). For both relapse-free and overall survival, three distinct components of the behavior of VEGF in relation to the linear predictor can be identified. The first component ‘‘\( a \)’’ includes VEGF values up to 90 pg/mg protein, and the curve is characterized by a slight positive trend. The second component ‘‘\( b \)’’ includes VEGF values from 100 to 1096 pg/mg protein, and the curve is characterized by a steep nonlinear positive trend. The third component ‘‘\( c \)’’ includes VEGF values greater than 1096 pg/mg protein and presents an inversion of tendency with linear predictor values.

Table 2. Univariate analysis on relapse-free and overall survival*

<table>
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multivariate analysis was carried out. In the initial model, the different outcome when compared with the reference category as 245-1096 pg/mg protein had a statistically significant difference,

\[
\text{Coefficient estimate value} = \text{reflects the pattern shown in Fig. 2.}
\]

When the median value of VEGF protein was 6523 pg/mg protein (range, 1096-6523 pg/mg protein). The protein was also a statistically significant prognostic factor for relapse-free survival. In the final model, only VEGF was a significant prognostic factor for relapse-free survival. For overall survival, the first-order interaction term of VEGF and estrogen receptor was also a statistically significant prognostic factor (Table 3).

**Discussion**

With the use of a highly sensitive enzyme immunoassay, we found that most of the primary invasive breast cancers had detectable concentrations of VEGF protein. The median VEGF cytosolic level found in the present series of patients (126.25 pg/mg protein) is comparable to that found by Toi et al. (34), who used a similar method but in samples of total tumoral tissue homogenate.

A previous study (17) assessed VEGF121 expression by the use of immunohistochemical methods and showed that this endothelial growth factor is mainly present in the cytoplasm of tumor cells and that approximately half of breast cancers are VEGF positive. A particularly high VEGF121 expression was found in tumors with elevated vascularization (16). Aberrant levels of VEGF have also been found in the serum of 10% of the patients with early stage breast cancer (40). However, the biologic significance of circulating endothelial growth factors is presently not known. In particular, it has not yet been demonstrated whether serum VEGF (40) or serum/urine basic fibroblast growth factor (41) act like hormones inducing neovascularization of tissues expressing the specific receptors. Aiello et al. (42) and Fava et al. (43) studied the biologic activity and the concentrations of VEGF in the ocular fluids of patients with retinal diseases and in the synovial fluids of arthritic tissues, respectively. These studies showed that the mean VEGF levels from patients with active proliferative diabetic retinopathy (42) or with rheumatoid arthritis (43), both being diseases characterized by abnormal neovascularization, are statistically significantly higher compared with the levels found in patients with other decreasing with the increase of VEGF values. If we consider the model with only three knots, the decreasing pattern observed after 1096 pg/mg protein disappears, but the corresponding model does not seem to be appropriate (as explained previously in “Methods” section). In any case, the patterns shown in Fig. 1 must be considered with caution, as the rather large confidence intervals show. The third component of the curve described above includes 18 patients, six of whom developed recurrence and two who died. The main characteristics of this subgroup of patients are as follows: median age, 57 years (range, 41-78 years); menopausal status, seven (39%) patients were premenopausal/perimenopausal, and 11 (61%) were postmenopausal; histologic type, ductal invasive was found in 11 (61%) case patients, lobular in two (11%) case patients, and other types in five (28%); tumor size: T1 in eight (44%), T2 in eight (44%), respectively. These studies showed that the mean VEGF levels from patients with active proliferative diabetic retinopathy (42) or with rheumatoid arthritis (43), both being diseases characterized by abnormal neovascularization, are statistically significantly higher compared with the levels found in patients with other decreasing with the increase of VEGF values. 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retinal disorders or other forms of arthritis, respectively. However, the concentrations of VEGF detected in these benign diseases are much lower than those we have found in breast cancer. Taken together, these data suggest that VEGF is implicated in the pathogenesis of some diseases where it promotes angiogenesis and vascular permeability (6). No apparent association was observed in our series of patients with node-negative breast cancers between cytosolic levels of VEGF protein and the conventional clinicopathologic prognostic factors. The lack of association of VEGF with the hormone receptors examined (i.e., those for estrogen and progesterone) suggests that this angiogenic peptide is likely to stimulate the growth of human breast cancer independently of the hormone pathways, via direct autocrine and/or paracrine stimulation on tumor cells or enhancing intratumoral vascular permeability, thus allowing more oxygen and nutrients to reach the tumor.

Previous studies have shown that the assessment of angiogenesis as determined by immunohistochemical techniques [e.g., to quantify microvessel density (25) or VEGF expression (17)] was of prognostic value in human breast cancer. However, this is the first study to report that intratumoral cytosolic levels of VEGF protein are associated with the clinical outcome of patients with a malignant tumor. The observation that most of the tumors studied had detectable VEGF suggests that the method we used is highly sensitive and more specific than immunohistochemical assays (34). In this series of patients with node-negative breast carcinoma, the relationship between VEGF levels and the risk of recurrence and death was not linear. For values of VEGF up to 1096 pg/mg protein, the probability of relapse-free and overall survival increased by the increment of the concentrations of this endothelial growth factor. However, beyond the value of 1096 pg/mg protein up to the highest values, the probability of risk decreased with the increase of the concentrations of VEGF.

Fig. 1. A = relapse-free survival; B = overall survival. Relationship between the values of concentrations of vascular endothelial growth factor (VEGF) protein with the linear predictor (logarithmic value of hazard ratio) (dark squares) of the patients to develop recurrence (A) or death (B). 95% confidence intervals are shown (fine lines).
Certain possible explanations for this observation can be proposed. In experimental solid tumors, it has been shown that the abnormal neovascularization accompanying tumor growth beyond 1 mm³ may paradoxically limit tumor progression because of enhanced vascular compression, interstitial pressure (epithelial tumors lack a functional lymphatic network), and necrosis (44,45). Another possibility is that in this subgroup of cancers that produce high levels of VEGF, the endothelial component lacks specific receptors for the soluble VEGF isoforms (22). Alternatively, in these tumors, the high production of angiogenic factors may be locally neutralized or overcome by the overexpression of endogenous angiogenesis inhibitors, such as thrombospondin-1 or angiostatin (1,3). Finally, a more trivial possibility is that because of the relatively short follow-up time, some patients in the subgroup with the highest values of VEGF are at high risk but they have not yet developed recurrence or died. However, we advise that the interpretation of this range of the data may be affected by the small number of cases and events. Therefore, additional studies with larger series of patients and longer follow-up are needed to verify which hypothesis, among those posed above, is the more tenable.

We found that assessment of VEGF protein levels by use of this enzymatic immunoassay is characterized by a low intra-assay coefficient of variation. The immunohistochemical methods, in general, are evaluated by subjective criteria. Therefore, we think that the enzymatic immunoassay we used is a valuable and objective method to detect VEGF. We suggest that additional studies are needed to properly evaluate this new assay, which should be standardized by quality controls as done in the past for the hormone-receptor assays (35).
The potential disadvantages of this enzymatic immunoassay are that it does not distinguish the different isoforms of VEGF and it does not permit the identification of the cellular sources of VEGF. In fact, some stromal cells have also been shown to be able to produce this endothelial growth factor (5,22).

Toi et al. (34) have recently reported, in a different series of patients with breast cancer, that levels of VEGF protein assessed by use of the same enzymatic immunoassay (on total tissue homogenates) were statistically significantly associated with VEGF expression by a more conventional immunohistochemical method. Additional studies are needed to prospectively evaluate, in the same series, the clinical value of the different methods available to assess VEGF (immunoenzymatic, immunohistochemistry, in situ hybridization, and northern and western blot analyses) and its related tyrosine kinase receptors.

The results of our study suggest that VEGF is a main angiogenic pathway in human breast cancer. However, it should be considered that more angiogenic peptides may be produced by the same tumor, that the coexpression of diverse endothelial growth factors has a synergistic effect on angiogenesis (46,47), and that, ultimately, neovascularization is the result of the net local balance between angiogenic stimuli and angioinhibitory pathways (1,48).

Another important clinical implication of our results, beyond prognosis, is that VEGF may be a potentially useful target for pharmacologic inhibition of angiogenesis (1,3). Independent experimental studies have shown that it is possible to suppress tumor growth in vivo through inhibition of angiogenesis by blocking the biologic functions of VEGF. For example, neutralizing antibodies to VEGF (49), cytotoxic conjugates of recombinant VEGF with the diphtheria toxin (50), and genetic approaches with the use of retroviruses encoding dominant-negative mutant KDR/flk-1 receptors capable of infecting and inhibiting the growth of the target endothelial cells (12) all brought about angiogenesis-related tumor regression in animal models. A humanized version of a high-affinity anti-VEGF monoclonal antibody is approaching clinical evaluation (22).

Moreover, because VEGF is the only angiogenic peptide known to act specifically on endothelial cells (6,22), another promising anticancer pharmacologic strategy could be the administration of agents blocking endothelial cell proliferation, such as AGM-1470 (TNP-470) (51) or angiostatin (52).

The development of angiogenesis inhibitors poses some challenges to the identification of the optimal study design, the criteria for eligibility of the patients, the modality and schedule of administration, and the criteria for evaluation of their biologic and therapeutic efficacy in cancer patients (53,54). In any case, at present, inhibition of angiogenesis represents one of the most promising novel therapeutic strategies to improve the cure of solid tumors in general and of breast cancer in particular (25).

In conclusion, our data suggest that VEGF is an important mediator of angiogenesis and is a valuable prognostic indicator in women with node-negative breast carcinoma.

Further studies are warranted to verify whether the determination of VEGF concentrations may be useful as a marker of breast cancer tumorigenesis. Indeed, it should be investigated whether the determination of VEGF protein may be used as a therapeutic target to identify the patients who are more likely to gain benefit from antiangiogenic agents capable of blocking VEGF functions and to monitor the efficacy of these treatments in time.

References


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Notes
Supported by the Associazione Italiana per la Ricerca sul Cancro, Milan, Italy. We thank Daniela Mazzocco for the preparation of the manuscript. Manuscript received July 3, 1996; revised October 15, 1996; accepted October 25, 1994.