The Short Form of the Alternatively Spliced flt-4 but not Its Ligand Vascular Endothelial Growth Factor C Is Related to Lymph Node Metastasis in Human Breast Cancers

Sarah P. Gunningham, Margaret J. Currie, Cheng Han, Bridget A. Robinson, Prudence A. E. Scott, Adrian L. Harris, and Stephen B. Fox

ABSTRACT
Angiogenesis is essential for tumor growth and metastasis. It is regulated by numerous angiogenic factors, one of the most important being vascular endothelial growth factor (VEGF). Recently, VEGF-C, a new VEGF family member, has been identified that binds to the tyrosine kinase receptors flt-4 [VEGF receptor (VEGFR) 3] and KDR (VEGFR2). Although the importance of VEGF has been shown in many human tumor types, the contribution of VEGF-C and its primary receptor flt-4 to tumor progression is less well understood. We have therefore measured the level of VEGF-C, flt-4, and KDR mRNA by RNase protection assay and the pattern of VEGF-C expression by immunohistochemistry in 11 normal breast tissue samples and 61 invasive breast cancers. No significant difference in VEGF-C expression was observed between normal and neoplastic breast tissues (P = 0.11). There was a significant correlation between VEGF-C and both flt-4 (P = 0.02) and KDR (P = 0.0002), but no association was seen between VEGF-C and either lymph node status (P = 0.66) or number of involved nodes (P = 0.88), patient age (P = 0.83), tumor size (P = 0.20), estrogen receptor status (P = 0.67), or tumor grade (P = 0.35). No significant relationship was present between VEGF-C and vascular invasion (P = 0.30), tumor vascularity (P = 0.21), VEGF-A (P = 0.62), or thymidine phosphorylase expression (P = 1.00). VEGF-C was expressed predominantly in the cytoplasm of tumor cells, although occasional stromal components including fibroblasts were also positive. We could demonstrate no association between lymph node metastasis and either VEGF-C (P = 0.66) or flt-4 (P = 0.4). However, we did observe a significant loss of the long but not the short isoform of flt-4 in tumors compared with normal tissues (P = 0.02 and P = 0.25, respectively), and this difference was largely accounted for by the reduction of long flt-4 in node-positive tumors. These findings strongly support a role for VEGF-C/flt-4 signaling in tumor growth by enhancement of angiogenesis and/or lymphangiogenesis and suggest that differential regulation of these processes may be controlled via flt-4 isoform transcription. They further suggest that the measurement of flt-4 isoform expression may identify a patient group that is likely to have node-positive disease and therefore benefit from additional treatment and also emphasize an additional ligand interaction that could be exploited by anti-VEGFR therapy.

INTRODUCTION
Angiogenesis is a complex dynamic process consisting of coordinated endothelial cell migration, proliferation, matrix remodeling, and anastomosis. These various processes are regulated by several families of angiogenic factors, one of the most important being VEGF. VEGF is a multifunctional homodimeric protein that exists in multiple isoforms. These bind to at least two receptors, flt-1 (VEGFR1) and KDR (VEGFR2), that are largely restricted to endothelium. VEGF increases vascular permeability, endothelial cell proliferation, and survival in addition to enhancing migration and vascular tube formation. VEGF is critical for normal vascular development in that even heterozygous deletion of one allele results in an embryonic lethal phenotype. Numerous studies have shown that VEGF and its receptors are expressed in many human tumor types, including in situ (4, 5) and invasive breast carcinomas (5, 6). Furthermore, several reports have now shown that VEGF is not only significantly associated with microvessel density (6–8) but also gives prognostic information in both node-negative and -positive breast cancer patients.

Recently, several new members of the VEGF family have been identified including VEGF-C (12, 13). The VEGF-C gene

3 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; RPA, RNase protection assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TP, thymidine phosphorylase; DCIS, ductal carcinoma in situ; flt-4, fms-like tyrosine kinase-4; KDR, kinase insert domain-containing receptor.

Received 5/5/00; revised 7/17/00; accepted 8/15/00.

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 grants from the Health Research Council of New Zealand, the Cancer Society of New Zealand, and the Canterbury Medical Research Foundation.

2 To whom requests for reprints should be addressed, at Department of Anatomical Pathology, Christchurch School of Medicine, Canterbury Health, Christchurch Hospital, Private Bag 4710, Christchurch, New Zealand. Phone: 64-3-364-0592; Fax: 64-3-364-0593; E-mail stephen.fox@chmeds.ac.nz.

3 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; RPA, RNase protection assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TP, thymidine phosphorylase; DCIS, ductal carcinoma in situ; flt-4, fms-like tyrosine kinase-4; KDR, kinase insert domain-containing receptor.
has been localized to chromosome 4q34 (14) and it produces a propeptide that is proteolytically cleaved, which, unlike other members of the VEGF family, is non-heparin binding. The tyrosine kinase flt-4 (VEGFR3), which has two isoforms that may differ in their signaling properties (15), appears to be the major receptor for VEGF-C, but the fully processed protein also binds to KDR (but not flt-1; Ref. 16). Although less potent than VEGF-A, VEGF-C also stimulates migration and proliferation of endothelial cells in addition to increasing vascular permeability (17). It is expressed during embryonic development in areas of lymphatic sprouting (18) and is expressed prominently in normal adult heart, placenta, ovary, intestine, and thyroid gland (12, 13). These studies, together with data from genetically engineered mice (19), strongly suggest a role for VEGF-C in lymphangiogenesis and lymphatic maintenance (20, 21).

VEGF-C may also play a role in both physiological and pathological angiogenesis (22, 23), specifically, in wound healing (24), tissue ischemia (17), and ovarian function (25). More recently, VEGF-C has been identified in several human tumor types including breast carcinoma (26, 27), mesotheliomas (28) ovarian carcinoma (29), prostate carcinoma (30), thyroid carcinoma (31) and gastric carcinoma (32). However, unlike the numerous studies demonstrating the importance of VEGF-A in human breast cancer, only limited data are available for VEGF-C. Indeed, to date, as few as 12 invasive breast tumors appear to have been examined for expression of this (lymph) angiogenic factor (26, 27). In view of the paucity of data, we examined the level of expression of VEGF-C and its receptors, flt-4 and KDR, by RPA and the pattern of VEGF-C expression by immunohistochemistry in a series of normal and malignant breast tissues. Our aims were to determine the role of this pathway in both normal and neoplastic breast tissue and to assess its contribution to breast tumor neovascularization and metastasis by correlation with angiogenic and clinicopathological characteristics.

**PATIENTS AND METHODS**

**Tumors and Patients**

VEGF-C, total flt-4, and KDR were measured in breast carcinomas (n = 61) and histologically normal tissues (n = 11) derived from patients undergoing surgery at the John Radcliffe Hospital (Oxford, United Kingdom) and Christchurch Hospital (Christchurch, New Zealand). Tumors were treated by simple mastectomy (n = 47) or lumpectomy (n = 7) with axillary node sampling. Fifty-one patients had axillary node status confirmed microscopically. Histological subtypes included 44 ductal carcinomas not otherwise specified, 4 lobular carcinomas, and 6 others. Grading of ductal carcinomas was performed according to the modified Bloom and Richardson method (33). The clinicopathological characteristics of the series are shown in Table 1. Clinical data were absent for seven patients. An additional 12 normal breast tissues and 19 breast tumors (the clinicopathological characteristics of which are shown in Table 2) together with normal colon, lung, and kidney tissue were also examined for expression of flt-4 isoforms.

### Table 1: Contingency table comparing VEGF-C mRNA expression quantified by RPA and densitometry with clinicopathological and angiogenic variables

<table>
<thead>
<tr>
<th>VEGF-C</th>
<th>Low</th>
<th>High</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs) &lt;50</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>≥51</td>
<td>18</td>
<td>18</td>
<td>0.83</td>
</tr>
<tr>
<td>Lymph nodes –</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>14</td>
<td>13</td>
<td>0.66</td>
</tr>
<tr>
<td>Grade I</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>8</td>
<td>0.35</td>
</tr>
<tr>
<td>Tumor size &lt;2 cm</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>≥2 cm</td>
<td>21</td>
<td>18</td>
<td>0.20</td>
</tr>
<tr>
<td>ERb &lt;10</td>
<td>11</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>14</td>
<td>13</td>
<td>0.67</td>
</tr>
<tr>
<td>Vessel invasion No</td>
<td>20</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td>8</td>
<td>0.3</td>
</tr>
<tr>
<td>Vascularity Low</td>
<td>12</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>12</td>
<td>7</td>
<td>0.05c</td>
</tr>
<tr>
<td>TP –</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>12</td>
<td>17</td>
<td>1.00</td>
</tr>
<tr>
<td>VEGF-A –</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>13</td>
<td>16</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Where total numbers for each variable do not equal 61, data are unavailable.

b fmol/mg protein.

c Significant (lost in multivariate analysis).

### Table 2: Table with clinicopathological characteristics of tumors used in the flt-4 isomorph expression studies

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Low</th>
<th>High</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥51</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph nodes –</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I/II</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size &lt;2 cm</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2 cm</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERb &lt;10</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor type Ductal</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobular</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vessel invasion Yes</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgical treatment Simple mastectomy</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumpectomy</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Where total numbers for each variable do not equal 19, data are unavailable.

b fmol/mg protein.
Preparation of RNA. Tissue was snap frozen after surgical resection, and total RNA was prepared by either the method of Chomczynski and Sacchi or the guanidinium thiocyanate lysis and cesium chloride gradient method (34).

Probes. A 415-bp VEGF-C probe corresponding to region 625–1040 of the VEGF-C gene [VEGF-C cDNA was provided by Dr. Kari Alitalo (The Ludwig Institute for Cancer Research and University of Helsinki, Helsinki, Finland)] in pCRII was linearized with BamHI and transcribed with T7 polymerase to generate an antisense probe. The flt-4 probe was generated from pBSK containing 300–1800 bases of flt-4 [PCR cloned using primers GGCAGCTACGTCTGCTACTA and CACGTTCTTGCAGTCGAGCA from a placental library (Dr. David Simmons, Oxford, United Kingdom)]. This was then linearized with EcoRI and transcribed with T3 polymerase to yield a 273-bp protected fragment.

An additional 275 bases of flt-4 were cloned from Y79 cells (American Type Culture Collection, Manassas, VA) to generate a probe to distinguish between the long and short isoforms (Fig. 1). The two sequences deviate at 3914 bases of the published short sequence and the reverse primer (CCGCGCGCGGCGGTGCCCTGCTGCA containing the NotI site) derived from the long form sequence (15) were cloned into pBSK, linearized with XhoI, and transcribed using T3 polymerase to create an antisense probe. The probe spanned the splice site (15) to give a protected fragment of 275 bp (3682–3914 bp plus 43 bases of the long form sequence; Ref. 15) for the long form and 232 bp (3682–3914 bp) for the short form.

The KDR probe was generated from pBSK containing 6–356 bases after cutting with BglII and transcribing with T3 polymerase. The KDR sequence was derived by the subcloning of bases 1–999 of the coding region obtained by PCR using primers ATGAGCAAGGTGCTGCTGGCC and GCCACTTCAAAGCAACAAA from a placental library (Dr. David Simmons).

GAPDH sense and antisense probes were used as described previously (35).

RPA Protocol. Each cDNA template (200 ng) was linearized as described above and transcribed using a Riboprobe Combination System T3/T7 Kit (Promega, Madison, WI). The probes were designed to generate different-sized hybridization products to allow several factors to be assayed within the same sample, thereby avoiding the problem of interassay variability.

---

**Table 3** The median (range) and SDs of VEGF-C, KDR, and flt-4 in normal and neoplastic breast tissues measured by RPA standardized to arbitrary units by GAPDH.

<table>
<thead>
<tr>
<th></th>
<th>VEGF-C</th>
<th></th>
<th>KDR</th>
<th></th>
<th>flt-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Median (range)</td>
<td>SD</td>
<td>n</td>
<td>Median (range)</td>
</tr>
<tr>
<td>Normal</td>
<td>11</td>
<td>4.9 (2.1–12.3)</td>
<td>3.0</td>
<td>11</td>
<td>5.9 (1.2–16.7)</td>
</tr>
<tr>
<td>Tumor</td>
<td>61</td>
<td>6.0 (0.0–99.8)</td>
<td>14.5</td>
<td>29</td>
<td>5.8 (1.6–28.8)</td>
</tr>
</tbody>
</table>

---

**Fig. 1** Design of a riboprobe to detect the long and short flt-4 isoforms.
and enabling the measurement and direct comparison of the levels of each factor in the same sample. The DNA template was removed with DNase I, and the reaction was purified using a mini Quick Spin RNA Column (Boehringer Mannheim). β-Emission was counted using a Tri-Carb Spectral Liquid Scintillation Counter (Packard, Canberra, Australia), and probe was added to achieve a concentration of 50,000 cpm/30 μl hybridization buffer. RNA samples (20 μg) were resuspended in hybridization buffer containing the respective labeled RNA probes, denatured and incubated overnight at 45°C. The RNA remaining after hybridization was digested with RNase A and RNase T1 (Boehringer Mannheim). RNases were digested with proteinase K, and proteins were removed by phenol/chloroform/isoamyl alcohol extraction. Hybridized RNA was ethanol precipitated and size separated on an 8% polyacrylamide gel. Gels were vacuum dried (Bio-Rad model 583) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY) between intensifying films at −80°C overnight. Probes subjected to RNase digestion and undigested probe were included in all assays to determine the efficacy of RNase digestion and probe integrity.

As we have described previously (35), internal controls can be variable. To avoid this, 20 μg of total RNA were loaded for each sample, and then, to control for any intraexperimental losses, a transcribed GAPDH sense probe and a labeled antisense GAPDH probe were also added to each sample and assayed. mRNA was quantified by scanning laser densitometry and standardized against this external GAPDH spike control. tRNA (20 μg) was used as a negative control for each experiment. Tumors were placed into positive or negative groups for each factor by stratifying according to the median values.

### Immunohistochemistry

Formalin-fixed paraffin-embedded sections (n = 25) were cut onto coated slides and stained with PG44c to identify TP (36), JC70 (anti-CD31) or QBEND10 (anti-CD34; Dako) to highlight endothelium, and VG1 (37) and affinity-purified goat polyclonal IgG (Santa Cruz Biotechnology) to identify VEGF-A and VEGF-C, respectively. These primary reagents were followed by standard streptavidin-biotin-peroxidase-diaminobenzidine immunohistochemical techniques (Dako, Medbio Enterprises, Ltd., New Zealand). Predigestion with 12.5 mg protease type XXIV (Sigma Chemical Co., Poole, United Kingdom/100 ml PBS for 20 min at 37°C) was required for JC70, and micro-waving pretreatment for 10 min in Tris-EDTA buffer was required for VG-1 and VEGF-C, but no treatment was necessary for TP. For VEGF-C, preincubation with its immunizing peptide using a 5-fold (by weight) excess of blocking peptide overnight at 4°C was performed to ensure specificity.

### Assessment of Immunohistochemical VEGF-A, VEGF-C, and TP Expression

VEGF-A (n = 49) and VEGF-C (n = 25) were measured by assessing the percentage of tumor cells with positive staining and placing tumors into three groups: (a) low (<30%); (b) intermediate (30–70%); and (c) high (>70%) (38). Tumors were considered positive when more than 30% of tumor cells stained positively for each antibody. For TP, tumors (n = 25) were assessed for the proportion and intensity of cell staining. Tumors were placed into groups with <25%, 25%–74%, and >75% of cells stained and further assessed for weak, moderate, and strong staining. Tumors were considered positive for TP when more than 25% of the tumor cells demonstrated moderate staining (39, 40).

### Assessment of Tumor Vascularity

Tumor vascularity (n = 53) blinded for clinicopathological data was assessed using two equivalent methods. The tumors of the Oxford series were counted by scanning at low power (×40–100) for the three areas of highest vascularity before using a 25-point Chalkley point eyepiece graticule (41) at ×400 (0.155 mm²) over these hot spots. The graticule was oriented so that the maximum number of points were on or within the areas of highlighted vessels. The mean of the three graticule counts was then generated and used in the statistical analysis. The upper third was used as a cut point for categorical analysis as determined previously (42). The Christchurch tumors were semiquantitatively placed into high and low/medium expression groups. Again, tumors blinded for clinicopathological data were
scanned at low power (×40–100) for the three areas of highest vascularity and then examined at high power (×250–400) and placed into the above-mentioned categories using a semiquantitative subjective score. This method of vascular assessment has been shown to be equivalent to the Chalkley method (41, 43).

### Statistical Analysis

Spearman rank correlation coefficients were used for studying the association between continuous variables. Tests of hypotheses on the location parameter (median) were done using rank statistics (Mann-Whitney, Kruskal-Wallis, and adjusted Kruskal-Wallis for ordered groups). The χ² test was used to test for independence of categorical variables including categorized continuous variables, and logistic regression/multivariate analysis was used to confirm any significant statistical associations. All tests were performed using the Stata package release 4.0 (Stata Corp., College Station, TX).

### RESULTS

#### VEGF-C, Flt-4, and KDR Expression in Normal and Neoplastic Breast Tissues

The medians (ranges) and SDs observed for VEGF-C, Flt-4, and KDR expression in normal tissues and tumors by RPA are given in Table 3 (Fig. 2). Although VEGF-C was higher in tumors than in normal breast tissues, this did not reach statistical significance (P = 0.11). Similarly, KDR expression was also higher in tumors than in normal tissues, and this was of borderline significance (P = 0.08). There was no significant difference in Flt-4 expression between normal and neoplastic breast tissues (P = 0.15).

#### Relationship between VEGF-C, Flt-4, and KDR and Clinicopathological Variables and Angiogenic Factors

There was a significant positive correlation in tumors between VEGF-C and both Flt-4 (P = 0.02) and KDR (P = 0.0002; two-sample t test). In the categorical analysis, a weak but significant negative association between VEGF-C and tumor vascularity (P = 0.05) was observed, but in logistic regression with VEGF-C as continuous variable, the P became P = 0.21 (odds ratio, 0.95), showing no statistical support for the initial observation. In the other categorical analyses, no association was observed between VEGF-C and lymph node status (P = 0.66), the number of involved nodes (P = 0.88), patient age (P = 0.83), tumor size (P = 0.20), estrogen receptor status (P = 0.67), and tumor grade (P = 0.35) or between VEGF-C and vascular invasion (P = 0.3), VEGF-A (P = 0.62), or TP expression (P = 1.00).

#### Flt-4 Isoform Expression in Normal and Neoplastic Tissues

Both the long and short forms of Flt-4 were identified in all normal breast tissues and in some breast tumor tissues (Fig. 3). However, the ratio observed in normal tissues was altered in...
the different clinical groupings. There was a significant reduction in the long isoform but not the short isoform in breast tumors compared with normal breast tissues \( (P = 0.02 \text{ and } P = 0.25, \text{ respectively; Table 4; Fig. 4, a and b}) \). There was a significant reduction in the long form of \( \text{flt-4} \) in node-positive tumors \( (P = 0.002) \) but not node-negative \( (P = 0.32) \) tumors compared with normal breast tissues, but no significant difference in short \( \text{flt-4} \) isoform expression was demonstrated between normal breast tissues and either node-negative \( (P = 0.16) \) or node-positive breast tumors \( (P = 0.37; \text{ Fig. 4, a and b}) \). Both the long and short isoforms were identified in normal lung and kidney, but expression of the long form of \( \text{flt-4} \) was negligible in normal colon (Fig. 5).

**Relationship between Node Status and VEGFR Expression by RPA.** There was a significant inverse relationship between lymph node status and \( \text{flt-4} \) \( (P = 0.02) \) but not KDR \( (P = 0.45) \). However, in a multivariate logistic regression analysis that takes into account the confounding variables of tumor size and \( \text{flt-1} \), the association between nodal status and \( \text{flt-4} \) was no longer retained \( (P = 0.4) \). This was due to a triangular relationship between the variables such that there is a strong positive correlation between \( \text{flt-4} \) and \( \text{flt-1} \) (Table 5).

**VEGF-C Expression by Immunohistochemistry.** VEGF-C immunoreactivity was observed in the cytoplasm of the inner ductal cells of acini of the terminal duct lobular unit from normal lobules and in the ducts. The myoepithelial cells were negative, and endothelial staining in normal tissues was not observed (Fig. 6).

In tumors, VEGF-C was generally homogenously expressed in the cytoplasm of neoplastic cells, although some heterogeneity within tumors was seen in occasional cases. There was increased intensity at the tumor-normal interface in one case, but no up-regulation of staining was observed adjacent to areas of necrosis. Staining of nonneoplastic tumor elements in matrix and inflammatory cells together with occasional weak endothelial cell staining was also seen. Staining could be abolished by preincubation with VEGF-C immunizing peptide. In some cases in which DCIS was present in the biopsies, the intraduct component of the malignancy was also demonstrated VEGF-C positivity (Fig. 6).

Three tumors were negative for VEGF-C, 7 cases were in the low expression group, 10 tumors were in the intermediate expression group, and 5 cases were in the high expression group. Fifteen tumors were considered positive for VEGF-C (>30% of tumor cells stained), and 10 tumors were negative for VEGF-C. There was no significant relationship between VEGF-C protein by immunohistochemistry and mRNA expression \( (P = 0.10; n = 25) \).

**DISCUSSION**

Although there are numerous studies demonstrating the importance of VEGF-A in breast cancer, to date there are only limited data examining the expression of VEGF-C in breast tissues (26, 27) and no clinicopathological data. In this study, we observed similar levels of VEGF-C, \( \text{flt-4} \), and KDR expression in both normal and neoplastic tissues and suggest a role for VEGF-C in physiological and pathological situations. Indeed, in vitro VEGF-C is regulated by estrogen (23, 44), in keeping with a normal role in the tissue alterations that occur during cyclical hormonal changes. The absence of up-regulation of receptors is in agreement with numerous receptor studies (5, 45–47) and is likely to be due to receptor heterogeneity (48).

In tumor samples, we observed no significant correlation between VEGF-C mRNA and most standard clinicopathological variables including lymph node status. Although this contrasts with the positive relationship between VEGF-C and node status in gastric (32) and prostate carcinomas (30), the ability of VEGF-C to synergize with VEGF and basic fibroblast growth factor (49), angiogenic factors that are highly expressed in human breast cancers (6, 50), together with its receptors expressed predominantly on vascular endothelium (27) suggest a significant role in tumor angiogenesis. The absence of an association between VEGF-C and tumor vascularity (the initial correlation observed did not hold up in the logistic regression analysis) is anticipated because breast cancers express numerous angiogenic factors (6) that act in concert to generate the tumor vasculature.

Nevertheless, although \( \text{flt-4} \), like its ligand, has been proposed as a lymphatic marker, we were unable to demonstrate a significant association between the level of total \( \text{flt-4} \) expression and lymph node status or the number of involved nodes (the initial inverse correlation between \( \text{flt-4} \) expression and nodes was due to its association with tumor size and \( \text{flt-1} \)). This is likely due to the expression of \( \text{flt-4} \) on vascular endothelium in breast cancers. It has been suggested that \( \text{flt-4} \) is required for remodeling the primary vascular plexus in development and that it later becomes restricted to lymphatic endothelium (reviewed in Ref. 51) and regulates lymphangiogenesis (19, 52). In the tumor context, neoplastic cells may cause associated vascular endothelial cells to revert to an embryological phenotype by up-regulating \( \text{flt-4} \) expression.

However, in view of the alternative splicing of \( \text{flt-4} \) to generate long and short isoforms, we also investigated the expression of the long and short isoforms in normal and tumor tissues
using a probe that spans the splice acceptor site. We observed a significant reduction in expression of the long isoform in breast tumors compared with normal breast that was largely due to node-positive tumors in which long flt-4 was almost undetectable. No significant difference in expression was observed for the short flt-4 isoform between tumors and normal tissues. These findings raise the possibility that isoform generation through alternative splicing is part of the mechanism by which angiogenesis and lymphangiogenesis are regulated. flt-4 isoforms differ at their COOH terminus, with the long form having an additional 65 amino acids containing three extra tyrosine residues, suggesting that the two isoforms may have different signaling properties (15, 53). Indeed, this is supported in vitro, where only the long form of flt-4 was able to support the growth of fibroblasts in soft agar (54). An additional level of control is likely to be present at the translation level because in leukemic cell lines, despite the presence of both the long and short mRNA transcripts, only the long flt-4 protein was detected (15, 55). Moreover, in a small survey of normal tissues, we also observed differential expression of flt-4 isoforms in a tissuespecific manner, suggesting that regulation of flt-4 isoforms also plays a role in normal tissue.

We observed cytoplasmic VEGF-C staining by immunohistochemistry in normal ductal and neoplastic epithelial cells, but, unlike VEGF-A, there was no increase in VEGF-C near areas of necrosis (the presumed regions of hypoxia). This is mirrored by the in situ mRNA expression profile (26) and is also consistent with the observation that hypoxia does not change VEGF-C expression (23, 56). We also observed weak endothelial cell staining in some vessels, which may be expressed in endothelial cells as reported in vitro (56) and/or, like other VEGF family members, receptor bound. There was no significant association between mRNA by RPA and protein by immunohistochemistry, suggesting that, as with VEGF-A, there is differential translation in some tumors (57). In some cases in

**Fig. 6** Immunohistochemistry using affinity-purified IgG goat polyclonal antibody against VEGF-C and streptavidin-biotin-peroxidase with diaminobenzidine substrate in normal and malignant breast tissues (A–I). Staining of inner ductal epithelial but not myoepithelial cells of acini and ducts in the terminal duct lobular unit was observed (arrows, A). Staining of tumor cells was usually homogenous and cytoplasmic in distribution (B) and was abolished by preincubation with an immunizing peptide (C). Expression was not increased in areas adjacent to necrosis (asterisk, D), and some tumors showed heterogeneity with strong staining immediately adjacent to areas that were weak or negative (asterisk, E). Weak endothelial positivity (arrow) was observed in the endothelium in a proportion of vessels (F), and up-regulation in malignant cells was present in some patients at the normal tissue-tumor interface (arrows, G). In some cases, coexistent DCIS was also positive for VEGF-C (H).
which DCIS was present in the biopsies, the intraductal component of the malignancy also demonstrated VEGF-C positivity, suggesting that this angiogenic factor is utilized early in tumor development. Indeed, a study of VEGF-C in different grades of DCIS is warranted to assess whether this angiogenic factor might be a marker for disease progression.

These findings strongly support a role for VEGF-C/Flt-4 signaling in tumor growth by enhancement of angiogenesis and/or lymphangiogenesis and suggest that differential regulation of these processes may be controlled via flt-4 isoform transcription. They further suggest that the measurement of flt-4 isoform expression may identify a patient group that is likely to have node-positive disease and therefore benefit from additional treatment and also emphasizes an additional ligand interaction that could be exploited by anti-VEGFR therapy.

ACKNOWLEDGMENTS

We thank Dr. Kari Alitalo for the kind donation of the VEGF-C cDNA and the staff of the Anatomical Pathology Laboratory (Christchurch Hospital) for cutting archival material and performing the immunohistochemistry.

REFERENCES


Lymph Node Metastasis in Human Breast Cancers

The Short Form of the Alternatively Spliced flt-4 but not Its Ligand Vascular Endothelial Growth Factor C Is Related to Lymph Node Metastasis in Human Breast Cancers

Sarah P. Gunningham, Margaret J. Currie, Cheng Han, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/6/11/4278

Cited articles
This article cites 54 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/6/11/4278.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/6/11/4278.full#related-urls

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

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

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/6/11/4278.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.