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Association of Vascular Endothelial Growth Factor Expression with Tumor Angiogenesis and with Early Relapse in Primary Breast Cancer

Masakazu Toi,[1] Seigo Hoshina, Toshiaki Takayanagi and Takeshi Tominaga

Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113

Angiogenesis is an independent prognostic indicator in breast cancer. In this report, the relationship between expression of vascular endothelial growth factor (VEGF; a selective mitogen for endothelial cells) and the microvessel density was examined in 103 primary breast cancers. The expression of VEGF was evaluated by immunocytochemical staining using anti-VEGF antibody. The microvessel density, which was determined by immunostaining for factor VIII antigen, in VEGF-rich tumors was clearly higher than that in VEGF-poor tumors ($P<0.01$). There was a good correlation between VEGF expression and the increment of microvessel density. Furthermore, postoperative survey demonstrated that the relapse-free survival rate of VEGF-rich tumors was significantly worse than that of VEGF-poor tumors. It was suggested that the expression of VEGF is closely associated with the promotion of angiogenesis and with early relapse in primary breast cancer.

Key words: Angiogenesis — Vascular endothelial growth factor — Breast cancer

Solid tumors require neovascularization for growth and metastasis. Many experimental results have demonstrated that secretion and activation of various endothelial growth factors, named angiogenic factors, by tumor cells play crucial roles in the formation of neovasculature.[1,2] Recent studies have indicated the prognostic value in solid tumors of the microvessel density as an overall marker of angiogenesis. It can be conveniently assessed by immunostaining for factor VIII.[3–6] In primary breast cancer, patients with high vessel density showed a poor prognosis compared to those with low vessel density.[3] We also demonstrated that the microvessel density is an independent prognostic indicator as potent as nodal status, which has been the most important prognostic indicator in primary breast cancer patients.[6] Furthermore, Harris's group documented the importance of angiogenesis, evaluated by measuring platelet/endothelial cell adhesion molecule (CD31), as an indicator of node metastases and survival.[4] However, little is known about which molecule, among many new angiogenic factors, plays a crucial role in the neovascularization. In this report, the association between expression of vascular endothelial growth factor (VEGF), which is thought to be a selective growth factor for endothelium,[7] and the microvessel density was examined in 103 primary breast cancer patients. The significance of VEGF expression as a prognostic factor is discussed.

[1] To whom correspondence should be addressed.

PATIENTS AND METHODS

Patients Tumor tissues used for VEGF and factor VIII staining were obtained from 103 primary breast cancer patients who had received extended, standard, or modified radical mastectomy with full dissection of axillary lymph nodes, from 1984 to 1992. Non-invasive tumor was excluded. Tissues were immediately frozen after their removal and stored at −80°C until processing. Immunoreactivities of VEGF and factor VIII were similar between old samples and recent samples in terms of staining pattern and positive rate.

Immunocytochemistry Expressions of VEGF and factor VIII antigen were assessed in frozen sections with the indirect immunoperoxidase technique. Anti VEGF monoclonal antibody and recombinant human VEGF were kind gifts from Genentech Ltd. (CA). Anti human factor VIII monoclonal antibody was purchased from Chemicon International Inc. (CA). Microvessel density was evaluated by immunostaining for factor VIII as described previously.[6] Briefly, in the areas which were considered to be most active for neovascularization, stained cells were counted in three 1.0 mm^2 regions of a ×100 microscopic field and the average was calculated. For VEGF, the immunoreactivities were graded as (−), (±), (+) and (++) according to the staining intensity. A tumor with more than grade (+) staining was designated as a VEGF-rich tumor. A tumor with (−) or (±) staining was regarded as a VEGF-poor tumor. Tumors that consisted of VEGF-positive subpopulations and VEGF-negative subpopulations were graded as (+).

These evaluations were done by an observer completely blinded as to the patients' characteristics.

ER assay Estrogen receptor (ER) was measured by the dextran-coated charcoal (DCC) method using [^{3}H-17]-estradiol and any tumor with more than 5 fmol/mg protein was determined as positive.

Detection of VEGF transcripts Human breast cancer cell lines, MCF7, T47D and MDA231, were cultured in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Total RNA was extracted from each cell line by a standard acid-guanidinium-phenol-chloroform method. VEGF transcripts were examined by the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Each cDNA was annealed with a random primer and reverse-transcribed with M-MuLV (Boehringer Mannheim Biochem., Mannheim, Germany). After first strand synthesis, the cDNA was subjected to 35 rounds of amplification. Cycles were 1 min (first cycle 3 min) at 94°C, 2 min at 50°C and 3 min (finally 10 min) at 72°C in a temperature cycler. VEGF cDNA was amplified with primers, set A (5′-GAAGTGGTGAAGTTCATGGATGTA-3′, 5′-GGATCCTTCTGTATCAGTCTTTCC-3′) and set B (5′-GGTACCTCCGAAACCATGAACTTT-3′, 5′-GGATCCTTCATTTCAGGTTTCTGG-3′), as shown in Fig. 1. PCR products were analyzed by electrophoresis on 1% agarose gel.

Statistics Survival curves were drawn by the Kaplan-Meier method and the univariate relationship between prognostic indicator and relapse-free survival rate was assessed by means of the logrank test.[8] In multivariate analysis, Cox's proportional hazards model was used.[9] The unpaired Student's t test and chi-square test were used for the evaluation of background factors.

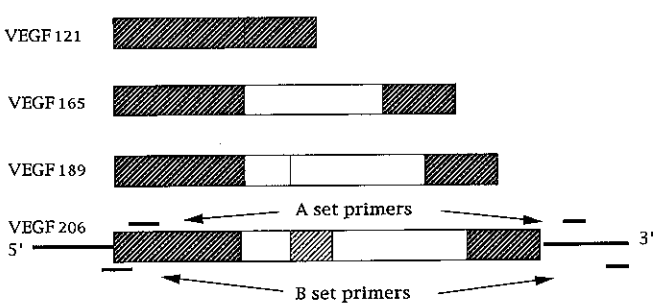

Fig. 1. Schematic model of 4 VEGF transcripts and the design of primers. Exons are represented by boxes. VEGF transcripts were examined by the RT-PCR method. VEGF cDNA was amplified with primers, set A (5′-GAAGTGGTGAAGTTCATGGATGTA-3′, 5′-GGATCCTTCTGTATCAGTCTTTCC-3′) and set B (5′-GGTACCTCCGAAACCATGAACTTT-3′, 5′-GGATCCTTCATTTCAGGTTTCTGG-3′).

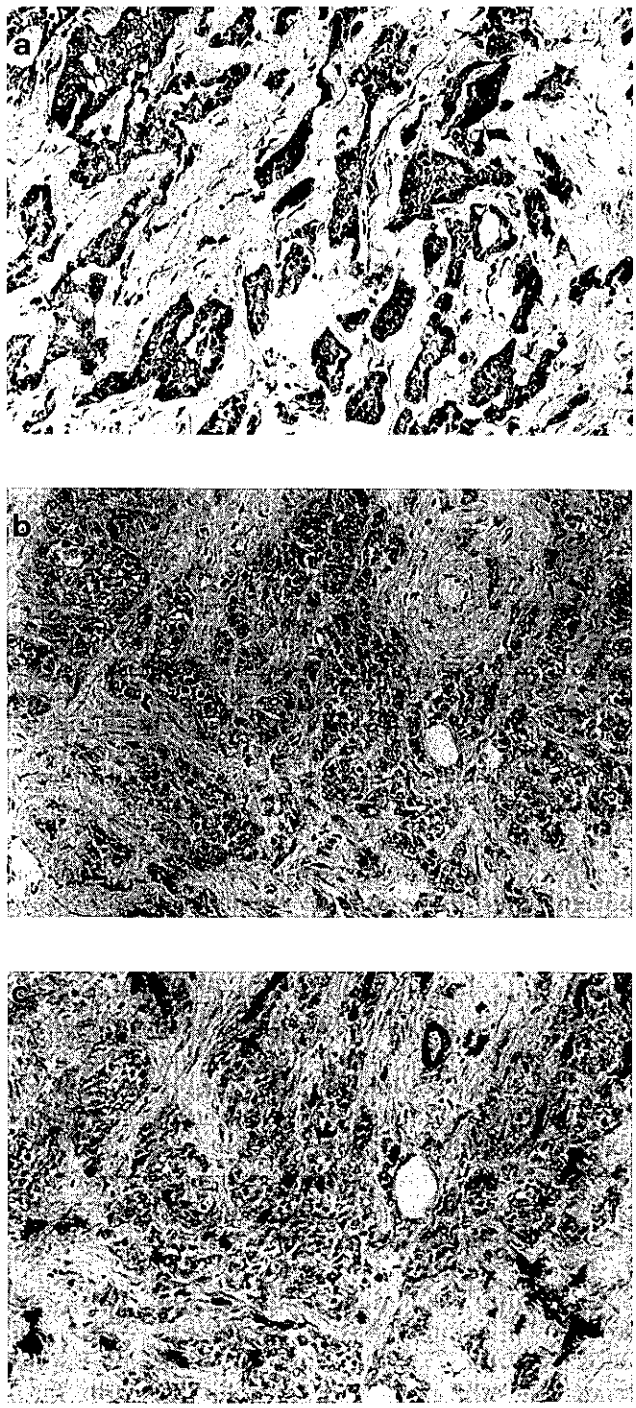

Fig. 2. Staining of VEGF in breast carcinoma tissues. a) A ductal carcinoma tissue with VEGF (++) staining. Cytoplasmic staining of VEGF was seen in tumor cells. A diffuse distribution of VEGF-positive tumor cells was apparent. b) VEGF (+) staining. c) Factor VIII staining in a serial section of the same VEGF (+) tumor specimen. One hundred and ten factor VIII-stained endothelial cells per 1.0 mm^2 in a ×100 microscopic field were recognized.

RESULTS

Relationship between VEGF expression and microvessel density The expression of VEGF was mainly identified in the cytoplasma of tumor cells. Representative cases of VEGF (++) and VEGF (+) stainings are shown in Fig. 2. VEGF staining was absorbed by the pretreatment of anti-VEGF antibody with recombinant human VEGF (data not shown). A weak VEGF positive staining was seen on endothelial cells. However, no direct correlation between staining intensity of tumor cells and that of endothelial cells was seen. In several tumor tissues, a heterogeneous distribution of VEGF stained tumor cells was seen. Out of 103 tumors, 29 (28.2%) were categorized as VEGF-rich tumors. In most of the remaining VEGF-poor tumors, a faint staining on tumor cells was observed.

Microvessel counts varied from 23 to 240 counts/1.0 mm^2 ($\times$100 microscopic field). The average count of 103 tumors was 81.1/1.0 mm^2. No correlation between the microvessel density and menopause, tumor size, nodal status or ER was found, though there was a close correlation between the microvessel density and the expression of VEGF ($P<0.01$). The microvessel density clearly increased in proportion to the increment of VEGF expression (Table I).

VEGF transcripts Three kinds of cDNAs, VEGF 189 (613 bp), VEGF 165 (541 bp) and VEGF 121 (408 bp) were detected in all 3 cell lines by RT-PCR with both sets of primers (Fig. 3). In a preliminary study, in 14 primary breast tumors, the same 3 transcripts were detected by the RT-PCR method (data not shown).

Survival Postoperative survey demonstrated that the relapse-free survival rate of patients with VEGF-rich tumors was significantly worse than that of patients with VEGF-poor tumors (Fig. 4, $P<0.01$ logrank test, median follow-up: 51 months). Among various background factors, there was no significant difference in various prognostic factors between VEGF-poor tumors and VEGF-rich tumors (Table II). A multivariate anal-

Table I. VEGF and Vessel Density

Vessel density (counts/×200 field)	VEGF (−), (±)	VEGF (+)	VEGF (++)
–49	44 (59.5)	3 (16.7)	0 (0)
50–99	19 (25.7)	6 (33.3)	5 (45.5)
100–149	9 (12.2)	5 (27.8)	4 (36.4)
150–	2 (2.7)	4 (22.2)	2 (18.2)
Total	74 (100)	18 (100)	11 (100)

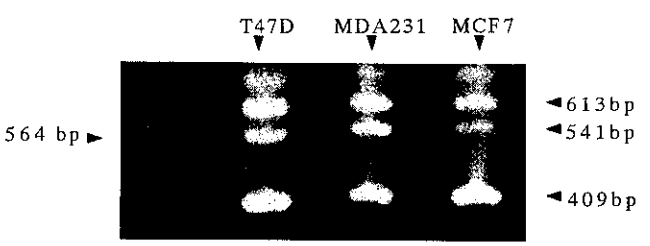

Fig. 3. VEGF transcrips. VEGF 189, VEGF 165 and VEGF 121 were detected in 3 kinds of cultured human breast cancer cells.

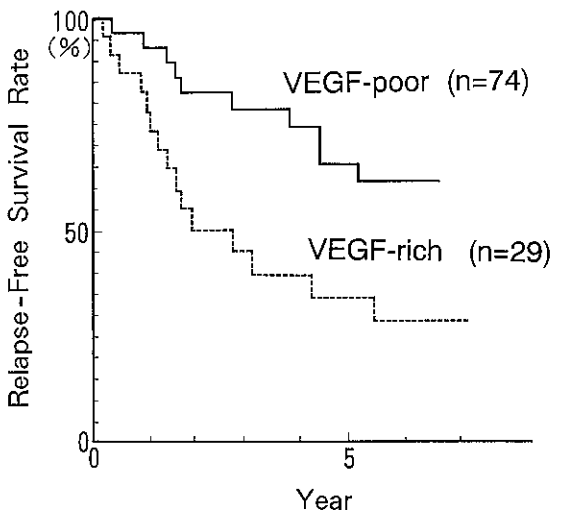

Fig. 4. Relapse-free survival rate stratified by VEGF status. The relapse-free survival rate of VEGF-rich tumors was significantly worse than that of VEGF-poor tumors ($P<0.01$, logrank test).

Table II. Background Factors

		VEGF-poor n=74	VEGF-rich n=29
Menopause	pre	33	10
	post	41	19
Tumor size	–2 cm	7	4
	2–5	46	16
	5–	21	9
Nodal metastases	0	30	8
	1–3	16	8
	4–	28	13
ER	−	33	13
	+	38	11
	unknown	3	5
Histology	pap-tub	27	6
	sol tub	17	12
	sci	26	10
	others	4	1

Table III. Multivariate Analysis

Parameter	Categories	*P* value
Menopause	pre, post	NS
Tumor size	<3 cm, >3 cm	NS
Nodal status	+, −	0.010
Vessel density	<100, >100	0.026
ER	+, −	NS
VEGF	rich, poor	0.039

NS: Not significant.

ysis showed that expression of VEGF is an independent prognostic indicator (Table III).

DISCUSSION

There have been few studies on the association of the expression of angiogenic factors and the microvessel density in human tumors. In this study, we found that the expression of VEGF was closely associated with increment of the microvessel density. The average vessel count in VEGF-rich tumors was significantly higher than that in VEGF-poor tumors. VEGF is known to be a specific growth factor for endothelial cells because its receptor (flt) is selectively expressed in endothelium.[10] It is also known that VEGF plays a crucial role in vasculogenesis.[11] The potent mitogenic activity of VEGF for endothelial cells has been characterized and is considered to be comparable to that of basic fibroblast growth factor (bFGF), which is throught to be the most potent mitogen for endothelium among many angiogenic factors.[12] VEGF possesses a signal peptide and it is largely free to diffuse in tissues after secretion,[13] but bFGF lacks it. VEGF is known to be expressed in a variety of tumor cell types, and also in tumor tissues such as glioblastomas, bladder and renal carcinomas, which have been clinically noted to be rich in neovascularization.[14, 15] On the other hand, the expression of bFGF in breast cancer cells is reported to be infrequent. Therefore, expression of VEGF might directly contribute to the promotion of angiogenesis.

Out of 4 VEGF transcripts, 3 shorter forms were detected in human cultured breast cancer cells and also in human breast cancer tissues by an RT-PCR method (data not shown). Although further cases should be examined, active forms of VEGF are produced in human breast cancer because the two shorter forms are known to be efficiently secreted and highly mitogenic for endothelial cells, while the longer ones are mostly cell-associated.[7] Recently, a rapid increase in the level of two smaller transcripts by estradiol was reported in the rat uterus.[16] Although no correlation was found between ER status and VEGF expression, it is of interest to investigate hormonal regulation of VEGF expression. In addition to estradiol, EGF stimulates the production of VEGF in glioma cells.[17] Since transforming growth factor(TGF)-α, which is a ligand for EGFr, is known to be expressed in human breast cancer cells involved in breast cancer growth,[18, 19] a TGF-α and EGFr system might be important not only for autocrine or paracrine growth, but also for endothelial proliferation.

In the postoperative survey, the relapse-free survival rate of VEGF-rich tumors was significantly worse than that of VEGF-poor tumors. A multivariate analysis suggested that VEGF status is an independent prognostic indicator in primary breast cancer patients.

Recently, it was reported that a monoclonal antibody specific for VEGF suppressed tumor growth in a human xenograft model.[20, 21] Furthermore, a low-molecular-weight angiogenesis inhibitor, AGM-1470, inhibited the growth of chemically induced mammary tumors in rats.[22] In addition to its role as a prognostic indicator, tumor angiogenesis is a potential target for therapy in patients with well vascularized tumors.

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