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Expression of Vascular Endothelial Growth Factor, Its Receptor, and Other Angiogenic Factors in Human Breast Cancer

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Abstract

Angiogenesis is essential for the growth and metastasis of solid tumors. In this study, we examined gene expression of vascular endothelial growth factor (VEGF); its receptor, flt-1; basic fibroblast growth factor; and transforming growth factors (TGFs) α and β in 18 paired cases of human breast carcinomas and the adjacent nonneoplastic tissues. In all of the paired cases, VEGF expression was markedly increased in the carcinomas. In contrast, an insignificant difference was observed in the expression of flt-1, basic fibroblast growth factor, TGF- α , and TGF- β between the malignant breast tissue and the nonneoplastic counterpart. Immunostaining showed variable VEGF positivity of the malignant cells, whereas the nonneoplastic breast epithelial cells were negative. The findings of this study suggest that VEGF is an important angiogenic factor in human breast cancer.

Introduction

The switch to an angiogenic phenotype is dependent upon the production of positive and negative regulators of angiogenesis (1, 2). To date, at least 20 angiogenic molecules have been identified (3). In human tumors, bFGF² and VEGF are the most commonly found angiogenic factors. bFGF is mitogenic for a variety of cell types, whereas VEGF acts exclusively on ECs (3–7). bFGF and VEGF act directly as angiogenic factors through EC surface receptors. At least two high-affinity receptors for FGF exists, *i.e.*, high-affinity receptors (FGFR1), which are involved in signal transduction, and low-affinity receptors (FGFR2), with a suggested role of storing FGF in the extracellular matrix (8). Cultured ECs express mainly FGFR1, but *in situ* hybridization studies have demonstrated few or no FGFR1 transcripts in vascular EC of human melanomas (8, 9). Two high-affinity receptors for VEGF have been identified, flt-1 and KDR/Flk-1, both of which possess tyrosine kinase activity (9–11). It has been suggested that flt-1 is involved in endothelial organization during embryonic development and is important for EC quiescence, whereas KDR is considered to be a major regulator of angiogenesis (4).

TGF- α stimulates angiogenesis *in vivo* and the development of capillary endothelial networks *in vitro* (12). Several clinical and experimental studies have demonstrated that TGF- α modulates malignant progression of mammary epithelial cells (13). TGF- β is a multifunctional protein with diverse effects on cell growth (14). It inhibits the formation of tube-like structures of ECs *in vitro* (15).

Although the presence of individual angiogenic factors has been reported in human breast cancer, this is the first study to quantitate

gene expression of four angiogenic factors in paired cases of human breast carcinomas and the adjacent nonneoplastic tissues. We have also examined the expression of flt-1, a high-affinity VEGF receptor with tyrosine kinase activity.

Materials and Methods

Human Breast Tissue Samples. Paired malignant and adjacent nonneoplastic breast tissue samples from 18 women were obtained from the National Disease Interchange/Cooperative Human Tissue Network (Philadelphia, PA). The malignant breast tissue samples included 15 infiltrating ductal carcinomas, 2 lobular carcinomas, and 1 mucinous carcinoma. Samples of the nonneoplastic adjacent breast tissue from the same breast were obtained in all of the cases. The paired samples were frozen in liquid nitrogen immediately after surgical removal and stored at -80°C . In some of these cases, paraffin-embedded breast tissues were available and were used for immunostaining of VEGF.

Northern Blot Analysis. Total RNA was extracted from the breast tissues using RNazol kit (TEL-TEST, Friendwood, TX), according to the procedure recommended by the supplier. RNA aliquots of 15 μg were electrophoresed in 1% agarose-formaldehyde gels, transferred to nylon membranes (Schleicher & Schuell, Keene, NH), and hybridized with 1×10^6 cpm/ml of random-primed, ³²P-labeled cDNA probes. The same RNA membrane was sequentially hybridized with cDNA probes of VEGF, flt-1, bFGF, TGF- α , and TGF- β , with removal of the probes after each hybridization. VEGF cDNA clone was isolated as described previously (16). Briefly, first-strand cDNA was synthesized with oligo-dT primer from total RNA of the HT1080 human fibrosarcoma cell line (American Type Culture Collection, Rockville, MD). PCR reaction was carried out with the following primer sequences: forward 5'-TCGGCCTCCGAAACCATGA-3' and reverse 5'-CCTGGTGAGAGATCTGGTTC-3' at 94°C for 1 min, 52°C for 1.5 min, and 72°C for 2 min for 30 cycles. The PCR product was recovered from a 3% agarose gel after electrophoresis and purified; then its sequence was confirmed. Isolation of the flt-1 cDNA clone used has been described previously (9). cDNAs for bFGF and TGF- α cDNAs were purchased from R&D Systems (Minneapolis, MN). cDNAs for TGF- β and β -actin were purchased from American Type Culture Collection. Densitometric analysis of gene expression was performed by measuring absorbance with a scanning densitometer (Scanmaster 3; Hudson, NH) and interpreted with Quantity One software (Protein Database, Huntington Station, NY). The level of gene expression was calculated after normalization of RNA with the β -actin control. Statistical analysis of intergroup differences was determined by the paired Student's *t* test and Welch *t* test.

Immunohistochemistry. Immunohistochemical staining for VEGF was performed on formalin-fixed, paraffin sections, using an avidin-biotin immunoperoxidase technique. A rabbit polyclonal antibody for human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:200 dilution.

Results

Northern Blot Analysis. Paired samples of malignant and nonneoplastic breast tissues from 18 patients were subjected to Northern blot analysis, using cDNA probes for VEGF, flt-1, bFGF, TGF- α , and TGF- β . In all of the cases, the two major VEGF transcripts of 4.3 and 3.9 kb were more abundant in the carcinomas than the adjacent nonneoplastic breast tissues. Representative samples from the Northern blot analysis are depicted in Fig. 1, and the results from the densitometric analysis of gene expression are shown in Table 1.

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² The abbreviations used are: bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; EC, endothelial cell; flt-1, fms-like tyrosine kinase-1; TGF, transforming growth factor.

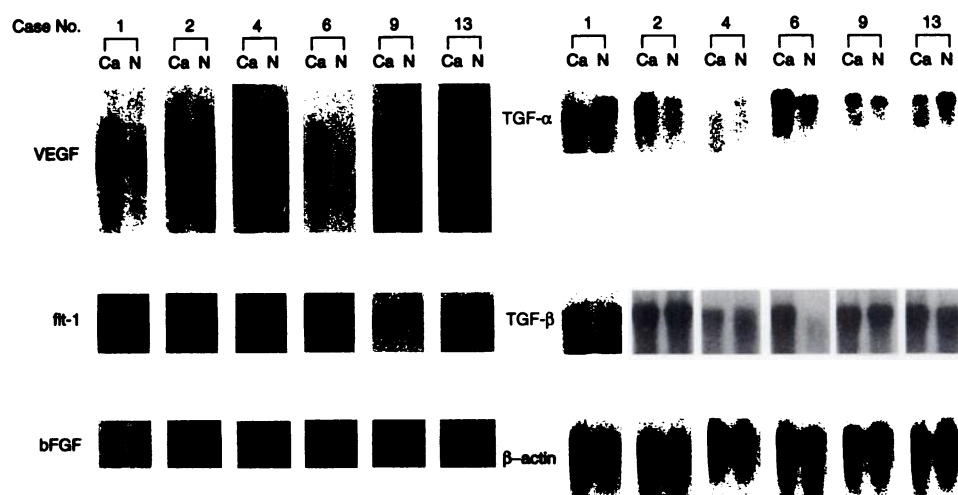


Fig. 1. Northern blot analysis of VEGF, flt-1, bFGF, TGF- α , and TGF- β gene expression in human breast samples. Each lane was loaded with 15 μ g of total RNA extracted from the carcinomas (Ca) and the adjacent nonneoplastic breast tissues (N).

Quantitation of the VEGF signal after normalization with β -actin revealed a mean density of 13.884 (range, 4.322–34.862) for the carcinomas and 1.974 (range, 0.381–3.621) for the nonneoplastic breast tissues ($P < 0.001$; Table 1; Fig. 2). In contrast to VEGF, the expression of its receptor, flt-1, was not increased in the breast carcinoma samples (Table 1; Fig. 2). Gene expression of other angiogenic factors, including bFGF, TGF- α , and TGF- β , was comparable in the malignant and the nonneoplastic tissues (Table 1; Figs. 1 and 2). The 7-kb bFGF signal was very faint in all of the samples and not clearly detectable until after 10 days of exposure (Table 1; Figs. 1 and 2). TGF- α was visualized as a 4.5-kb transcript and TGF- β as a 2.5-kb transcript (Fig. 1). The level of expression for TGF- α and TGF- β was not different in the malignant and the adjacent nonneoplastic breast tissues (Table 1; Fig. 2).

Immunohistochemistry. Paraffin-embedded histological sections, which were available on some of the paired breast cases, were immunostained using a VEGF-specific antibody. The immunoreactivity of VEGF was almost exclusively restricted to the tumor cells (Fig. 3), although rare fibroblasts within the tumor stroma appeared to be positive as well. The degree of VEGF immunostaining of the malignant cells within the same tumor was variable, ranging from very weak to intense staining. In contrast to the malignant tumors, none of

the nonneoplastic breast tissue sections exhibited VEGF immunopositivity.

Discussion

In this study analyzing gene expression of four known angiogenic factors in paired samples of breast carcinoma and the adjacent nonneoplastic tissues, VEGF was the only one preferentially expressed in the carcinomas. RNA expression of the VEGF receptor, flt-1, however was not increased in the carcinomas.

Up-regulation of VEGF expression has been reported in a variety of malignant human tumors (17–19). An *in situ* hybridization study of human breast samples showed high VEGF expression in the tumor cells but not the normal duct epithelium (20). Similarly, our immunostaining of the breast tissues showed that most of the positive VEGF staining was associated with the tumor cells. There were also scattered VEGF-positive, fibroblast-like cells in the tumor stroma. This observation concurs with findings published recently of increased VEGF expression in mammary fibroblasts cultured under hypoxic conditions (21), as well as in stromal cells of gastrointestinal adenocarcinomas (20). Apart from being an angiogenic factor, VEGF is also known as a vascular permeability factor (20, 22). As such, it

Table 1 Summary of gene expression in 18 paired cases of breast carcinomas and the adjacent nonneoplastic tissues

Case no.	Gene expression ^a										Age (yrs)	Histological type	Grade	LN ^b metastasis
	VEGF		flt-1		bFGF		TGF- α		TGF- β					
	Ca ^b	N ^b	Ca	N	Ca	N	Ca	N	Ca	N				
1	9.830	0.755	1.424	0.239	0.628	0.628	3.561	3.154	3.649	3.566	51	Ductal carcinoma	II	+
2	12.962	3.120	0.619	0.722	0.684	0.540	2.666	1.234	5.841	5.996	45	Ductal carcinoma	III	+
3	16.930	2.212	0.586	0.602	0.584	0.562	2.861	1.986	6.201	5.841	57	Ductal carcinoma	II	+
4	34.862	3.456	0.410	0.586	0.596	0.621	0.581	0.843	2.526	2.167	49	Ductal carcinoma	High	+
5	11.465	2.121	1.000	0.936	0.741	0.686	1.864	2.102	5.321	4.864	71	Ductal carcinoma	II	-
6	7.891	0.621	0.608	0.594	0.689	0.698	3.252	1.122	4.733	1.422	64	Ductal carcinoma	II	-
7	4.322	0.426	1.202	0.784	0.542	0.484	3.461	2.864	5.012	4.941	46	Lobular carcinoma	High	+
8	10.494	2.862	0.642	0.662	0.644	0.602	5.314	3.201	3.861	3.842	43	Ductal carcinoma	IV	-
9	8.648	2.652	0.596	0.322	0.135	0.082	1.274	0.844	4.216	4.708	73	Ductal carcinoma	III	-
10	8.841	3.106	0.942	0.860	0.622	0.564	3.454	3.861	2.841	2.012	51	Ductal carcinoma	III	+
11	9.810	0.896	0.881	0.840	0.701	0.804	4.261	4.081	4.104	4.810	48	Ductal carcinoma	II	+
12	24.252	3.081	0.910	0.874	1.212	1.081	2.290	1.940	3.986	4.012	75	Mucinous carcinoma	IV	+
13	26.446	3.621	0.886	0.714	1.042	0.852	1.322	1.628	4.896	4.624	44	Ductal carcinoma	III	+
14	20.224	2.061	0.687	0.741	0.432	0.401	3.610	2.946	2.941	2.861	55	Ductal carcinoma	II	-
15	6.424	0.381	0.865	0.884	1.862	1.252	0.962	1.212	5.321	4.864	75	Lobular carcinoma	II	+
16	9.424	1.386	0.742	1.001	0.469	0.510	1.868	1.741	4.208	4.101	48	Ductal carcinoma	III	+
17	8.624	0.921	1.234	1.111	0.584	0.523	2.654	2.502	3.842	3.512	52	Ductal carcinoma	II	-
18	18.454	2.861	0.446	0.402	0.796	0.689	1.248	1.059	2.864	2.741	78	Ductal carcinoma	II	-

^a Gene expression presented after normalization with β -actin.
^b LN, lymph node; Ca, carcinoma; N, adjacent nonneoplastic tissue.

stimulates extravasation of plasma proteins, such as fibrin, which when deposited in the extracellular matrix, may serve as a foundation for the formation of tumor stroma and new capillary network (23).

Studies on the VEGF receptors, flt-1 and KDR, indicate that they have different signal transduction pathways (24). KDR, but not flt-1, undergoes ligand-dependent tyrosine phosphorylation in intact cells (25, 26). In colon cancer, VEGF and KDR expression was higher in metastatic than nonmetastatic tumors (20), and in nude mouse transplants of VEGF-transfected melanoma cells, KDR was overexpressed, whereas flt-1 expression was at a background level (27). Conversely, both flt-1 and KDR were found to be up-regulated in the EC lining of tumor vasculature, compared to the surrounding normal vessels (17, 20). We were unable to get a KDR cDNA for the present study, but flt-1 expression was found to be comparable in the malignant and nonneoplastic breast tissues. This finding is consistent with features described recently of flt-1 that its expression is comparable in proliferating and quiescent EC, and that flt-1 does not seem to be associated with mitogenic or chemotactic EC response (24, 25). The emerging evidence from recent literature, therefore, suggests that KDR is critical for acquiring the full spectrum of the VEGF-mediated angiogenic response.

bFGF is thought to be one of the more potent angiogenic factors and has been shown to be up-regulated in malignancy (28). In this study, however, the level of bFGF transcripts was very low in both the carcinomas and the nonneoplastic breast tissue samples. In another study on bFGF expression in human breast cancer, the level of bFGF mRNA transcripts was higher in the benign lesions than the malignant tumors (29). Findings similar to ours were described in renal cell carcinomas, showing a marked increase in VEGF mRNA expression of the tumors, compared to the adjacent normal tissues, and very low bFGF expression in both types of tissues (19). Although our findings suggest that bFGF is not a major angiogenic factor in human breast cancer, it should be kept in mind that bFGF and VEGF have synergistic effects on angiogenesis (30, 31). Also, mobilization of an extracellular reservoir of matrix-bound bFGF by tumor-derived proteinases, such as heparanases and collagenases, may contribute to tumor neovascularization.

It is known that TGF- α and TGF- β regulate the growth of breast cancer cells *in vitro* (32, 33). Both are expressed in human breast cancer (34, 35), but there are contradictory reports on the prognostic significance of these growth factors (34–36). In our study, insignificant differences in TGF- α and TGF- β RNA expression were observed between the malignant and the adjacent nonneoplastic breast tissues.

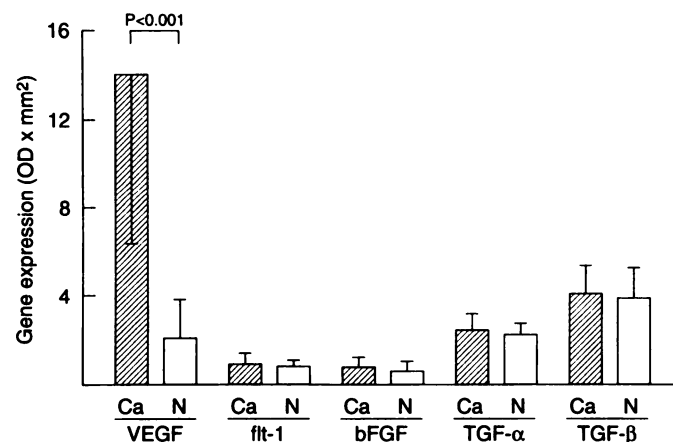


Fig. 2. Quantitative comparison of VEGF, flt-1, bFGF, TGF- α , and TGF- β gene expression in paired carcinomas (Ca) and nonneoplastic (N) human breast samples. Columns, mean; bars, SD; $n = 18$.

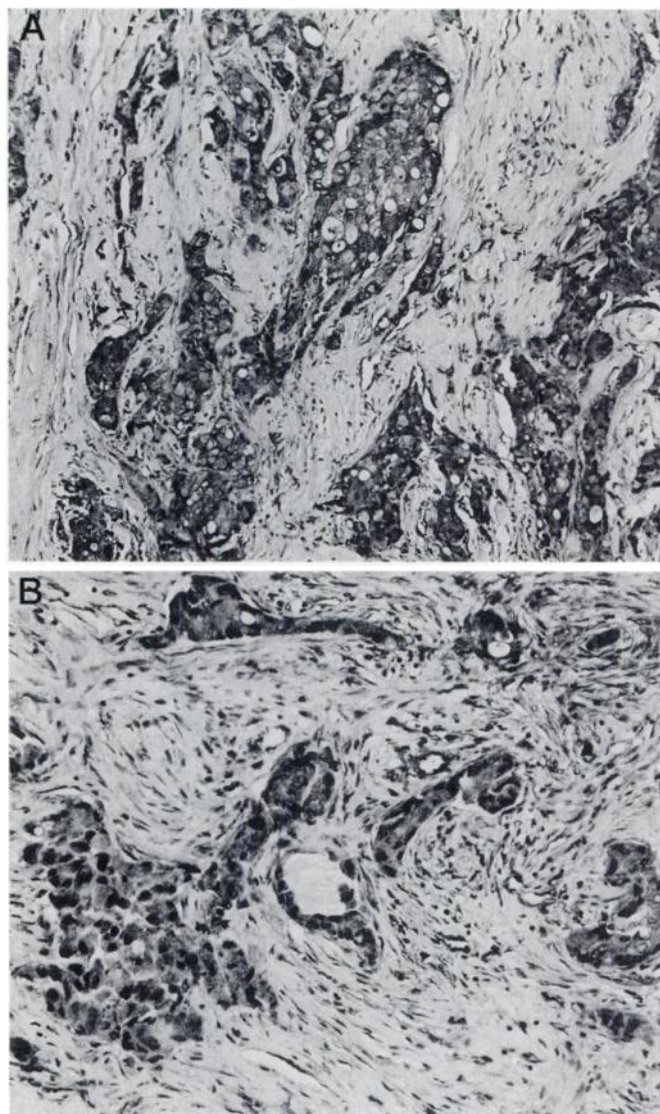


Fig. 3. Immunohistochemical localization of VEGF protein expression in infiltrating ductal carcinomas. A, the strongest VEGF staining intensity is seen at the periphery of the invasive tumor nests. B, nests of VEGF-positive tumor cells are surrounded by very cellular tumor stroma. Although not clearly visualized in this photograph, some of the spindle-shaped stromal cells showed moderate cytoplasmic VEGF immunoreactivity.

In summary, in analyzing gene expression of VEGF, bFGF, TGF- α , and TGF- β in human breast cancer, only VEGF was overexpressed in the carcinomas compared with the adjacent nonneoplastic tissues. However, expression of the VEGF receptor, flt-1, was not different. These results suggest that VEGF may play a crucial role in the promotion of angiogenesis in human breast cancer.

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