Jpn. J. Cancer Res. **90**, 977–981, September 1999

Expression of Vascular Endothelial Growth Factor (VEGF) Family Members in Breast Cancer

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Vascular endothelial growth factor (VEGF)-A is known to play an important role in tumor angiogenesis. Three additional members of the VEGF family, VEGF-B, -C and -D, have recently been discovered. VEGF-C and VEGF-D are ligands for VEGF receptor-3, which is expressed in the endothelium of lymphatic vessels. The expression of VEGF-C is known to be associated with the development of lymphatic vessels. Therefore, it is conceivable that VEGF-C and VEGF-D might play a role in the development of lymphatic vessels in solid tumors. To obtain some clue as to this role, we developed a semi-quantitative reverse transcription-polymerase chain reaction method to investigate the mRNA expression levels of each VEGF family member in breast cancer. All the VEGF family members were expressed at different levels in seven human breast cancer cell lines explored. Although VEGF-A and VEGF-B expressions were detected in both node-positive and node-negative breast tumors, VEGF-C expression was detected only in node-positive tumors. VEGF-D expression was detected only in an inflammatory breast cancer and a tumor which developed an inflammatory skin metastasis. These findings suggest a possible relationship between the expression level of VEGF-C and/or VEGF-D and the development of lymphatic tumor spread.

Key words: VEGF-C — VEGF-D — Lymph node metastasis — Inflammatory breast cancer

Angiogenesis, the formation of new vessels, is a key process in the growth and metastasis of solid tumors.[1)] Vascular endothelial growth factor (VEGF)-A, also known as vascular permeability factor, is considered to play an important role in tumor angiogenesis.[2)] Recently, three new members of the VEGF family, VEGF-B, -C and -D, have been discovered and characterized.[3–6)] It has been suggested that VEGF family members are expressed in a variety of human tumors in different ways,[7–10)] but their precise roles remain to be elucidated.

VEGF-C is a ligand for VEGF receptor-3 (VEGFR-3, Flt-4), a tyrosine kinase receptor which is predominantly expressed in the endothelium of lymphatic vessels.[4)] Experimental results with the VEGF-C-transgenic mouse have shown that the expression of VEGF-C is associated with hyperplasia of lymphatic vessels.[11)] The newest member of the VEGF family, VEGF-D, is 48% identical with VEGF-C.[5, 6)] VEGF-D also can bind to and activate VEGFR-3.[6)] To date, no other growth factor involved in the development of lymphatic vessels has been reported. Therefore, it is conceivable that VEGF-C and VEGF-D might play a crucial role in the development of lymphatic vessels in solid tumors.

To obtain some clue as to this role, we developed a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method to investigate the mRNA expression levels of the VEGF family members in breast cancer. Firstly, we investigated the expression levels of VEGF family members in seven human breast cancer cell lines and found that all the VEGF family members were expressed in cancer cells at different levels. Next, normal mammary glands and breast cancer specimens were analyzed by the same method.

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MATERIALS AND METHODS

Cell lines and tumor samples The KPL-1, KPL-3C and KPL-4 cell lines were established in our laboratory and their characterization has been reported elsewhere.[12–14)] These cell lines were derived from the malignant pleural effusion of three different Japanese patients with recurrent breast cancer. The KPL-1 and KPL-3C cell lines are estrogen receptor (ER)-positive, but the KPL-4 cell line is not. Four other human breast cancer cell lines, the MCF-7 (early passage), T-47D, MDA-MB-231 and SkBr-3 cell lines were kindly provided by Dr. Robert B. Dickson (at the Lombardi Cancer Center, Georgetown University Medical Center, DC). All of the seven cell lines, except for the KPL-3C line, were routinely cultured in Dulbecco's modified Eagle's medium (ICN Biochemicals, Costa Mesa, CA) supplemented with 5% fetal bovine serum (ICN Biochemicals Japan, Osaka). KPL-3C cells were cultured in RPMI-1640 medium (GIBCO BRL, Bethesda, MD) supplemented with 5% fetal bovine serum and 1 n*M* phorbol-12-myristate-13-acetate (Sigma Chem. Co., St. Louis, MO).

A total of 20 breast cancer specimens were selected from the tumor bank of our department. Eight tumors were derived from patients with node-negative breast cancer, and the other 12 tumors were derived from patients with node-positive or inflammatory breast cancer. The histology of each tumor sample, particularly the grade of vascular invasion, was reviewed by a pathologist [Y. M.] according to the criteria described by Pinder *et al*.[15] In brief, vascular invasion was assessed in the breast tissue adjacent to the infiltrating margin of the primary tumor. In each case, sections including the largest dimension of the main tumor were examined. Both lymphatic spaces and capillary-sized small blood vessels were designated as vascular because they were indistinguishable from each other.

Additionally, to investigate the expression levels of the VEGF family members in normal mammary tissues, three normal mammary tissues were selected from the tumor bank of our department. The samples were resected from mastectomy specimens and located more than 5 cm away from breast tumors. Histologic examination of the samples revealed neither cancer tissues nor fibrocystic changes.

RT-PCR analysis Total cellular RNA from the human breast cancer cell lines and breast tumor specimens was extracted with a TRIzol RNA extraction kit (GIBCO BRL Life Technologies, Gaithersburg, MD) according to the manufacturer's recommendations. One microgram of total RNA and oligo(dT)$_{18}$ primer (final concentration: 1 μM) in 12.5 μl of diethyl pyrocarbonate-treated water was heated to 70°C for 2 min followed by cooling on ice for 1 min. cDNA synthesis was initiated with 200 units of recombinant Molony-murine leukemia virus reverse transcriptase (CLONTECH Laboratories, Inc., Palo Alto, CA) under the conditions recommended by the manufacturer, and the reaction was allowed to proceed at 42°C for 1 h. The reaction was terminated by heating at 94°C for 5 min. cDNA was finally dissolved to a final volume of 100 μl by adding 80 μl of diethyl pyrocarbonate-treated water and the solution was frozen at −20°C until use. Oligonucleotide primers for RT-PCR were designed using a published sequence of each target gene and synthesized by the solid-phase triester method. The primers used in this study and the expected sizes from the reported cDNA sequence are shown in Table I. Each RT-PCR reaction contained 1/100 of cDNA (equivalent to the cDNA from 10 ng of initial total RNA), 200 n*M* concentrations of each primer, 200 μM deoxynucleotide triphosphates, 10 m*M* Tris-HCl (pH 8.8), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.08% Nonidet P40, and 1 unit of recombinant *Thermus aquaticus* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) in a final volume of 20 μl. After an initial denaturation at 94°C for 4 min, various cycles of denaturation (94°C for 15 s), annealing (60°C for β-actin and 56–60°C for other genes for 15 s), and extension (72°C for 30 s) for the respective target genes were performed on a DNA Thermal Cycler 2400 (PC-960G Microplate Gradient Thermal Cycler, Mortlake, Australia). The final extension was performed for 5 min. The number of cycles in the RT-PCR was determined so as to obtain logarithmic amplification of each gene for semi-quantitative analysis of the expression levels of the genes (Table I). After visualization of the RT-PCR products by 1.2% agarose gel electrophoresis with ethidium bromide staining, gel images were obtained using the FAS-II UV-image analyzer (Toyobo Co., Ltd., Tokyo), and the densities of the products were quantified using the Quantity One version 2.5 (PDI Inc., Huntington Station, NY). The relative expression levels were calculated as the density of the product of the respective target genes divided by that of β-actin from the same cDNA.

Statistical analysis The relative mRNA expression levels of each VEGF family member in node-positive and -negative tumors were compared using the unpaired Student's *t* test. The criterion of significance was set as $P<0.05$.

RESULTS

Expression of mRNAs of all the VEGF family members was detected in all seven human breast cancer cell lines explored by our RT-PCR analysis, as shown in Fig. 1.

Table I. PCR Primers and Conditions

Genes	Primers	T_a[a)] (°C)	No. of cycles	Product size (bp)
VEGF-A	5′-GCAGAATCATCACGAAGTGG-3′	58	35	212
	5′-GCATGGTGATGTTGGACTCC-3′			
VEGF-B	5′-CCTTGACTGTGGAGCTCATG-3′	60	35	246
	5′-TGTCTGGCTTCACAGCACTG-3′			
VEGF-C	5′-AGACTCAATGCATGCCACG-3′	57	35	435
	5′-TTGAGTCATCTCCAGCATCC-3′			
VEGF-D	5′-GCTGTTGCAATGAAGAGAGC-3′	56	40	313
	5′-TCTTCTGTTCCAGCAAGTGG-3′			
β-Actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′	60	35	610
	5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′			

a) Annealing temperature.

However, the relative expression level of each VEGF family member in comparison with β-actin expression differed remarkably among the cell lines (Table II). A higher relative expression level of VEGF-A was observed in KPL-3C cells (0.90), of VEGF-B in KPL-1 cells (1.14), of VEGF-C in KPL-1 (0.75) and MDA-MB-231 cells (0.90), and of VEGF-D in KPL-4 (0.75) and KPL-3C cells (0.72).

Table II. VEGF Family Member Expression in Human Breast Cancer Cell Lines

Cell line	Relative expression levels[a)]			
	VEGF-A	VEGF-B	VEGF-C	VEGF-D
KPL-1	0.78	1.14	0.75	0.65
KPL-3C	0.90	0.31	0.18	0.72
KPL-4	0.43	0.16	0.43	0.75
MDA-MB-231	0.58	0.29	0.90	0.57
MCF-7	0.26	0.33	0.63	0.22
T47D	0.14	0.33	0.61	0.13
SkBr3	0.17	0.35	0.57	0.08

a) Calculated as the density of the product of the respective target genes divided by that of β-actin from the same cDNA.

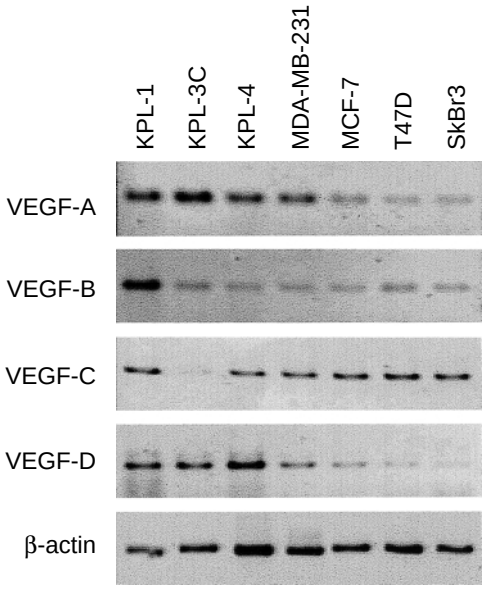

Fig. 1. RT-PCR analysis of the VEGF family members in seven human breast cancer cell lines. The gel images are shown in inverted presentation. Primers and PCR conditions are shown in Table I.

Table III. Patients' Characteristics and Expression Levels of the VEGF Family Members

Sample No.	Age (years)	Histology	Tumor size (cm)	No. of positive nodes	Lymphatic invasion	Vascular invasion	Inflammatory skin metastasis	Relative expression level[a)]			
								VEGF-A	VEGF-B	VEGF-C	VEGF-D
1	44	IDC[b)]	1.5	0	No	No	No	0.11	ND[c)]	ND	ND
2	47	IDC	3.3	0	No	No	No	2.12	ND	ND	ND
3	44	IDC	2.2	0	No	No	No	0.99	0.54	ND	ND
4	38	IDC	2.5	0	No	No	No	0.18	1.43	ND	ND
5	61	IDC	2.5	0	No	No	No	0.51	0.32	ND	ND
6	55	IDC	3.0	0	No	No	No	0.95	0.10	ND	ND
7	67	Mucinous	2.5	0	No	No	No	1.32	0.53	ND	ND
8	58	IDC	3.3	0	No	No	No	0.34	0.22	ND	ND
9	60	Inflammatory	9.0	NA[d)]	Yes	No	No	0.47	0.97	0.17	0.19
10	69	IDC	2.0	6	No	No	No	1.73	13.43	0.60	ND
11	58	IDC	5.5	15	Yes	No	Yes	1.65	0.16	0.09	ND
12	44	Inflammatory	6.0	7	Yes	No	No	3.74	2.80	ND	ND
13	54	IDC	2.2	16	No	Yes	No	1.39	0.61	0.36	ND
14	44	IDC	2.3	8	No	Yes	No	0.16	ND	ND	ND
15	42	IDC	4.5	5	No	No	No	0.55	1.58	0.41	ND
16	53	IDC	3.2	1	Yes	No	Yes	0.70	0.52	0.11	0.39
17	56	IDC	2.5	6	Yes	No	No	0.29	ND	0.08	ND
18	57	IDC	4.0	12	Yes	No	No	0.04	0.10	0.03	ND
N1	43	Normal	NA[e)]	NA	NA	NA	NA	2.90	ND	ND	ND
N2	49	Normal	NA	NA	NA	NA	NA	ND	ND	ND	ND
N3	39	Normal	NA	NA	NA	NA	NA	0.43	ND	ND	ND

a) Calculated as the density of the product of the respective target genes divided by that of the β-actin from the same cDNA.
b) Invasive ductal carcinoma.
c) Not detectable.
d) Not assessable because preoperative chemotherapy caused pathologic complete response.
e) Not assessable.

As shown in Table III, VEGF-A expression was detected in all the breast tumor samples. No significant difference between node-positive tumors and -negative tumors was observed in the relative expression level of VEGF-A (mean±SE, 0.96±0.30 and 0.82±0.24, P=0.73). VEGF-B expression was detected in 16 (80%) out of the 20 tumor samples. It was undetectable in two (25%) out of eight node-negative tumors and in two (17%) out of 12 node-positive tumors. The relative expression level of VEGF-B in node-positive tumors and -negative tumors (2.04±1.07 and 0.39±0.17, P=0.23) was not significantly different. Surprisingly, VEGF-C expression was detected in 9 (75%) out of 12 node-positive tumors, but 0 (0%) out of 8 node-negative tumors. The relative expression level of VEGF-C in the node-positive tumors was significantly higher than that in the node-negative tumors (0.23±0.09, P=0.04). VEGF-D expression was detected in 2 (17%) out of 12 node-positive tumors, but 0 (0%) out of 8 node-negative tumors. Interestingly, VEGF-D expression was detected in two (33%) out of six inflammatory-type breast cancers (primary tumors exhibited an inflammatory skin change in two cases and an inflammatory skin metastasis was observed in the remaining four cases).

VEGF-A mRNA expression but not mRNA expression of VEGF-B, -C and -D was detected in the normal mammary tissues (Table III). The result of RT-PCR analysis of normal mammary tissues and representative tumor samples is shown in Fig. 2.

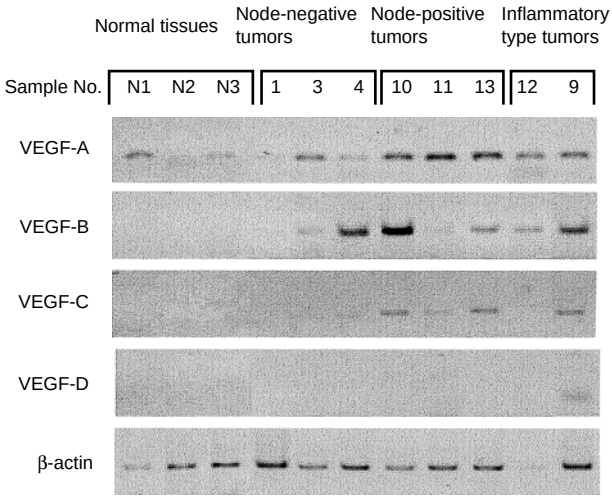

Fig. 2. RT-PCR analysis of the VEGF family members in three normal mammary tissues and eight representative tumor samples. The gel images are shown in inverted presentation. Primers and PCR conditions are shown in Table I.

DISCUSSION

Expression analysis using semi-quantitative RT-PCR in this study suggested a possible relationship between the expression level of VEGF-C and/or VEGF-D and the development of lymphatic tumor spread. VEGF-C expression was detectable by our RT-PCR analysis in 75% of node-positive tumors, but 0% of node-negative tumors. VEGF-D expression was detectable in 33% of inflammatory-type tumors, but 0% (0 out of 14) of other non-inflammatory-type tumors. In addition, the KPL-1 cell line, which causes frequent lymphatic metastasis in nude mice,[12] expressed a higher level of VEGF-C mRNA and the KPL-4 cell line, which was derived from a patient with an inflammatory skin metastasis,[14] expressed a higher level of VEGF-D. In contrast, VEGF-A and VEGF-B, which are known to stimulate the formation of blood vessels in tumors, were expressed in most tumor samples explored regardless of nodal status.

It should be noted that our RT-PCR analysis is semi-quantitative and that the protein levels of the VEGF family members were not investigated in this study. However, the expression level of each member was normalized with respect to that of a housekeeping gene, β-actin, in each sample and the relative expression levels were compared with each other. In addition, the localization of mRNA of each VEGF family member was not investigated in this study. However, it has been reported that both mRNA and the protein product of VEGF-B are localized to tumor cells and that VEGF-C mRNA is localized to tumor cells.[8] No information is available with regard to the localization of VEGF-D mRNA in tumor tissues. In addition, we have shown in this study that all seven human breast cancer cell lines explored express all the VEGF family members. These findings suggest that all the VEGF family members might be preferentially expressed in cancer cells. Further analysis is clearly needed to investigate the protein expression levels of the VEGF family members.

The grade of angiogenesis, which is expressed as the microvessel density in tumors, is reported to be a strong prognostic indicator in a variety of malignancies.[16] However, lymph node status is still the strongest prognostic factor in breast cancer. It has been speculated that lymph node metastasis is promoted by tumor cell aggressiveness, such as abilities of invasiveness, motility and proliferation. Otherwise, no specific factor that promotes lymphatic tumor cell spread has been discovered. Very recently, however, two such candidates, VEGF-C and VEGF-D, have been put forward. These factors might promote the development of lymphatic vessels in tumors and the entry of tumor cells into lymphatic vessels. Although no causative factor inducing an inflammatory skin change in breast cancer has been discovered, VEGF-C and/or VEGF-D might promote lymphatic tumor cell spread into subdermal lymphatic vessels and might induce an inflammatory skin change. Our expression analysis in the present study can-

not cast light on these hypothetical issues because the causal relationship between VEGF-C and/or -D expression and lymphatic tumor spread remains to be investigated. Neutralizing antibodies against VEGFR-3 may be useful to clarify the causal relationship and might be useful for the treatment of inflammatory-type breast cancer.

Because of a lack of methodology to recognize specifically lymphatic vessels, the precise mechanisms of the development of lymphatic vessels in tumors have not been investigated. However, monoclonal antibodies against a specific antigenic marker for lymphatic endothelial cells, VEGFR-3, to which VEGF-C and VEGF-D bind, have been produced and these monoclonal antibodies can distinguish lymphatic vessels from blood vessels.[17] Further studies on the roles of the VEGF family members using the VEGFR-3 antibodies may disclose the mechanisms of lymphatic metastasis as well as of inflammatory skin change in breast cancer.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan and by Research Project Grants (No. 10-113, 10-307) from Kawasaki Medical School.

(Received April 7, 1999/Revised May 20, 1999/2nd Revised June 14, 1999/Accepted June 23, 1999)

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