The Korean Journal of Pathology
2005; 39: 99-105

Expression of Vascular Endothelial Growth Factors A, C and D in Gastric Adenocarcinoma

Myoung-Ja Chung[1,3]
Jin-Wook Lee[1] · Ki-Hoon Yu[1]
Doo Hyun Yang[2] · Kyu Yun Jang[1]
Woo Sung Moon[1]
Myoung-Jae Kang[1]
Dong-Geun Lee[1]

[1]Department of Pathology, [2]Surgery, Chonbuk National University Medical School, [3]Research Institute of Clinical Medicine, Jeonju, Korea

Received : November 5, 2004
Accepted : January 18, 2005

Corresponding Author
Myoung-Ja Chung, M.D.
Department of Pathology, Chonbuk National University Medical School, 634-18 Keumam-dong, Dukjin-gu, Jeonju 561-712, Korea
Tel: 063-250-1227
Fax: 063-270-3135
E-mail: mjchung@chonbuk.ac.kr

*This paper was supported by research funds of Research Institute of Clinical Medicine.

Background : Vascular endothelial growth factor (VEGF)-C and VEGF-D are novel growth factors that regulate lymphatic vessel growth. This study was designed to examine whether the expression of three VEGF family members, VEGF-A, VEGF-C and VEGF-D are associated with the clinicopathologic parameters, especially with lymph node metastasis, in advanced gastric carcinomas. **Methods :** Immunohistochemical staining was performed for VEGF-A, VEGF-C, and VEGF-D in the surgically resected specimens from 102 patients with advanced gastric carcinoma. The mRNA expressions of the three VEGF family members were assessed in 16 cases of tumor tissues and their corresponding non-neoplastic tissues. **Results :** Of the 102 gastric carcinomas, 74 (73%), 82 (80%), and 34 (33%) cases showed cytoplasmic immunoreactivity for VEGF-A, VEGF-C and VEGF-D, respectively. Both VEGF-A and VEGF-C expressions were associated with lymphatic invasion and lymph node metastasis ($p<0.05$), but the VEGF-D expression was not associated with them ($p>0.05$). In the tumor tissue, VEGF-C mRNA expression was greater, while VEGF-D mRNA expression was lower than in the non-neoplatic tissue adjacent to the tumor. **Conclusions :** VEGF-A and VEGF-C may play important roles for the lymphatic spread of gastric carcinoma. We suggest that neutralizing both VEGF-A and VEGF-C may be reguired to block lymph node metastasis.

Key Words : Vascular endothelial growth factor-A; Vascular endothelial growth factor-C; Vascular endothelial growth factor-D, Stomach, Adenocarcinoma

For cancer to metastasize, the tumor cells have to invade the blood or lymphatic vessels and they have to disseminate through them. A high number of vessels in a tumor can facilitate tumor metastasis. Vascular endothelial growth factor (VEGF)-A is a homodimeric glycoprotein that is a mitogen for endothelial cells, and it is also a potent inducer of vascular permeability.[1] The biologic activities of VEGF-A are exerted via a binding to the tyrosine kinase receptors, vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR-2.[2] Numerous proteins that are closely related to the primary structure of VEGF-A have been reported in recent years, and these proteins may also play important roles in vascular biology. VEGF-C was initially identified as a ligand for tyrosine kinase VEGFR-3 (Flt4),[3] a receptor that is expressed by endothelial cell precursors in 8.5-day-old mouse embryos. It is expressed later in the development of venous and lymphatic endothelium with its expression being restricted to lymphatic endothelial cells in the adult.[4] Because the expression of VEGFR-3 is largely restricted to the lymphatic endothelium, the major function of VEGF-C appears to be the regulation of lymphatic vessel growth.[3] VEGF-C is also a ligand for VEGFR-2,[3] but the *in vivo* functional significance of this potential interaction is unknown. VEGF-D (also known as *c-fos*-induced growth factor, FIGH), is another member of the VEGF family of secreted glycoproteins and it activates VEGFR-2 and VEGFR-3.[5] It is closely related to VEGF-C in primary structure. The lymphatic system serves as the primary conduit for the metastasis of most carcinomas, and the detection of lymphatic invasion by tumor cells is one of the most important indicators of tumor metastasis for the majority of malignant tumors. Yet little effort has been directed toward understanding the regulatory mechanism of lymphatic vessel growth and function in both the physiological and pathological conditions. Several studies have failed to iden-

tify the functional lymphatics within tumors,[6] leading to the hypothetical concept that lymphangiogenesis may not play a major role in tumor metastasis.[7] However a series of recent articles has demonstrated that intratumoral lymphatics are formed in solid organ cancers and lymphangiogenesis in a murine model can be stimulated for a variety of experimental cancers. Although the expression of VEGF-A in gastric carcinoma has been considerably studied, only limited data for VEGF-C and VEGF-D is available.[8,9] Especially, VEGF-D has been functionally implicated in lymphangiogenesis, but the relation between the expression of VEGF-D and lymphatic metastasis in gastric carcinoma remains poorly understood.

In this study, we have examined the expression of VEGF-A, VEGF-C and VEGF-D in advanced gastric carcinomas, and investigated the relationship of these factors to the clinicopathologic parameters including lymph node metastasis and tumor recurrence.

MATERIALS AND METHODS

Case selection

The study population was composed of 102 advanced gastric carcinoma patients. All the patients underwent gastrectomy along with lymph node dissection. The resected stomach and lymph nodes were histologically examined by H&E staining. The patients' ages ranged from 31 to 79 years (average age: 60.5 years), and 40 patients were women and 62 patients were men. The clinicopathological characteristics of the series of tumors are summarized in Table 1. For the evaluation of lymphatic invasion, lymphatic capillaries were generally distinguished light microscopically by the absence of erythrocytes in their lumens and more irregular and wider lumen than the blood capillaries. Also, lymphatic vessels were typically devoid of pericytes and smooth muscle cells, although the larger lymphatic vessels generally processed a thin muscular layer. The lymphatic invasion was significantly correlated with a lymph node metastasis ($p<0.05$). Follow-up data were available for 67 of the 102 patients; the median follow-up period for recurrence was 34 months (range: 6-59 months). Immediately after resection of the stomach, tissues were taken from 16 pairs of primary tumor and the corresponding non-neoplastic tissues, and were stored at -80℃ for reverse transcription-polymerase chain reaction (RT-PCR).

Cell lines and culture

Three gastric adenocarcinoma cell lines, KATO III, SNU-216,

Table 1. Correlation between clinicopathologic characteristics and expression of VEGF-A, VEGF-C and VEGF-D in advanced gastric carcinomas

	No	VEGF-A				VEGF-C				VEGF-D		
		-	1+	2+	p	-	1+	2+	p	-	1+	p
Size (cm)	102	5.4±2.7	4.6±2.6	5.8±3.0	0.11	6.4±3.4	4.8±2.6	5.3±2.7	0.80	5.2±2.4	6.3±3.6	0.08
Histologic grade					0.19				0.12			0.78
Well & Moderately	41	8	17	16		4	14	23		28	13	
Poorly & Signet	61	20	16	25		16	16	29		40	21	
Lauren classification					0.50				0.19			0.81
Intestinal	31	5	12	14		3	11	17		22	9	
Mixed	18	5	5	8		2	7	9		12	6	
Diffuse	53	18	16	19		15	12	26		34	19	
Growth Pattern					0.14				0.04			0.15
Expanding	9	2	2	5		2	3	4		8	1	
Mixed	29	4	9	16			11	18		16	13	
Infiltrative	64	22	22	20		18	16	30		44	20	
Depth of Invasion					0.045				0.26			0.52
Proper muscle	21	4	4	13		1	9	11		12	9	
Subserosa	29	12	11	6		7	9	13		21	8	
Extraserosa	52	12	18	22		12	12	28		35	17	
Lymphatic Invasion					0.046				0.04			0.88
Negative	29	13	7	9		8	12	9		19	10	
Positive	73	15	26	32		12	18	43		49	24	
Lymph node metastasis					0.04				0.04			0.77
Negative	35	15	9	11		8	15	12		24	11	
Positive	67	13	24	30		12	15	40		44	23	

and MKN-28, and two colon adenocarcinoma cell lines, KM12C and KM12SM, were obtained from the Korean cell line bank (Seoul, Korea).

KM12C and KM12SM are two cell lines with different metastatic potential. KM12C was characterized as a cell line with a low metastatic potential and KM12SM was a cell line with a high metastatic potential. These three stomach cell lines were routinely cultured in RPMI 1640 medium (GIBCO BRL, Bethesda, MD, USA) supplemented with 10% fetal bovine serum (FBS, ICN Biochemicals Osaka, Japan). The two colon cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, ICN Biochemicals, Costa Mesa, CA, USA).

Immunohistochemical staining

Immunohistochemical studies with monoclonal mouse antihuman VEGF-A (G153-694, BD PharMingen, CA, USA), goat antihuman VEGF-C (AF752, R&D system, MN), and goat antihuman VEGF-D (AF286, R&D system, MN, USA) were performed. Thin sections (5 μm) of the representative formalin fixed and paraffin embedded samples were used for the immunohistochemistry. The indirect avidin-biotin peroxidase method was applied by means of a Ventana ES automated immunohistochemistry instrument (Ventana Medical Systems, Inc., Tucson, AZ, USA), and peroxidase activity was visualized using 3-amino 9-ethylcarbazole as a substrate. For the immunohistochemical staining for VEGF-C and VEGF-D, sections were pretreated in a citrate buffer at a pH 6.0 in a microwave oven for 30 min. Sections from the gastric carcinomas that had been repeatedly found to express VEGF-A, VEGF-C and VEGF-D were incubated as the positive controls. Negative control slides were run using an isotype-matched mouse IgG (Sigma, St. Louis, MO, USA) at the same concentration as that of the primary antibody.

RT-PCR

The total cellular RNA was extracted from the frozen gastric tumor specimens and from the adenocarcinoma cell lines by using a Tri reagent (Molecular Research Center, OH, U.S.A) according to the standard acid-guanidium-phenol-chloroform method. RT-PCR was performed using a RT-PCR Kit (GeneAmp RNA PCR kit, Perkin-Elmer, CA, U.S.A). Briefly, 1 μg of the total RNA in diethyl pyrocarbonate-treated water (DEPC) was denatured at 65℃ for 10 min. This was chilled on ice for 5 min and the denatured RNA was then reverse-transcribed at 42℃ for 60 min in a reaction mixture containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 1 mM dNTP, 2.5 μM random hexamers, 1 U/mL RNase inhibitor and 2.5 U/mL MULV reverse transcriptase. The cDNA was next incubated at 95℃ for 10 min to inactivate the reverse transcriptase and this served as the template for PCR amplification. PCR was performed after adding 50 μL of the PCR mixture (50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.5 mM each dNTP, 1.3 pM of each sense and the antisense primers and 1U Taq polymerase). After the denaturation for 5 min at 94℃, 35 cycles of denaturation (for 30 sec at 95℃), annealing (for 30 sec at 50℃), and extension (for 30 sec at 72℃) were repeated on a DNA thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer, CA, U.S.A). The final extension was performed for 7 min at 72℃. The PCR products were electrophoresed in a 1.0% agarose gel, and the product was detected by an ethidium bromide staining. The primers used in this study and the expected sizes of the reported cDNA sequences are shown in Table 2.

Table 2. PCR primers and conditions

Genes	Primers	Ta (℃)	No. of cycles	Product size (bp)
VEGF-A	5′-GCAGAATCATCACGAAGTGG-3′ 5′-GCATGGTGATGTTGGACTCC-3′	57.0	35	212
VEGF-C	5′-GTCTGTGTCCAGTGTAGATG-3′ 5′-AGGTAGCTCGTGCTGGTGTT-3′	57.0	35	360
VEGF-D	5′-CAGTGAAGCGATCATCTCAGTC-3′ 5′-TACGAGGTGCTGTGTTCATAC-3′	60.0	35	397
ACTIN	5′-CTTCTACAATGAGCTGCGTG-3′ 5′-TCATGAGGTAGTCAGTCAGG-3′	57.0	35	305

Ta, annealing temperature.

Assessment of immunohistochemical VEGF-A, VEGF-C, and VEGF-D expression

VEGF-A, VEGF-C and VEGF-D staining was mainly located in the tumor cell cytoplasm, and the cytoplasmic staining was recorded by a semiquantitative grading system that considered both the intensity of the staining and the proportion of the stained tumor cells. The intensity was recorded as 0 (no staining) to 3 (strong staining). The percentage of the stained cytoplasmic area of the tissue sample was recorded as 0 (no tumor cells were positive), 1 (positive staining in <10% of tumor cells), 2 (positive staining in 10-50% of the tumor cells), or 3 (positive staining in >50% of the tumor cells). A staining index was calculated as the sum of the staining intensity and the stained area. A staining index greater than 2 was considered positive, and then the positive cases were categorized into two groups as 1+ (a staining index of 3 or 4) and 2+ (a staining index >4).

Statistical analysis

Statistical significance was evaluated with SPSS 10^R statistical software using the χ^2 test, and one way ANOVA testing was used to determine the significance of the association between the independent groups. The results were considered significant if p-value was less than 0.05.

RESULTS

VEGF-A, VEGF-C and VEGF-D expression by immunohistochemistry

In non-neoplastic gastric mucosa, the cells from the neck and base of the gastric glands, (especially the endocrine cells and parietal cells), and the inflammatory cells were immunopositive for VEGF-A, VEGF-C and VEGF-D. However, the foveolar epithelial cells and the smooth muscle cells did not show immunoreactivity for the three different VEGFs.

The correlation between VEGF-A, VEGF-C and VEGF-D expressions and the clinicopathologic characteristics of the gastric cancers are summarized in Table 1. VEGF-A was homogenously expressed in the cytoplasm of the neoplastic cells (Fig. 1A). Seventy-four out of 102 cases of advanced gastric carcinomas (73%) expressed the VEGF-A protein. Thirty-three of 74 positive cases were 1+ and 41 cases were 2+. The VEGF-A expression was significantly correlated with the depth of invasion, lymphatic invasion and lymph node metastasis ($p<0.05$). Eighty-two out of 102 cases of advanced gastric carcinomas (80%) expressed the VEGF-C protein. Thirty of 82 cases were 1+ and 52 cases were 2+. In the carcinoma tissue, VEGF-C was diffusely expressed in the cytoplasm (Fig. 1B). Cancer cells in the lymphatic vessels frequently showed intracytoplasmic VEGF-A and VEGF-C immunoreactivity. The VEGF-C expression was significantly correlated with the growth pattern, lymphatic invasion and lymph node metastasis ($p<0.05$). No significant correlations were seen between the VEGF-C expression and tumor size, depth of invasion, histologic grade or Lauren classification. VEGF-C was usually homogenously expressed in the tumor cells, but an increased intensity at the tumor-normal tissue interface was seen in occasional cases. VEGF-C immunoreactivity was also observed in the inflammatory cells. The VEGF-D was expressed in 34 out of 102 cases (33%). In the carcinoma cells, VEGF-D immunoreactivity was weak only in 20-30% of the tumor cells (Fig. 1C). All of the positive cases were 1+. The VEGF-D expression was not significantly correlated with lymph node metastasis, lymphatic invasion and the other clinicopathologic characteristics.

Follow-up data were available for 67 of the 102 cases of gastric adenocarcinomas: there were 16 cases (24%) of metastasis that occurred most frequently in the liver or the peritoneum. The relationship between tumor recurrence and the expressions of VEGF-A, VEGF-C or VEGF-D is shown in Table 3. No significant correlations were seen between tumor recurrence and the expression of VEGF-A, VEGF-C and VEGF-D. However,

Table 3. Correlation between recurrence and the expression of vascular endothelial growth factor (VEGF)-A and VEGF-C and VEGF-D in gastric adenocarcinoma

Recurrence	VEGF-A			p	VEGF-C			p	VEGF-D		p
	-	1+	2+		-	1+	2+		-	1+	
No	16	9	26	0.23	8	13	30	0.23	29	22	0.19
Yes	3	1	12		2	1	13		12	4	

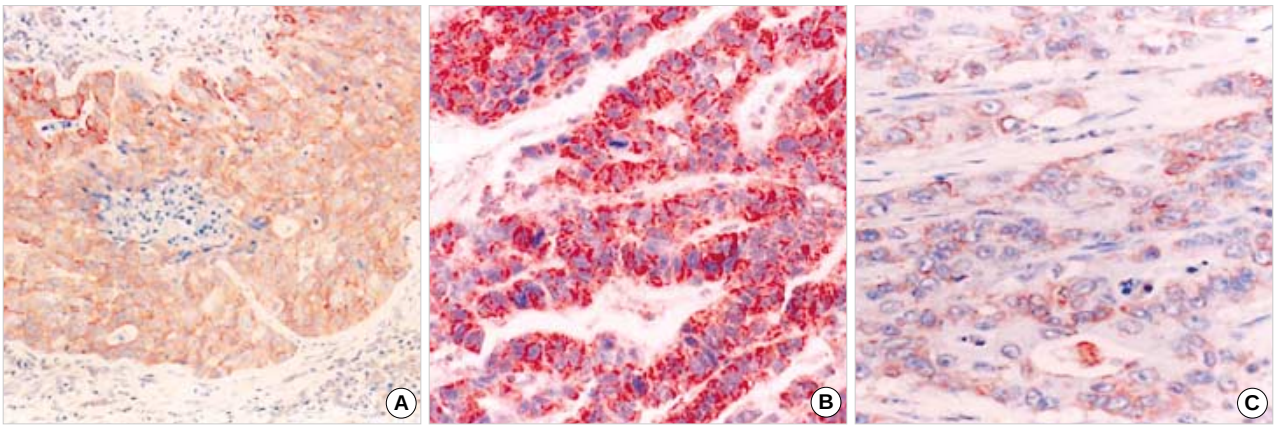

Fig. 1. Gastric adenocarcinoma, stained for VEGF-A, VEGF-C and VEGF-D. (A, B) tumor cells show strong diffuse immunoreactivity for VEGF-A and VEGF-C (A: VEGF-A, B: VEGF-C). (C) VEGF-D immunoreactivity is weak in tumor cells.

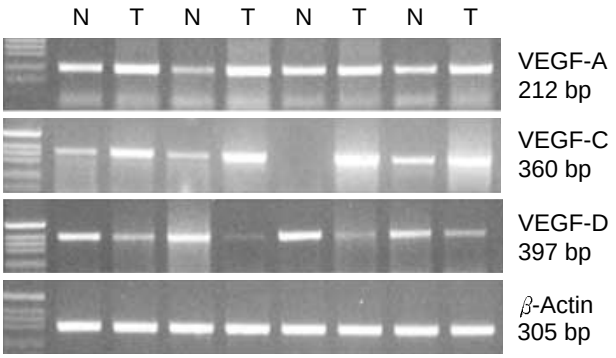

Fig. 2. RT-PCR analysis of the VEGF-A, VEGF-C and VEGF-D in gastric carcinoma tissue and corresponding non-neoplastic tissue. N, non-neoplastic tissue; T, tumor tissue.

those cases with tumor recurrence showed a tendency towards the diffuse and strong expression for VEGF-A and VEGF-C. Diffuse and strong expressions of VEGF-A and VEGF-C were seen in 13 (81%) and 12 (75%) cases of the 16 cases with tumor recurrence, respectively. On the other hand, 12 (75%) cases of the 16 cases with tumor recurrence were negative for VEGF-D.

Expression of VEGF-A, VEGF-C, and VEGF-D by RT-PCR

Sixteen pairs of carcinoma tissue and their corresponding non-neoplastic gastric tissue were examined to verify the mRNA expression of the three VEGF family members. A representative gel indicating VEGF-A, VEGF-C and VEGF-D mRNA expression is shown in Fig. 2. VEGF-A mRNA was detected in all 16 cases of carcinoma and their normal tissues. VEGF-C gene expression was also found both in the tumor tissue (86%) and in the normal tissue (86%), and the mRNA levels were higher in the carcinoma tissues than in the normal tissues. On the contrary, VEGF-D gene expression was down regulated in the carcinoma tissues compared with the normal tissues. Eleven carcinoma (69%) and 14 non-neoplastic tissues (88%) expressed VEGF-D mRNA.

Expression of the mRNAs of the three VEGF family members from the adenocarcinoma cell lines are shown in Fig. 3. VEGF-A and VEGF-C mRNAs were detected in all 3 of the gastric adenocarcinoma cell lines. VEGF-D mRNA was detected at a low level in the two gastric adenocarcinoma cell lines. In the two colon adenocarcinoma cell lines, the expressions of VEGF-A and VEGF-C mRNAs were higher in the KM12SM cell line (a cell line with a high metastatic potential) than in the KM12C cell line (a cell line with a low metastatic potential). However, VEGF-D mRNA was detected only in the KM12C cell line.

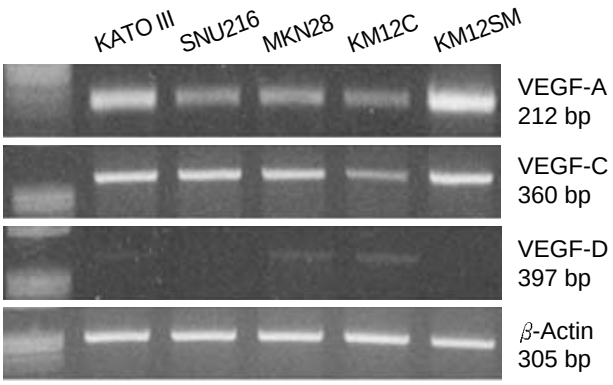

Fig. 3. RT-PCR analysis of the VEGF-A, VEGF-C and VEGF-D in three gastric adenocarcinoma cell lines (KATO III, SNU-216 and MKN-28) and two colon adenocarcinoma cell lines (KM12C and KM12SM).

DISCUSSION

This study examined the expression of three VEGF family members in clinical specimens of advanced gastric carcinoma and also in 5 adenocarcinoma cell lines, and we then examined their correlation with the clinicopathologic characteristics. We demonstrate increased expressions of VEGF-A and VEGF-C in advanced gastric carcinomas. VEGF-A and VEGF-C protein expressions were associated with lymphatic invasion and lymph node metastasis, but VEGF-D protein expression was not associated with the any of the clinicopathologic characteristics of the gastric carcinomas. VEGF-D mRNA expression was observed to be higher in the normal gastric tissue than in the carcinoma tissue. In the different cell lines, the expression level of VEGF-D mRNA was low.

Strong angiogenesis, which is reflected by high vascular density, may indirectly increase a cancer's lymphatic spread. Pre-existing lymphatics can be invaded by tumor cells when the tumor reaches the vessels via an outgrowth driven by angiogenesis, or this might be accomplished by the formation of new lymphatic vessels (lymphangiogenesis). VEGF-A is the most powerful endothelial cell-specific mitogen that has been associated with tumor neovascularization, and this growth factor has been implicated in the growth and metastatic spread of tumors. A number of investigators have reported that an over-expression of VEGF-A is associated with a poor prognosis of neoplasms.[10,11] In this study, the VEGF-A expression was significantly correlated with the depth of invasion, lymph node metastasis and the lymphatic invasion.

Although the significance of preexisting peri-tumoral lymphatics that are available as conduits for tumor cell dissemination has been well recognized, it remains unclear whether a tumor

can stimulate lymphangiogenesis and whether a tumor's metastasis necessitates the molecular activation of the lymphatic system.[12] The overexpression of VEGF-C has recently been reported to induce lymphangiogenesis and to increase lymph node metastasis.[8,13] This study examined the relationship between VEGF-C protein expression and the clinicopathologic parameters of gastric carcinoma. The VEGF-C expression was significantly correlated with lymphatic invasion, lymph node metastasis and tumor growth patterns. The correlation between the VEGF-C expression and the rate of metastasis to the lymph nodes has been reported on for breast, gastric, lung and prostate cancer,[8,14-17] although contrasting results have also been seen.[18] The mechanism for the increased lymph node metastasis of MCF-7-VEGF-C tumors was explained by the increased lymphangiogenesis and not by an increased growth rate or larger tumor volumes.[13] A strong correlation between VEGF-C expression and the lymph node status, lymphatic invasion and poor prognosis was previously demonstrated for gastric carcinoma.[8] This suggests that cancer cells producing VEGF-C may induce the proliferation and dilatation of lymphatic vessels, and this results in the development of invasion by cancer cells into lymphatic vessels and lymph node metastasis.[19] Human VEGF-D was isolated as a VEGF-related transcript and the mouse homolog (called c-fos-induced growth factor), has been cloned independently.[20] Recent studies have demonstrated the important role of VEGF-D for tumor lymphangiogenesis and lymph node metastasis.[21,22] However, only a few studies have been done on the expression of VEGF-D in clinical specimens and the role of VEGF-D regarding tumor metastasis is largely unknown. In this study, the VEGF-D expression was weak and it was not correlated with lymph node metastasis, lymphatic invasion and the other clinicopathologic characteristics. Tumor with lymph node metastasis has recently been associated with a pattern of low VEGF-D and high VEGF-A, VEGF-B and VEGF-C.[23] In contrast, the strong widespread expression of VEGF-D was associated with tumor recurrence, death and lymph node involvement for colorectal carcinoma.[22] Tumors that expressed VEGF-D show increased intra-tumoral and/or peritumoral lymphatic vessels and lymphatic spread induced by VEGF-D was blocked with an antibody specific for VEGF-D.[21] In this study, the VEGF-D mRNA levels were lower in the carcinoma tissues than in the normal tissues. The KM12SM cell line showed a higher VEGF-A and VEGF-C mRNA expression than did the KM12C cell line, whereas VEGF-D mRNA was undetectable in the KM12SM cell line. According to these results, we suggest that VEGF-D does not act to directly increase the lymphatic spread of cancer. Similar results were revealed for head and neck carcinoma and for colorectal carcinoma.[24,25] These studies suggested that the decrease in VEGF-D occurring in carcinomas might allow the higher expression of VEGF-A and VEGF-C to more readily bind to the VEGF receptors, and this produces the angiogenic switch that is required for tumor growth.[24] We thought that our results support this hypothesis. However, there were reports that VEGF-D expression was higher in carcinoma tissue than in normal colorectal tissue, and this correlated with lymph node metastasis;[22] therefore, the role of VEGF-D expression in tumor and its physiologic significance need further evaluation.

This study was designed to examine whether the expression of three VEGF family members, VEGF-A, VEGF-C and VEGF-D, is associated with the clinicopathologic parameters of advanced gastric carcinoma. In this study we have demonstrated that VEGF-A and VEGF-C protein expressions were significantly correlated with lymphatic invasion and lymph node metastasis. These results support the idea that VEGF-A and VEGF-C may play important roles for the lymphatic spread of gastric carcinoma; thus, neutralizing both VEGF-A and VEGF-C may be required to block lymph node metastasis. VEGF-D is down regulated in carcinoma tissue and its role in gastric carcinoma needs to be clarified with further experimentation and evaluation.

REFERENCE

1. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983; 219: 983-5.
2. Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene 2000; 19: 5598-605.
3. Joukov V, Pajusola K, Kaipainen A, *et al*. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996; 15: 290-8.
4. Kaipainen A, Korhonen J, Mustonen T, *et al*. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 1995; 92: 3566-70.
5. Achen MG, Jeltsch M, Kukk E, *et al*. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 1998; 95: 548-53.
6. Jain RK. Transport of molecules in the tumor interstitium: a review. Cancer Res 1987; 47: 3039-51.
7. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases.

Nature 2000; 407: 249-57.
8. Yonemura Y, Endo Y, Fujita H, *et al*. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. Clin Cancer Res 1999; 5: 1823-9.
9. Ichikura T, Tomimatsu S, Ohkura E, Mochizuki H. Prognostic significance of the expression of vascular endothelial growth factor (VEGF) and VEGF-C in gastric carcinoma. J Surg Oncol 2001; 78: 132-7.
10. Kido S, Kitadai Y, Hattori N, *et al*. Interleukin 8 and vascular endothelial growth factor - prognostic factors in human gastric carcinomas? Eur J Cancer 2001; 37: 1482-7.
11. Sauter ER, Nesbit M, Watson JC, Klein-Szanto A, Litwin S, Herlyn M. Vascular endothelial growth factor is a marker of tumor invasion and metastasis in squamous cell carcinomas of the head and neck. Clin Cancer Res 1999; 5: 775-82.
12. Leu AJ, Berk DA, Lymboussaki A, Alitalo K, Jain RK. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. Cancer Res 2000; 60: 4324-7.
13. Mattila MM, Ruohola JK, Karpanen T, Jackson DG, Alitalo K, Harkonen PL. VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. Int J Cancer 2002; 98: 946-51.
14. Kurebayashi J, Otsuki T, Kunisue H, *et al*. Expression of vascular endothelial growth factor (VEGF) family members in breast cancer. Jpn J Cancer Res 1999; 90: 977-81.
15. Akagi K, Ikeda Y, Miyazaki M, *et al*. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br J Cancer 2000; 83: 887-91.
16. Ohta Y, Nozawa H, Tanaka Y, Oda M, Watanabe Y. Increased vascular endothelial growth factor and vascular endothelial growth factor-c and decreased nm23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. J Thorac Cardiovasc Surg 2000; 119: 804-13.
17. Tsurusaki T, Kanda S, Sakai H, *et al*. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer 1999; 80: 309-13.
18. Gunningham SP, Currie MJ, Han C, *et al*. 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. Clin Cancer Res 2000; 6: 4278-86.
19. Swartz MA, Skobe M. Lymphatic function, lymphangiogenesis, and cancer metastasis. Microsc Res Tech 2001; 55: 92-9.
20. Orlandini M, Marconcini L, Ferruzzi R, Oliviero S. Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc Natl Acad Sci USA 1996; 93: 11675-80.
21. Stacker SA, Caesar C, Baldwin ME, *et al*. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 2001; 7: 186-91.
22. White JD, Hewett PW, Kosuge D, *et al*. Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. Cancer Res 2002; 62: 1669-75.
23. Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S. Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. Clin Cancer Res 2000; 6: 2431-9.
24. George ML, Tutton MG, Janssen F, *et al*. VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. Neoplasia 2001; 3: 420-7.
25. O-charoenrat P, Rhys-Evans P, Eccles SA. Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis. Cancer 2001; 92: 556-68.