Vascular Endothelial Growth Factor C Stimulates Progression of Human Gastric Cancer via Both Autocrine and Paracrine Mechanisms

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Abstract

Purpose: Vascular endothelial growth factor (VEGF)-C induces lymphangiogenesis by activating the VEGF receptor (VEGFR)-3, which is expressed by lymphatic endothelial cells. VEGFR-3 has also been detected on several malignant cells, but the significance of VEGFR-3 expression on malignant cells remains unclear. In this study, we examined the expression and function of VEGFR-3 in gastric carcinoma cells.

Experimental Design: We examined the expression of VEGFR-3 by four human gastric carcinoma cell lines and in 36 surgical specimens of gastric carcinoma. We also used cDNA microarrays to examine the effect of VEGF-C on gene expression in VEGFR-3-expressing KKLs cells. To stimulate VEGF-C/VEGFR-3 signaling in an autocrine manner, the VEGF-C expression vector was transfected into KKLs cells, and stable transfectants were established. These cells were then transplanted into the gastric walls of nude mice.

Results: Two of the four gastric carcinoma cell lines expressed VEGFR-3 mRNA. In 17 of 36 gastric carcinoma specimens, VEGFR-3-specific immunoreactivity was detected on tumor cells. In vitro treatment of KKLs cells with VEGF-C stimulated cell proliferation and increased expression of mRNAs encoding cyclin D1, placental growth factor, and autocrine motility factor. Following inoculation of VEGF-C-transfected and control cells into the gastric walls of nude mice, tumor growth of the VEGF-C-transfected cells was greatly accelerated in comparison with that of control cells. Greater angiogenesis and lymphangiogenesis were also detected in VEGF-C-transfected tumors than in control tumors.

Conclusions: Gastric carcinoma cells express VEGF-C and VEGFR-3. VEGF-C may play a role in the progressive growth of human gastric carcinoma through both autocrine and paracrine mechanisms.

The process of cancer metastasis is sequential and selective and consists of a series of interlinked independent steps (1). To produce a metastatic lesion, tumor cells must complete all of the steps, which include angiogenesis, motility, invasion, survival in the circulation, adhesion, extravasation, and proliferation (2, 3). Several sets of growth factors and their cognate receptors have been reported to be important in the regulation of metastasis. For example, cancer cells attach to the vascular endothelium via adhesion molecules, invade the tissue via motility factors, grow in the tissue with the help of growth factors, and establish their blood supply by the participation of angiogenic factors. Thus, disruption of a growth factor/receptor axis is a current strategy for the development of anticancer drugs (4).

The vascular endothelial growth factor (VEGF) family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF; refs. 5, 6). The importance of VEGF and VEGF receptor (VEGFR) expression in tumor angiogenesis and lymphangiogenesis is supported by several lines of evidence. VEGF-A is one of the most potent stimulators of angiogenesis identified thus far, affecting endothelial cell proliferation and motility and vascular permeability (7). VEGF-A exerts its angiogenic functions through activation of the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed primarily by vascular endothelial cells (8). However, VEGFRs are also expressed by a wide variety of cancer cell lines. VEGF-A and VEGF-1/2 are coexpressed in a number of cancers, including cancers of the breast (9), prostate (10), colon (11), and pancreas (12, 13), suggesting that VEGF-A may directly influence tumor cell growth via an autocrine mechanism.

VEGF-C induces lymphangiogenesis by activating VEGFR-3, which is expressed by lymphatic endothelial cells (14). We
Translational Relevance

Many studies in experimental models of cancer have shown that the vascular endothelial growth factor (VEGF)-C/VEGFR-3 signaling system is a key regulator of tumor lymphangiogenesis. In the present study, we showed that tumor cells express not only VEGF-C but also VEGFR-3 in human gastric carcinoma. In vitro and in vivo experiments showed VEGF-C acts as a growth factor for carcinoma cells, in addition to acting as a lymphangiogenic or angiogenic factor. Therefore, VEGF-C may play a role in the progressive growth of human gastric carcinoma through both autocrine and paracrine mechanisms. We propose that interruption of the VEGF-C/VEGFR-3 axis may be a therapeutic approach for controlling disease progression. Reagents that block this pathway could provide benefit for patients with gastric carcinoma in the clinic.

reported previously that expression of VEGF-C correlates with lymph node metastasis in several malignancies, including esophageal (15), gastric (16), and colorectal (17) carcinomas. VEGFR-3 has also been detected on malignant cells, including lung adenocarcinoma (18), head and neck carcinomas (19), prostate carcinoma (20), and leukemia cells (21). These observations suggest that VEGF-C may directly affect cancer cells. Su et al. (18) reported that the VEGF-C/VEGFR-3 axis plays an important role in promoting invasion and metastasis of human lung adenocarcinoma cells. However, unlike the well-characterized axes of VEGF-A and VEGFR-1/2, the biological significance of the activation of the VEGF-C/VEGFR-3 axis in epithelial tumor cells is not well understood. The expression status and significance of VEGFR-3 on gastric carcinoma cells remain unclear. In the present study, to characterize the VEGF-C/VEGFR-3 axis in gastric carcinoma, we examined the expression and function of VEGFR-3 on gastric carcinoma cell lines and tissue specimens.

Materials and Methods

Patients and tumor specimens. Thirty-six patients who underwent surgical resection for gastric carcinoma without preoperative treatment at Hiroshima University Hospital, Hiroshima, Japan, were enrolled in this study. The patient group comprised 32 men and 4 women; median age was 66 y. Informed consent was obtained from all patients for participation in the study. Paraffin-embedded archival specimens from the patients were examined by immunohistochemistry. Pathology reports and clinical histories were reviewed for accurate staging at the time of surgery. Criteria for staging and histologic classification were those proposed by the Japanese Research Society for Gastric Cancer (22). All patients had invasive gastric carcinoma in which the tumor invasion was beyond the submucosa (21 patients stage II; 15 patients stage III).

Cell cultures. Four cell lines established from human gastric carcinomas were maintained in RPMI 1640 (Nissui Co.) with 10% fetal bovine serum (FBS; MA BioProducts). The TMK-1 cell line (a poorly differentiated adenocarcinoma) was provided by Dr. Y. Takahashi of Chiba University, and MKN-28, from a well-differentiated adenocarcinoma) was provided by Dr. E. Tsujimoto of the clinic. VEGF-C reverse, GTAGCTCGTGCTGGTGTTCA (VEGF-C reverse, 28 cycles).

Cell proliferation assays. In vitro growth was measured with a Cell Proliferation Biotrak ELISA System, version 2 (Amersham Biosciences), according to the manufacturer's instructions to determine whether recombinant human VEGF-C (rhVEGF-C; R&D Systems) would stimulate proliferation of KKLs cells. Cells were seeded in a 96-well plate at a density of 1 × 10^3 cells per well and incubated overnight in 200 μl culture medium containing 10% FBS. After incubation for 24 h, cells were cultured in serum-free culture medium containing 10 μmol/L bromodeoxyuridine with or without rhVEGF-C for 24 h, and cell proliferation was measured in a plate reader (Microplate Manager 5.2.1; Bio-Rad) at 450 nm.

Microarray analysis. KKLs cells were cultured in RPMI 1640 without FBS for 6 h and then cultured with or without rhVEGF-C (20 ng/ml) for 8 h. These cells were collected and stored at −80°C until use. Microarray analysis was done with the Human Cancer CHIP (version 4, Takara Shuzo), which contains 637 human cancer–related genes (listed on the home page of Takara Shuzo)5 spotted on glass plates. A fluorescent probe synthesized by reverse transcription of 1 μg of the above mRNA with 50 U AMV reverse transcriptase (Takara Shuzo) was added to each reaction mixture. Cy3- and Cy5-labeled probes were prepared from mRNAs isolated from control cells and rhVEGF-C-treated cells, respectively; both were mixed in the reaction buffer [6× SSC, 0.2% SDS, 5× Denhardt's solution, 0.8 mg/ml poly(dA), and 1 mg/ml yeast tRNA]. The mixture was hybridized to cdNA chip at 65°C overnight. The chip was washed twice with 2× SSC/0.2% SDS solution at 55°C for 30 min and then with the same solution at 65°C for 5 min. Finally, the chip was washed with 0.05× SSC at room temperature for 10 min. Signals on the hybridized chip were visualized and quantified with the Scan-Array 5000 (GSI Lonomoids) and normalized to the averaged signals of housekeeping genes. Genes were excluded from further investigation when the intensities of both Cy3 and Cy5 were below 1,000 fluorescence units. Those with Cy3/Cy5 signal ratios >2.0 were regarded as up-regulated.

Semi-quantitative and quantitative reverse transcription-PCR. Total RNA was extracted from gastric carcinoma cell lines with an RNasea Kit (Qiagen) according to the manufacturer's instructions. cdNA was synthesized from 1 μg total RNA with a first-strand cdNA synthesis kit (Amersham Biosciences). After reverse transcription of RNA into cdNA, quantitative reverse transcription-PCR (RT-PCR) was done with a LightCycler-FastStart DNA Master SYBR-Green 1 Kit (Roche), and quantitative RT-PCR was used to monitor gene expression and was done with a LightCycler system and LightCycler Data Analysis Software ver. 3.5 (Roche) in accordance with standard procedures. PCR reactions were carried out in triplicates. To correct for differences in both RNA quality and quantity between samples, the data were normalized to those of β-actin. Primers for PCR were designed with specific primer analysis software (Primer Designer, Scientific and Educational Software), and the specificity of the sequences was confirmed by FASTA (EMBL Database). Primer sequences, annealing temperatures (Ta), and PCR cycles were as follows: VEGF-C forward, GAGGACGATTACGGTCTGT and VEGF-C reverse, GTAGCTCGTGCTGGTGTTCA (VEGF-C PCR product, 371 bp; Ta, 59°C; 28 cycles); VEGFR-3 forward, GGTTCCT-CCAGGATGAAGAC and VEGFR-3 reverse, 213 bp; Ta, 59°C; 28 cycles); AMF forward, ACTTCCTGGTCCTGAG; and AMF reverse, GTGAAAACTCGTCGTAGAG (AMF PCR product, 213 bp; Ta, 59°C; 40 cycles); PIGF forward, ATGTTCCAGGCTTCCCTGTG and

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IGF reverse, CTTCATCTCTCCGCAGAG (IGF PCR product, 201 bp; Ta, 59°C; 40 cycles); GAPDH forward, ATCATCCCTGCCCCTACTGG and GAPDH reverse, CCCCAGGAGCCGCTTACAC (GAPDH PCR product, 188 bp; Ta, 55°C; 28 cycles); and β-actin forward, GGACCTCGAGCAAGAGATGG and β-actin reverse, AGCAGTTGTTGGCGTACAG (β-actin PCR product, 234 bp; Ta, 55°C; 35 cycles). After amplification, PCR products were resolved on 5% nondenaturing polyacrylamide gels in Tris-borate-EDTA buffer. RT-PCR in the absence of reverse transcriptase showed no specific bands.

**Immunohistochemical staining.** Immunohistochemistry for VEGF-C, VEGFR-3, Lyve1, and Ki-67 was done on fresh-frozen specimens cut into 8-μm sections. Retrieval Solution (Dako). Immunohistochemical staining of CD31 were pretreated by microwave twice for 5 min in Dako REAL Target Retrieval Solution (Dako). Immunohistochemical staining of CD31 was done on fresh-frozen specimens cut into 8-μm sections, mounted on positively charged slides and stored at -80°C. Frozen tissue sections were fixed in cold acetone for 10 min. VEGF-C was detected with a goat antihuman polyclonal antibody (1:200; R&D Systems), and VEGFR-3 was detected with a goat antihuman polyclonal antibody (1:50; R&D Systems). Lyve1 was detected with the rat antimouse monoclonal antibody (1:25, Dako). CD31 was detected with the mouse antihuman polyclonal antibody (1:200; Pharmingen). The primary antibodies were applied to the slides and incubated overnight in humidified boxes at 4°C. After incubation for 1 h at room temperature with peroxidase-conjugated corresponding secondary antibodies, a positive reaction was detected by exposure for 5 to 10 min to stable 3,3'-diaminobenzidine. Slides were counterstained with hematoxylin for visualization of the nucleus.

**Gene transfection and cloning of transfected cell lines.** A full-length VEGF-C cDNA (a 1.9-kbp EcoRI-EcoRI fragment) was inserted into the EcoRI-EcoRI site of pBR322 (Invitrogen). The resultant plasmid was digested with XhoI-BamHI and cloned into the XhoI-BamHI site of the pEGFP-N1 expression vector (BD Biosciences) to yield the VEGF-C expression vector. Expression of VEGF-C cDNA was under the control of the cytomegalovirus promoter. The KKLs cell line was transfected with either the VEGF-C expression vector or the pEGFP-N1 vector alone with Lipofectin (Life Technologies) according to the manufacturer’s instructions. After transfection, cells were grown in selective medium (10% PBS-RPMI 1640 containing 800 μg/ml G418). The selective medium was changed every 3 d. G418-resistant clones (KKLs/VEGF-C and KKLs/EGFP) were selected and then expanded for additional studies. G418 was from Sigma.

**ELISA for VEGF-C protein.** For generation of conditioned media, tumor cells were plated at 1.0 × 10⁷/mL per 10-cm dish (Becton Dickinson Labware), and the supernatants were collected after 48 h, centrifuged to remove floating cells, and stored at -80°C. We used the Quantikine Human VEGF-C Immunoassay (R&D Systems) according to the manufacturer’s instructions to measure VEGF-C levels.

**Western blot analysis.** Expression of VEGF-C, VEGFR-3, and cyclin D1 was evaluated by Western blot analysis. The phosphorylation status of VEGFR-3 or Akt was also evaluated by Western blot analysis. To evaluate VEGFR-3 or Akt phosphorylation of KKLs cells stimulated with VEGF-C, cells were seeded in 10-cm dishes (1.5 × 10⁵ cells per dish) in growth medium and allowed to attach overnight before the growth medium was replaced with serum-free medium for an additional 24-h incubation. The cells were then stimulated with or without VEGF-C (20 ng/mL) for 5 or 10 min at 37°C. To evaluate VEGFR-3 or Akt phosphorylation in KKLs cells transfected with expression vector, cells

Fig. 1. Expression of VEGFR-3 and VEGF-C in human gastric carcinoma tissues. Immunoreactivity of VEGFR-3 was detected in tumor cells in 17 of 36 (47.2%) gastric carcinoma specimens. A, staining of VEGFR-3 on lymphatic vessels (red arrowhead). B, homogeneous staining of VEGFR-3 (black arrow) was observed in 7 of 17 specimens. Normal mucosa did not show VEGFR-3 staining (black arrowhead). C, in 10 of 17 specimens, VEGFR-3 expression was heterogeneous. Immunoreactivity for VEGFR-3 was more intense at the deepest invasive site (black arrow) than at the central portion or superficial part. D, VEGF-C immunoreactivity was detected on tumor cells.
were seeded in 10-cm dishes (1.5 × 10⁵ cells per dish) in growth medium and allowed to attach overnight before the growth medium was replaced by serum-free medium for an additional 24-h incubation. After three washes with cold PBS containing 1 mmol/L sodium orthovanadate, cells were lysed. Protein samples of the cell lysates (total protein 20 μg) or 1 μL of culture medium were separated by SDS-PAGE and transferred to nitrocellulose transfer membranes (Whatman GmbH). After being blocked with 1% or 3% skim milk in TBS, the membranes were incubated with primary antibodies, namely, polyclonal goat antihuman VEGF-C antibody, polyclonal goat antihuman VEGF-R-3 antibody, polyclonal mouse antihuman cyclin D1 (Dako), polyclonal rabbit antihuman phospho-VEGF-R-3 antibody (Calbiochem), polyclonal rabbit antibody to phospho-Akt (phosphorylated at Ser473, Cell Signaling Technology), or β-actin (Sigma), and were diluted in TBS at 4°C overnight. The membranes were then washed in TBS-T (0.1% Tween 20 in TBS) and incubated with the secondary antibodies specific for each primary antibody at room temperature for 1 h. To confirm equivalent protein loading, membranes were stripped with an ECL Plus Kit (Amersham Life Science).

**Immunofluorescence staining for pVEGFR-3.** To confirm the activation of VEGF-R-3 by VEGF-C, KKLS cells were cultured in RPMI 1640 without FBS for 24 h and then stimulated with or without rhVEGF-C (20 ng/mL) for 10 min. Tumor cells were stained with pVEGFR-2, 3 (1:1,000; Calbiochem).

**Animal models.** Male athymic BALB/c nude mice were obtained from Charles River Japan. The mice were maintained under specific pathogen-free conditions and used at 5 wk of age. This study was carried out after permission was granted by the Committee on Animal Experimentation of Hiroshima University.

**Orthotopic (gastric mucosa) xenograft model.** For implantation, subconfluent KKLS/VEGF-C and KKLS/EGFP cells were harvested by brief treatment with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid, and resuspended to a final concentration of 2.0 × 10⁷ cells/mL. Hanks’ solution. Using a 30-gauge needle attached to a 1-mL syringe, cells (1.0 × 10⁶/0.05 mL) were implanted into the gastric walls of nude mice under observation with a zoom stereomicroscope. After 4 wk, the mice were sacrificed, and the tumors were resected for study. The tumors were fixed in 10% buffered formalin or formalin-free IHC Zinc Fixative (Pharmingen) for immunohistochemistry.

**Quantitation of lymphatic vessel density, microvessel density, and Ki-67 labeling index.** Lymphatic vessel density and microvessel density were determined from counts of Lyve1-positive vessels and CD31-positive vessels, respectively. Vessel counts were assessed by light microscopy in immunohistochemistry-stained areas of the intratumoral and peritumoral regions containing the highest numbers of capillaries and small venules (23). Highly vascularized areas were first identified by scanning tumor sections at low power (×40 and ×100). The vessel count was determined for six such areas at ×400 (×40 objective and ×10 ocular), and the mean of the six counts was calculated. A vessel lumen was not necessary for a structure to be defined as a blood microvessel (24). In slides immunolabeled for Lyve1, only vessels with typical morphology (including a lumen) were counted as lymphatic vessels because of occasional weak antibody cross-reactivity with fibroblasts (24). The Ki-67 labeling index was determined by light microscopy at the site of the greatest number of Ki-67–positive cells. The sites were identified by scanning tumor sections at low power (×40). For the Ki-67 labeling index, the number of positive cells among ≈1,000 tumor cells was calculated as a percentage. Staining of cells was evaluated by two independent observers (M.K. and Y.K.) blinded to the patient’s status.

**Statistical analysis.** Results are expressed as mean ± SE. Fischer’s exact test or χ² test was used for the analysis of categorical data. Wilcoxon/Kruskal-Wallis analysis was used for comparison of continuous variables. A P value of <0.05 was considered statistically significant.

**Results**

**Immunolocalization of VEGFR-3 in human gastric carcinoma tissues.** We analyzed 36 human gastric cancer specimens with a polyclonal antibody specific for human VEGF-R-3. We detected VEGF-R-3-specific immunoreactivity on lymphatic endothelial cells (Fig. 1A). In 17 of 36 (47.2%) gastric carcinoma specimens, VEGF-R-3-specific immunoreactivity was detected on tumor cells (Fig. 1B and C). Immunoreactivity for VEGF-R-3 was more intense at the site of deepest invasion than at the central portion or superficial part of the tumor (Fig. 1C). In 12 of 36 (33.3%) gastric carcinomas, tumor cells expressed both VEGF-C and VEGF-R-3 (Fig. 1C and D). VEGF-R-3 immunoreactivity was not detected in normal gastric epithelial cells (Fig. 1B).

**Expression of VEGF-C and VEGF-R-3 in gastric carcinoma cell lines.** We next analyzed VEGF-C and VEGF-R-3 expression in gastric carcinoma cell lines. Gastric carcinoma cell lines constitutively expressed VEGF-C mRNA at various levels. Two
of the four gastric carcinoma cell lines (KKLS and MKN-1) expressed VEGFR-3 mRNA. Of these two cell lines, the KKLS cell line overexpressed VEGFR-3 mRNA (Fig. 2A). Expression of VEGF-C and VEGFR-3 by gastric carcinoma cells was confirmed at the protein level. Culture supernatants were assayed for VEGF-C by ELISA (Fig. 2B). VEGFR-3 protein expression was analyzed by Western blotting (Fig. 2C). KKLS cells expressed high levels of VEGFR-3 protein, but the amount of VEGF-C protein was below the limit of detection, which was not consistent with the level of mRNA. For this reason (high endogenous VEGFR-3 and low endogenous VEGF-C in KKLS cells), we used KKLS cells for further studies.

VEGF-C stimulates proliferation of KKLS cells. To investigate the possibility of autocrine tumor cell growth stimulation by VEGF-C, we treated KKLS cells with rhVEGF-C and analyzed phosphorylation of VEGFR-3, Akt, and mitogen-activated protein kinase in KKLS cells and the effect on cell proliferation. VEGF-C treatment induced phosphorylation of VEGFR-3 and Akt (Fig. 3A and B) and increased cell proliferation of KKLS cells in a dose-dependent manner (Fig. 3C). Phosphorylation of mitogen-activated protein kinase was not detected (data not shown). In contrast, VEGF-C had no effect on cell proliferation of TMK-1 cells (VEGFR-3 negative cell line; Fig. 3D).

VEGF-C up-regulates expression of genes associated with disease progression in KKLS cells. To study the downstream effector genes of VEGF-C/VEGFR-3 signaling, we did microarray analysis using the Human Cancer CHIP (Takara Shuzo). Expression of various mRNAs in untreated KKLS cells was compared with expression in KKLS cells treated with rhVEGF-C for 8 hours. Under the highly stringent conditions we used, 52 genes were classified as genes showing significantly increased expression in response to VEGF-C. Among these genes, we confirmed the increased expression of cyclin D, PlGF, and autocrine motility factor receptor (AMFR) by quantitative RT-PCR or Western blotting. Treatment with VEGF-C increased expression of AMFR mRNA in a dose-dependent and time-dependent manner (Fig. 4A). We also investigated expression of the mRNA encoding AMF, the ligand of AMFR. Treatment with VEGF-C also increased expression of AMF mRNA (Fig. 4B). Levels of PlGF mRNA were similarly increased in VEGF-C-treated KKLS cells (Fig. 4C). In addition, VEGF-C increased expression of cyclin D1 protein in a dose-dependent manner (Fig. 4D).

Transfection of the VEGF-C gene into KKLS cells. To stimulate autocrine VEGF-C/VEGFR-3 signaling, a VEGF-C expression vector was transfected into KKLS cells. After transfection with the VEGF-C expression vector or the control vector (pEGFP-N1), we selected a stable clone (KKLS/VEGF-C) that overexpressed VEGF-C and a control clone (KKLS/EGFP) for subsequent assays. Overexpression of VEGF-C mRNA and VEGF-C protein was confirmed by quantitative RT-PCR (Fig. 5A) and Western blotting.
respectively (Fig. 5B). Phosphorylation of Akt in KKLS/VEGF-C cells was also confirmed by Western blotting (Fig. 5B).

In vitro and in vivo proliferation of VEGF-C-transfected gastric carcinoma cells. Under culture conditions of 0.5% FBS, cell proliferation was stimulated by transfection with VEGF-C expression vector (Fig. 5C). To investigate the role of the VEGF-C/VEGFR-3 axis in an animal model, we implanted KKLS/VEGF-C and control cells into the gastric walls of nude mice. At the end of the 4-week experimental period, in vivo growth of KKLS/VEGF-C cells was significantly greater than that of control cells (Fig. 5D).

We next used immunohistochemistry for Lyve1, CD31, and Ki-67 to investigate lymphatic vessel density, microvessel density, and Ki-67 labeling index. Greater lymphangiogenesis (Fig. 6A) and angiogenesis (Fig. 6B) were observed in mice implanted with VEGF-C-transfected KKLS cells than in those implanted with control KKLS cells. Statistical analysis showed that the number of Ki-67-positive cells, as well as lymphatic vessel density and microvessel density, was significantly higher in the KKLS/VEGF-C tumors than in the control tumors (P < 0.01; Fig. 6C).

Discussion

Until recently, studies of VEGF family members have focused primarily on their functions as paracrine stimulators of angiogenesis or lymphangiogenesis. Promotion of tumor metastasis by VEGF-C is reported to be due to the induction of tumor lymphangiogenesis via effects of activated VEGFR-3 on lymphatic endothelial cells (25). Association of VEGF-C with tumor lymphangiogenesis and with lymph node metastasis has been observed in many human carcinomas, including thyroid, prostate, esophageal, gastric, colorectal, and lung cancers (15, 16, 26, 27). Furthermore, several studies have shown that overexpression of VEGF-C induces lymphangiogenesis and promotes tumor metastasis in mouse tumor models (28, 29).

After the discovery of VEGFRs on malignant cells, it was reported that VEGF-A can act as an autocrine growth factor for various types of cancer cells (13, 30, 31). Unlike the well-characterized VEGF-A/VEGFR-2 axis, there may be many undefined functions and molecular mechanisms involved in
tumor progression mediated by the VEGF-C/VEGFR-3 axis. Results regarding the expression of VEGF-3 on tumor cells are controversial. Some studies did not detect expression of VEGF-3 on tumor cells (32–34), whereas other studies found expression of VEGF-3 on tumor cells (18–21, 35–37). These contradictory findings suggest that expression of VEGFR-3 on cancer cells may differ between malignancies or cell lines. Expression of VEGF-C and VEGFR-3 correlates significantly with poor prognosis of specific types of cancer (18, 19, 21, 37–41). Marchio et al. (40) reported that tyrosine phosphorylation of VEGFR-3 is increased in Kaposi sarcoma cells treated with recombinant VEGF-C protein, and they found that activation of the VEGF-C/VEGFR-3 axis in Kaposi sarcoma cells is involved in the regulation of cellular functions, such as proliferation and migration. The VEGF-C/VEGFR-3 axis has also been found to play a role in the growth of malignant mesothelioma cells (41) and leukemic cells (21). It has become clear that the VEGF-C/VEGFR-3 axis plays an important role in promoting invasion and metastasis of human lung adenocarcinoma cells (18). It has also been reported, however, that inhibition of VEGFR-3 signaling by the soluble fusion protein VEGFR-3-immunoglobulin did not change the growth of lung cancer cells (42). These findings indicate that the effects and interactions of the VEGF-C/VEGFR-3 system in cancer biology are complex and may differ between malignancies. Previously, we reported that VEGF-C immunoreactivity is associated not only with lymphatic invasion and lymph node metastases but also with greater depth of tumor invasion in gastric cancer (16).

Fig. 5. Establishment of a clonal cell line overexpressing VEGF-C, and in vitro and in vivo growth of VEGF-C-transfected gastric carcinoma cells. A, expression of VEGF-C mRNA was examined by RT-PCR. B, Western blot analyses of VEGF-C in culture medium of transfected cells and phosphorylation of VEGFR-3 and Akt in lysates of transfected cells. C, cells (1 × 10⁵) were seeded in 24-well plates and cultured in medium containing 0.5% FBS. Cell number was determined in triplicate cultures. D, orthotopic (gastric mucosa) xenograft model. Tumor weights at 4 wk after implantation of a VEGF-C overexpressing clone (KKLS/VEGF-C) or cells transfected with the corresponding vector control (KKLS/EGFP). *P < 0.05; bars, SE.
This fact suggests that VEGF-C may directly influence tumor cell growth or motility via an autocrine mechanism. Therefore, we conducted the present study to clarify whether gastric cancer cells express functional VEGFR-3 and to evaluate the biological significance of VEGFR-3 expression in gastric cancer progression.

In the present study, we found that VEGFR-3 is expressed by tumor cells as well as lymphatic endothelial cells in gastric carcinoma tissues. Approximately half of our gastric cancers (17 of 36) contained tumor cells that expressed VEGFR-3 protein. On the basis of the lack of VEGF-C and VEGFR-3 expression in normal gastric epithelial cells, we concluded that de novo expression of VEGF-C and VEGFR-3 seems to be associated with the process of malignant transformation. Treatment of cultured KKLs cells with VEGF-C induced tyrosine phosphorylation of VEGFR-3 and then increased proliferation. It also induced expression of cyclin D1, PI GF, AMF, and AMFR. PI GF and AMF are growth factors known to regulate angiogenesis and tumor cell motility, respectively. We previously reported that VEGF-C expression is higher at the site of deepest

![Image](image-url)

**Fig. 6.** Immunohistochemistry for Lyve1 (A), CD31 (B), and Ki-67 (C) in KKLs cells growing in the stomach of nude mice. VEGF-C-transfected KKLs tumors showed increased density of Lyve-1– and CD31–positive vessels and higher numbers of Ki-67–positive cells than control tumors. Right panels, quantification of Lyve-1–positive vessels, CD31–positive vessels, and Ki-67–positive cells in these tumors. **P < 0.01; bars, SE.
penetration of the invasive tumor than in the superficial portions (16). In the present study, heterogeneous intratumoral staining was observed for VEGFR-3 and VEG-C, with the highest levels of expression at the invasive edges. Expression of PlGF and AMF/AMFR induced by VEGF-C/VEGFR-3 signaling may play a role in progression of gastric cancer cells to an aggressive phenotype.

Makinen et al. proved the critical role of VEGF-C/VEGFR-3 signaling in the growth and survival of lymphatic endothelial cells (43). They found VEGF-3 induces a protein kinase C–dependent p42/p44 mitogen-activated protein kinase activation and wortmannin-sensitive phosphorylation of Akt. However, the biochemical signaling pathways activated via VEGF-3 are unknown in tumor cells. In the present study, we found treatment with VEGF-C resulted in phosphorylation of Akt but not mitogen-activated protein kinase in KKLS cells. Further studies using Akt inhibitor will be needed to clarify whether Akt indeed plays a role as a signaling molecule of the VEGF-C/VEGFR-3 axis.

To stimulate VEGF-C/VEGFR-3 signaling in an autocrine manner, we transfected a VEGF-C expression vector into KKLS cells, established stable transfecants, and transplanted VEGF-C–transfected cells and control cells into the gastric walls of nude mice (orthotopic site). Lyve1-positive vessel-like structures were found at a much higher density in KKLS/VEG-C tumors than in control tumors. These findings are consistent with the published direct evidence for the role of VEGF-C in tumor lymphangiogenesis (28, 29). However, VEGF-C secreted by the tumor did not promote lymphatic metastasis in our present experiments. Lymph node metastasis was not observed in the KKLS/VEGF-C or the control mice. For lymphatic metastasis, tumor cells must complete multiple steps, which include lymphangiogenesis, motility, invasion, survival in the circulation, adhesion, extravasation, and proliferation (2, 3). He et al. (42) reported that tumors of lung carcinoma cells overexpressing VEGF-C contain more lymphatic vessels than vector-transfected tumors but do not have increased metastatic ability. Therefore, lymphangiogenesis induced by VEGF-C may not be the only metastasis rate-limiting factor. To survive in the lymph circulation and colonize lymph nodes, another growth factor, such as VEGF-A (44) or platelet-derived growth factor-BB (45), may be needed.

In addition to lymphangiogenesis, we observed greatly accelerated angiogenesis and in vivo growth of KKLS/VEG-C cells compared with that of control cells in the present study. VEGF-C and VEGF-D can exert angiogenic activity through VEGF-2 (46), and previous data clearly link VEGF-2 to angiogenesis and progression of gastric cancer (47). On the other hand, Mandriota et al. reported that VEG-C induces lymphangiogenesis, but not angiogenesis in double-transgenic mice (48). Although there is no explanation for the discrepancy, this might be due to differences in VEG-C proteolytic processing in the different models. The stepwise proteolytic processing of VEG-C generated several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C could activate VEGFR-2 (49). VEG-F-3 has been detected in both blood vessels and lymphatic vessels in tumor tissues (27, 50, 51). There are several studies that showed that antibody interference with VEG-FR-3 function can inhibit tumor growth by inhibiting neoangiogenesis in various human tumor xenografts in immunocompromised mice (34, 52). We found in the present study that VEG-C induces the expression of PlGF by KKLS cells. Expression of PlGF by KKLS cells may also play a role in tumor angiogenesis.

Beside roles in lymphangiogenesis and angiogenesis, we identified an additional role of VEGF-C/VEGFR-3 in tumor growth. Treatment with VEG-C increased expression of cyclin D1 (a cell cycle regulator) and stimulated growth of KKLS cells in vitro, and we found a higher number of Ki-67–positive cells in KKLS/VEGF-C tumors than in vector control tumors. He et al. (53) reported that treatment of ectopic xenografts of lung carcinoma cells with VEGF-3-immunoglobulin inhibits tumor growth without a reduction in microvessel density. These findings support the existence of autocrine stimulation of tumor growth by the VEGF-C/VEGFR-3 axis.

In summary, our results show that VEGF-C is an important growth factor, in addition to acting as a lymphangiogenic or angiogenic factor. Thus, we propose that interruption of the VEGF-C/VEGFR-3 axis may be a therapeutic approach for controlling disease progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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