Vascular Endothelial Growth Factor-C Expression and Invasive Phenotype in Ovarian Carcinomas

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Abstract

Purpose: To investigate the biological correlation between vascular endothelial growth factor (VEGF)-C expression and invasive phenotype in ovarian carcinomas.

Experimental Design: Gene and protein expression levels of VEGF-C in 10 ovarian carcinoma cell lines were correlated with invasive activity of the cells. The correlation between immunohistochemical expression of VEGF-C and tumor aggressiveness in 73 ovarian carcinomas was also examined with respect to clinicopathologic features and patient outcome.

Results: VEGF-C gene and protein expression differed remarkably among the cell lines, and there was a statistical correlation among VEGF-C expression, in vitro invasive activity, and matrix metalloproteinase-2 (MMP-2) gene expression and its activity. Anti-VEGF-C and anti-MMP-2 antibodies inhibited the invasive activity of tumor cells. VEGF-C expression in clinical tissue samples was well correlated with clinical stages, retroperitoneal lymph node metastasis, MMP-2 expression, angiogenesis, lymphangiogenesis, and low apoptotic index (AI). The patients whose tumors had strong VEGF-C expression and low AI underwent a poorer prognosis than did those with weak VEGF-C expression and high AI.

Conclusion: VEGF-C expression is closely related to invasive phenotype and affects the patient’s survival in ovarian carcinomas.

Growth of solid tumors depends on angiogenesis, the process by which new blood vessels develop from the endothelium of a preexisting vasculature (1). Tumors promote angiogenesis by secreting various angiogenic factors, and newly formed blood vessels induce tumor cell proliferation and invasiveness. Various peptide growth factors, such as vascular endothelial growth factor (VEGF; refs. 2, 3), basic fibroblast growth factor (4, 5), and thymidine phosphorylase (6–8), have been found to stimulate the proliferation and motility of endothelial cells, thus inducing new blood vessel formation. VEGF-A, also known as vascular permeability factor, is considered to play an important role in tumor angiogenesis (3). Recently, three new members of the VEGF family, VEGF-B, VEGF-C, and VEGF-D, have been discovered and characterized (9–11). It has been suggested that VEGF family members, basic fibroblast growth factor, and thymidine phosphorylase are expressed in a variety of human tumors in different ways.

VEGF-C is a ligand for VEGF receptor-3 (Flt-4), a tyrosine kinase receptor that is predominantly expressed in the endothelium of lymphatic vessels (10). Experimental results with the VEGF-C transgenic mouse have shown that VEGF-C expression is associated with hyperplasia of lymphatic vessels (12). It is conceivable that VEGF-C might play a crucial role in lymphatic proliferation and also in spread of solid tumors. Recently, some investigators have shown that VEGF-C expression was closely associated with tumor invasion and lymph node metastasis in gastric (13), breast (14), thyroid (15), and cervical (16) carcinomas. Moreover, Van Trappen et al. (17) reported that gene expression of VEGF-C is strongly coexpressed with that of matrix metalloproteinases (MMP) in cervical carcinoma tissues. However, there have been very few reports on the correlation between VEGF-C gene expression and invasive phenotype in ovarian carcinomas.

In the present study, we investigated VEGF-C gene and protein expression levels in various ovarian carcinoma cell lines and correlated them with invasive activity of the cells. Moreover, we examined the correlation between VEGF-C expression and tumor aggressiveness in ovarian carcinomas with respect to clinicopathologic features and patient outcome. Our findings suggest that VEGF-C expression is closely related to invasive phenotype and affects the patient’s survival in ovarian carcinomas.

Materials and Methods

Cell culture. Experiments were conducted using three human ovarian serous cystadenocarcinoma (SHIN-3, HOC-21, and HTOA), two mucinous cystadenocarcinoma (MN-1 and OMC-3), four clear cell adenocarcinoma (RMG-I, RMG-II, HUOCA-II, and HAC-2), and one endometrioid adenocarcinoma (HMOA) cell lines. The OMC-3 cell line
was established in our laboratory. The SHIN-3 and MN-1 cell lines (19) were kindly provided by Dr. Yasuhiko Kiyozuka (Kansai Medical College, Osaka, Japan). The RMG-I (20) and RMG-II (21) cell lines were kindly provided by Dr. Shiro Nozawa (Keio University, Tokyo, Japan). The HOC-21 (22) and HAC-2 (23) cell lines were provided by Dr. Naotake Tanaka (Chiba University, Chiba, Japan). The HUOCA-II (24), HTOA (25), and HMOA (26) cell lines were provided by Dr. Isamu Ishiwata (Ishiwata Hospital, Mito, Japan). The OMC-3, RMG-I, RMG-II, HUOCA-II, HTOA, and HMOA cell lines were maintained as monolayer cultures in Ham’s F-12 (Flow Laboratories, Irvine, United Kingdom) supplemented with 10% fetal bovine serum (Mitsubishi Chemical Co., Tokyo, Japan) at 37°C in a humidified incubator with 5% CO2 in air. The SHIN-3, MN-1, HOC-21, and HAC-2 cell lines were cultured in DMEM (Life Technologies, Bethesda, MD) supplemented with 10% fetal bovine serum as described above. The cells were grown in 75 cm2 tissue culture flasks (Nunc, Roskilde, Denmark), washed with PBS, and then harvested after a brief treatment with 0.1% trypsin solution containing 0.02% EDTA (Flow Laboratories). The cell viability was determined by trypsin blue dye exclusion before use.

Tumor conditioned medium (TCM) was prepared from the culture supernatant of the cells. Briefly, confluent monolayers of tumor cells grown in 6 cm2 plastic dishes (Corning 25010, Iwaki Glass, Tokyo, Japan) were rinsed twice with serum-free Ham’s F-12 or DMEM and incubated at 37°C for 48 hours with 4 mL serum-free medium. Protein solution of ovarian carcinoma cells was also prepared from each cell line as described previously (27). Briefly, confluent monolayers of tumor cells grown in 10 cm2 plastic dishes (Iwaki Glass) were rinsed twice with cold PBS and then lysed with modified radioimmunoprecipitation assay buffer (2 mmol/L sodium orthovanadate, 50 mmol/L NaF, 20 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 5 mmol/L sodium pyrophosphate, 10% glycerol, 0.2% Triton X-100, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonlfuoride, 10 mg/mL leupeptin, 10 mg/mL aprotinin). The protein concentration of TCM and protein solution thus obtained was determined by using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) and then stored at −80°C until use.

RNA isolation and cDNA preparation. RNA was extracted from the cells by SV-total RNA isolation kit (Promega Corp., Madison, WI) according to the supplier’s recommendation. Contaminating residual genomic DNA was removed by digestion with RNase-free DNase (Promega). cDNAs were prepared using at least 2 μg of total RNA and SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD) with random hexamers as primers, finally dissolved in diethyl pyrocarbonate-treated water, and then frozen at −20°C until use.

Quantitative reverse transcription-PCR analysis. Quantitative PCR amplification was done with a LightCycler (Roche Diagnostics, Tokyo, Japan) according to the method reported by Yamada et al. (28) with some modifications. The following primers were used: 5’-CCACAGGAT-CACCCCTAAT-3’ and 5’-AAATGAGGGACACAGAGC-3’ for VEGF-C, 5’-GTTGATCGCCCTTATACGTG-3’ and 5’-AGGACGCTAGC-CAGTCCGAT-3’ for MMP-2, and 5’-CCAACCCGAGGAAGATGAC-3’ and 5’-GGAAGAGCCGTGGAAGATGAC-3’ for β-actin as an internal control. cDNA aliquots (2 μL) were subjected to amplification in a 20 μL reaction mixture containing 2 μL LightCycler FastStart Mix (Taq DNA polymerase, reaction buffer and deoxynucleotide triphosphate mix, Roche Molecular Biochemicals, Mannheim, Germany), 1 μL sense and antisense primers (10 pmol/μL), 1 μL hybridization probe R (8 pmol/μL), 1 μL hybridization probe F (4 pmol/μL), 3 mmol/L MgCl2, and sterile distilled water. After an initial denaturation at 95°C for 15 minutes, 40 cycles of denaturation at 95°C for 10 seconds, annealing at 62°C for 10 seconds, and extension at 72°C for 10 seconds for the respective target genes were carried out on a Roche Diagnostics LightCycler System. A standard curve was generated using fluorescent data from the serial dilutions of the plasmid, including a single PCR product. The gene expression levels were evaluated as 1,000 × VEGF-C or MMP-2/β-actin. Reverse transcription-PCR products were visualized by 1.5% agarose gel electrophoresis with ethidium bromide staining if necessary. Each analysis was done in triplicate.

Sandwich ELISA for vascular endothelial growth factor-C. Protein solution of each cell line was assayed for VEGF-C expression of tumor cells according to the method reported by Weich et al. (29) with some modifications. Briefly, anti-human VEGF-C rabbit IgG 103 (10 μg/mL, IBL, Gunma, Japan) was used for coating and the antigen affinity-purified and peroxidase-conjugated antibody 408 (IBL) at 1 μg/mL was used as a detector antibody. As a standard, recombinant human VEGF-C (IBL) was used over a concentration range between 0.1 and 6.25 ng/mL. For visualization of the detector, tetramethylbenzidine (Roche Molecular Biochemicals) was used. After stopping the reaction with 1 mol/L H2SO4, the absorbance was measured at 450 nm by a microplate reader (Tosoh model MPR-A4, Tokyo, Japan). Generally, the samples were analyzed in different dilutions, measuring each dilution in triplicate.

Haptotaxis assay. The invasive activity of tumor cells was assayed in Chemotaxicell culture chambers (Kurabo, Osaka, Japan) according to the method reported by Albini et al. (30) with some modifications as described previously (16). Polyvinylpyrrolidone-free polycarbonate filters with 8.0 μm pore size were precoated with 10 μg fibronectin in a volume of 50 μL PBS on the lower surface and dried overnight at room temperature under a hood. The Matrigel diluted to 500 μg/mL with cold PBS was then applied to the upper surface of the filters (5 μg/filter) and dried again. Log-phase cell cultures of tumor cells were harvested with 0.1% trypsin containing 0.02% EDTA and resuspended to 106/mL in growth medium. Cell suspension (200 μL) was added to the upper compartment, and growth medium (600 μL) was immediately added to the lower compartment. The chamber was then incubated for 24 hours at 37°C in a 5% CO2 air. The filters were fixed with ethanol and stained with hematoxylin. The cells on the upper surface of the filters were removed by wiping with a cotton swab. The cells that had migrated to various areas of the lower surface were manually counted under a microscope at ×400 magnification. Each assay was done in triplicate.

Zymograms. The proteolytic activity of TCM was examined by electrophoresis in a gelatin-embedded polyclaramide gel based on the methods described by Heussen and Dowdle (31). The equal protein concentration of each TCM sample was mixed with SDS sample buffer containing 1 mmol/L phenylmethylsulfonlfuoride and applied, without heating or reducing, to polycaramide gels containing 1 mg/mL gelatin. After electrophoresis, the gels were washed twice with 2.5% Triton X-100 for 6 minutes to remove the SDS, incubated in the incubation buffer containing 0.15 mol/L NaCl, 50 mmol/L Tris-Cl, 10 mmol/L CaCl2, 0.05% NaN3, for 40 hours, and then stained in 0.1% Coomassie blue. Gel images of unstained bands were obtained using the ATTO densitograph (ATTO, Tokyo, Japan), and their densities were quantified using ATTO densitometry software version 2. The relative gelatinolytic levels were calculated as the density of each unstained band divided by that of purified MMP-2 simultaneously examined. Each assay was done in triplicate.

Effects of anti–vascular endothelial growth factor-C and anti–matrix metalloproteinase-2 antibodies on vascular endothelial growth factor-C on invasive phenotype of tumor cells. Effects of anti-VEGF-C antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) on the invasive and proteolytic activity of tumor cells were examined. Log-phase cell cultures of MN-1 cells were harvested and resuspended to a final concentration of 3.0 × 106/mL in growth medium. Cell suspension (200 μL), with or without various amounts of anti-VEGF-C antibody, was added to Chemotaxicell culture chambers and the cells that had invaded were counted as described above. Gelatinolytic levels of TCM samples from MN-1 cells incubated with or without anti-VEGF-C antibody were analyzed by zymograms as described above. Effects of anti-MMP-2 antibody (Fuji, Toyama, Japan) on the invasive activity and VEGF-C gene expression of MN-1 cells were also examined. In addition, effects of VEGF-C (R&D Systems, Inc., Minneapolis, MN) on the invasive activity and the gelatinolytic activity of OMC-3 cells were examined as described above. Each assay was done in triplicate. Moreover, FITC antibody expression in MN-1 and OMC-3 cells was examined by conventional reverse transcription-PCR analysis as

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described previously (32) using the primers 5'-AGCCATTCATCACAAGCCT-3' and 5'-GGCAACAGCTGGATGTCATA-3'. Flt-4 protein expression in these cells was also detected by Western blot analysis as described previously (33) using anti-Flt-4 antibody (Genzyme/Techne, Minneapolis, MN).

**Patients and tissue samples.** A total of 73 primary ovarian carcinomas, which had been resected in our department, were used in this study. Patients had received neither chemotherapy nor radiation therapy before surgery. All patients underwent abdominal simple total hysterectomy with bilateral adnexectomy and omentectomy. Retroperitoneal lymphadenectomy was done for 50 of 73 patients. Clinical stages and pathologic diagnosis were decided according to the classification of the International Federation of Gynecology and Obstetrics (34). Of these patients, 21 had stage I, 2 had stage II, 48 had stage III, and 2 had stage IV disease. Data concerning patient outcome, including overall survival and development of metastasis, were available for all 73 patients. Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin wax. Histologic features in resected tumors were assessed using standard H&E-stained sections. Serial sections, including the greatest diameter of the tumors from the operative specimens, were used.

**Immunohistochemical staining for vascular endothelial growth factor-C and matrix metalloproteinase-2.** An immunohistochemical study for VEGF-C and MMP-2 was done using the avidin-biotin-peroxidase complex method. Dewaxed and rehydrated tissue sections were incubated overnight at 4°C with goat polyclonal anti-VEGF-C antibody (Santa Cruz Biotechnology) or mouse monoclonal anti-MMP-2 antibody (Fuji) at a 1:50 dilution and then washed with PBS. Biotinylated rabbit anti-goat or horse anti-mouse immunoglobulin (DAKO, Kyoto, Japan) was then added to the sections for 30 minutes at room temperature. Peroxidase-conjugated avidin (DAKO) was applied after the sections were washed with PBS. Peroxidase activity was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The sections were counterstained with hematoxylin and mounted with a cover glass.

**Correlation between VEGF-C gene expression and the number of invaded tumor cells.** A correlation analysis was performed using the Spearman rank correlation test.

**Fig. 1.** Correlation between VEGF-C gene expression and the number of invaded tumor cells (A) or MMP-2 gene expression (B), the number of invaded tumor cells and MMP-2 gene expression (C) or activity (D), and VEGF-C protein expression and the number of invaded tumor cells (E) or MMP-2 activity (F). Points, mean of triplicates.
was detected by exposure of the sections to the solution of 0.05% 3,3′-diaminobenzidine and 0.01% H2O2 in Tris-HCl buffer (3,3′-diaminobenzidine solution) at pH 7.6 for 3 to 6 minutes at room temperature. The sections were counterstained with hematoxylin. Normal goat or mouse IgG was used as a substitute for the primary antibody for the negative controls. The immunoreactivity of VEGF-C and MMP-2 is expressed as a percentage of positively stained cancer cells per total number of cancer cells and assigned to one of three subgroups (−, <10%; +, 10-50%; and +++, >50%).

Determination of intratumoral microvessel or lymph vessel density. To highlight vascular or lymphatic endothelial cells, dewaxed and rehydrated tissue sections were incubated overnight at 4°C with mouse monoclonal anti-CD34 antibody (QB-END/10, Novocastra Laboratory, Newcastle, United Kingdom) or rabbit polyclonal anti-LYVE1 antibody (Abcam Ltd., Cambridge, United Kingdom) at 1:50 dilution and then washed with PBS. The following steps were the same as those used for the anti-VEGF-C or MMP-2 protocol. For the determination of intratumoral microvessel or lymph vessel density (IMVD or ILVD), the five most vascular or lymphovascular areas within a section were selected and counted under a light microscope with a 200-fold magnification as described by Weidner et al. (35) or Beasley et al. (36), respectively. The average numbers were recorded as IMVD or ILVD for each case.

Determination of apoptotic index. DNA breaks were detected in situ by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling according to the method of Gavrieli et al. (37) with some modifications as described previously (38). Dewaxed and rehydrated tissue sections were digested with 20 μg/mL proteinase K (Sigma, St. Louis, MO) for 15 minutes at room temperature and then washed with distilled water and subsequently with PBS. The tissues were incubated with a solution containing 2% H2O2 in PBS to inhibit endogeneous peroxidase activity and then washed with PBS. TdT buffer solution [100 mmol/L potassium cacodylate, 2 mmol/L cobalt chloride, 0.2 mmol/L DTT (pH 7.2)] containing 0.3 units/μL TdT (Intergen, Purchase, NY) and 0.04 nmol/μL digoxigenin-dUTP (Intergen) were added to cover the tissues, which were then incubated in a humidified atmosphere for 60 minutes at 37°C. The tissues were washed with buffer solution containing 300 mmol/L NaCl and 30 mmol/L sodium citrate for 30 minutes at 37°C to terminate the reaction and then washed with PBS. They were subsequently incubated with anti-digoxigenin-peroxidase complex for 30 minutes at room temperature and stained with a 3,3′-diaminobenzidine solution. The sections were counterstained with hematoxylin. Negative controls were obtained by omitting TdT from the buffer solution. The apoptotic index (AI) was calculated as the ratio of TdT-mediated dUTP nick end labeling–positive cancer cells to total number of cancer cells and obtained in more than five >200 microscopic fields.

Statistical analysis. All statistical calculations were carried out using StatView statistical software. The Spearman rank correlation coefficient was used to analyze the relation between two different values. The clinical characteristics, MMP-2 expression, IMVD, ILVD, and AI of the patients were compared with VEGF-C expression in the tumor cells and assigned to one of three subgroups (−, <10%; +, 10-50%; and +++, >50%). As shown in Fig. 1A, the protein expression levels of VEGF-C evaluated by sandwich ELISA were well correlated with the number of invaded tumor cells (Fig. 1E) and MMP-2 activity (Fig. 1F), with a coefficient correlation of 0.909 (P = 0.0003) and 0.725 (P = 0.0178), respectively. Effects of anti–vascular endothelial growth factor-C and anti–matrix metalloproteinase-2 antibodies or vascular endothelial growth factor-C on invasive phenotype of tumor cells. As shown in Fig. 2A, the invasive activity of MN-1 cells with higher gene expression levels of VEGF-C and MMP-2 into reconstituted basement membrane components was inhibited by the

Results

Correlation between vascular endothelial growth factor-C gene or protein expression and invasive phenotype in ovarian carcinoma cell lines. The gene expression levels of VEGF-C and MMP-2 in comparison with β-actin expression and the gelatinolytic activities of TCM from the cells differed remarkably among 10 ovarian carcinoma cell lines. The SHIN-3 and MN-1 cells had higher expression levels of the VEGF-C and MMP-2 genes and MMP-2 activity. As shown in Fig. 1A, there was a statistically significant correlation between VEGF-C gene expression and the number of invaded tumor cells, with a coefficient correlation of 0.929 (P = 0.0001). VEGF-C gene expression in 10 ovarian carcinoma cell lines was well correlated with MMP-2 gene expression with a coefficient correlation of 0.904 (P = 0.0003; Fig. 1B). MMP-2 gene expression and activity were also closely associated with the number of invaded tumor cells (Fig. 1C and D), with a coefficient correlation of 0.849 (P = 0.0019) and 0.780 (P = 0.0078), respectively. Moreover, the protein expression levels of VEGF-C evaluated by sandwich ELISA were well correlated with the number of invaded tumor cells (Fig. 1E) and MMP-2 activity (Fig. 1F), with a coefficient correlation of 0.909 (P = 0.0003) and 0.725 (P = 0.0178), respectively.

Fig. 2. Effects of anti-VEGF-C antibody on the invasive activity of MN-1 cells into reconstituted basement membrane components (A) and the gelatinolytic activity of TCM from the cells (D). Effects of anti-MMP-2 antibody on the invasive activity (B) and VEGF-C gene expression (E) of MN-1 cells. Effects of VEGF-C on the invasive activity (C) and the gelatinolytic activity (F) of OMC-3 cells. Flt-4 gene and protein expression in MN-1 and OMC-3 cells (G). Numbers, molecular weight of the proteinase bands. Bars, SE.
presence of 1 to 10 µg/mL anti-VEGF-C antibody in a concentration-dependent manner. Moreover, as can be seen in Fig. 2D, the gelatin zymography showed that the treatment of MN-1 cells with anti-VEGF-C antibody resulted in a decrease of MMP-2 activity in a concentration-dependent manner. Anti-MMP-2 antibody also inhibited the invasive activity of MN-1 cells (Fig. 2B) but did not affect VEGF-C gene expression levels of the cells (Fig. 2E). In contrast, the invasive and gelatinolytic activities of OMC-3 cells with lower gene expression levels of VEGF-C and MMP-2 were stimulated by the presence of 1 to 10 µg/mL VEGF-C in a concentration-dependent manner (Fig. 2C and F), respectively. Flt-4 gene and protein expression was detected in MN-1 and OMC-3 cells as shown in Fig. 2G.

Expression of vascular endothelial growth factor-C and matrix metalloproteinase-2, angiogenesis, lymphangiogenesis, and apoptosis in ovarian carcinomas. The immunohistochemical expression of VEGF-C and MMP-2 was observed in the cytoplasm of tumor cells (Fig. 3A and B). In contrast to the immunoreactivity of VEGF-C and MMP-2, that of anti-CD34 or anti-LYVE1 antibody was located only on the cytoplasm of vascular or lymphatic endothelial cells and not on tumor cells or interstitial cells (Fig. 3C or D), respectively. Intense TdT-mediated dUTP nick end labeling signals were observed in the apoptotic nuclei of some tumor cells (Fig. 3E). The negative control slides for VEGF-C, MMP-2, CD34, LYVE1, and TdT-mediated dUTP nick end labeling exhibited no specific staining. IMVD, ILVD, and AI ranged widely in the 73 tumors examined. Median values of 66.7 for IMVD, 5.2 for ILVD, and 5.8 for AI were taken as the cutoff points for discrimination of the 73 patients into two subgroups.

Correlation between vascular endothelial growth factor-C expression and clinicopathologic features, matrix metalloproteinase-2 expression, angiogenesis, lymphangiogenesis, and apoptosis. The clinicopathologic data, MMP-2 expression, IMVD, ILVD, and AI from 36 patients whose tumors had 2+ VEGF-C-positive staining and 37 whose tumors had negative or 1+ VEGF-C-positive staining were compared (Table 1). The immunoreactivity of VEGF-C was strongly correlated with clinical stages, retroperitoneal lymph node metastasis, MMP-2 expression, and lymphangiogenesis (P < 0.0001). VEGF-C expression in serous tumors was higher than that in nonserous tumors (P = 0.0184). There was also close correlation between VEGF-C expression and omental metastasis (P = 0.0003), peritoneal dissemination (P = 0.0016), hypervascularity (P = 0.0459), and low AI (P = 0.0423).

Correlation among peritoneal dissemination, apoptotic index, and tumor vascularity. As shown in Fig. 4A, the IMVDs in primary tumors with peritoneal dissemination were statistically higher than those without peritoneal dissemination (P = 0.0416). Interestingly, as can be seen in Fig. 4B, regression analysis with the Spearman rank correlation coefficient on plots of IMVD versus AI on a per case basis revealed a significant inverse correlation between them (R = 0.469, P < 0.0001).

Vascular endothelial growth factor-C expression or apoptotic index and patient outcome. Of the 73 patients examined in this study, 36 (49.3%) patients experienced recurrences and 33 (45.2%) had died due to relapse of the disease during follow-up period. The overall survival rates for 36 patients whose tumors had 2+ VEGF-C-positive staining were significantly lower than those for 37 whose tumors had negative or 1+ VEGF-C-positive staining (P = 0.0241; Fig. 5A). The prognosis of 42 patients whose tumors had low AI (<5.8) was poorer than that of 31 patients whose tumors had high AI (>5.8; P = 0.0049; Fig. 5B). When these analyses were conducted on a single histologic type of primary ovarian lesion with 47 serous adenocarcinomas, the patients whose tumors had strong VEGF-C expression and low AI again underwent a poorer prognosis than did those with weak VEGF-C expression and high AI (P = 0.0195 and 0.0423; Fig. 5C and D), respectively.
There was no statistical difference in the clinical outcome between 11 patients whose tumors had strong VEGF-C expression and high AI and 17 patients whose tumors had weak VEGF-C expression and low AI.

Table 1. Correlation between VEGF-C expression and clinicopathologic features in 73 ovarian carcinomas

<table>
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<tr>
<th>Variable</th>
<th>2+ expression</th>
<th>−/+ expression</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>(n = 36)</td>
<td>(n = 37)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
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<td></td>
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<tr>
<td>I + II</td>
<td>2 (8.7)</td>
<td>21 (91.3)</td>
<td>&lt;0.0001</td>
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<tr>
<td>III + IV</td>
<td>34 (68.0)</td>
<td>16 (32.0)</td>
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</tr>
<tr>
<td>Histology</td>
<td></td>
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<td></td>
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<tr>
<td>Serous</td>
<td>28 (59.6)</td>
<td>19 (40.4)</td>
<td>0.0184</td>
</tr>
<tr>
<td>Nonserous</td>
<td>8 (30.8)</td>
<td>18 (69.2)</td>
<td></td>
</tr>
<tr>
<td>Retroperitoneal lymph node metastasis*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>4 (12.5)</td>
<td>28 (87.5)</td>
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<td>Positive</td>
<td>16 (88.9)</td>
<td>2 (11.1)</td>
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<tr>
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<tr>
<td>Negative</td>
<td>9 (26.5)</td>
<td>25 (73.5)</td>
<td>0.0003</td>
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<tr>
<td>Positive</td>
<td>27 (69.2)</td>
<td>12 (30.8)</td>
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<tr>
<td>Peritoneal dissemination</td>
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<tr>
<td>Negative</td>
<td>12 (31.6)</td>
<td>26 (68.4)</td>
<td>0.0016</td>
</tr>
<tr>
<td>Positive</td>
<td>24 (68.6)</td>
<td>11 (31.4)</td>
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<tr>
<td>−/+ expression</td>
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<td>26 (89.7)</td>
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<tr>
<td>2+ expression</td>
<td>33 (75.0)</td>
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<tr>
<td>IMVD Hypo (&lt;66.7)</td>
<td>13 (37.1)</td>
<td>22 (62.9)</td>
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<td>23 (60.5)</td>
<td>15 (39.5)</td>
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<td>27 (77.1)</td>
<td>&lt;0.0001</td>
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<td>10 (26.3)</td>
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<td>AI Hypo (&lt;5.8)</td>
<td>25 (59.5)</td>
<td>17 (40.5)</td>
<td>0.0423</td>
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<td>Hyper (&gt;5.8)</td>
<td>11 (35.5)</td>
<td>20 (64.5)</td>
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*Fifty of 73 cases underwent retroperitoneal lymphadenectomy; (n) %.

Metastatic spread of the solid tumor depends on a critical cascade of events that includes tumor cell adhesion to a distant site, extracellular matrix degradation, migration, proliferation, and ultimately neovascularization (39). The primary tumor with a high proportion of angiogenic cells is likely to cause metastatic implants that are already angiogenic, enabling them to grow in lymph nodes and distant organs (35). Tumors that produce a higher level of angiogenic factors may have a more aggressive behavior than tumors negative for those factors in the process of invasion and metastasis.

In the present study, expression of VEGF-C mRNA differed remarkably among 10 ovarian carcinoma cell lines, and there was a statistical correlation between VEGF-C gene expression and the number of invaded tumor cells into reconstituted basement membrane. In addition, gene expression levels of VEGF-C were well correlated with those of MMP-2, and MMP-2 gene expression and its activity of the cells were again closely related to the number of invaded tumor cells. Moreover, there was a close correlation between VEGF-C protein expression and the number of invaded tumor cells. MMP-2 (72-kDa type IV collagenase) has been considered to play a central role in the process of invasion and metastasis of gynecologic tumors (16, 40, 41). Moreover, anti-VEGF-C antibody inhibited the invasive and proteolytic activities of MN-1 cells with higher gene expression levels of VEGF-C and MMP-2, whereas VEGF-C stimulated those of OMC-3 cells with lower gene expression levels of VEGF-C and MMP-2. In addition, VEGF receptor-3 (Flt-4) gene and protein expression was detected in MN-1 and OMC-3 cells. Anti-MMP-2 antibody also inhibited the invasive activity of MN-1 cells but did not affect VEGF-C gene expression levels of the cells. Ovarian carcinoma cells that produce VEGF-C may have a higher invasive and metastatic potential because of their capacity to pass through tissue barriers by degrading extracellular matrix with MMP-2 possibly by an autocrine loop of VEGF-C.

Abundant evidence supports the concept that tumors can induce angiogenesis through a variety of angiogenic molecules (2–8), and the grade of angiogenesis, expressed as IMVD, is reported to be a strong prognostic indicator in a variety of malignancies (42). VEGF-A and basic fibroblast growth factor have emerged as central regulators of the angiogenic process in physiologic and pathologic conditions (2–5), and their

Fig. 4. Correlation between IMVD and peritoneal dissemination or AI in primary ovarian carcinomas. A, relationship of IMVD to peritoneal dissemination of the tumor. B, regression analysis with the Spearman rank correlation coefficient on plots of IMVD versus AI on a per case basis (R = 0.469, P < 0.0001). Points, mean of triplicates; bars, SE.
expression in tumor cells has been considered to reflect the aggressive biological characteristics of the tumor. Several workers have found that the expression of thymidine phosphorylase also shows a significant correlation with tumor angiogenesis, and we have shown that the expression of thymidine phosphorylase gene and protein is closely associated with angiogenesis and invasive phenotype in cervical carcinomas (38, 40). However, as far as we are aware, there has been no report describing the possible association among VEGF-C expression, tumor aggressiveness, and patient outcome in ovarian carcinomas.

Our present results showed that VEGF-C expression was strongly correlated with clinical stages, retroperitoneal lymph node metastasis, MMP-2 expression, and lymphangiogenesis in ovarian carcinoma tissues. Tumor cells producing both VEGF-C and MMP-2 may have a higher potential for degradation of extracellular matrix and intravasation into the lymphatic vessels, resulting in tumor development and lymph node involvement. Interestingly, there was a close correlation between Immunohistochemical expression of VEGF-C and tumor vascularity. The IMVDs in primary tumors were also closely related to peritoneal dissemination. VEGF-C requires proteolytic cleavage to produce the fully active form of the factor, that of the central VEGF homology domain (43). The stepwise proteolytic processing of VEGF-C generates several VEGF-C forms with increased activity toward VEGF receptor-3, and the fully processed VEGF-C can bind to and activate VEGF receptor-2 (KDR/Flk-1; refs. 10, 43). In certain circumstances, proteolytic processing would release mature VEGF-C. VEGF-C secreted by tumor cells may also act as a potential angiogenic factor for blood vessels via activating VEGF receptor-2 on the cell membrane of endothelial cells in ovarian carcinomas.

Recent investigations have revealed that the incidence of spontaneous apoptosis in tumor cells is inversely related to the extent of neovascularization and that tumor angiogenesis may contribute to a reduction of apoptosis in tumors (38, 44). The present finding that the AIs in the hypervascular condition were significantly lower than those in the hypovascular condition indicates that apoptotic cells concomitantly decrease when intratumoral microvessels increase. The present study also showed that the patients whose tumors had strong VEGF-C expression and low AI underwent a poorer prognosis than those with weak VEGF-C expression and high AI \((P = 0.0195 \text{ and } 0.0423; C \text{ and } D)\), respectively.

**References**


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