Enhancement of Angiogenesis, Tumor Growth, and Metastasis by Transfection of Vascular Endothelial Growth Factor into LoVo Human Colon Cancer Cell Line

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ABSTRACT

The expression of vascular endothelial growth factor (VEGF), a highly potent angiogenic molecule, is thought to be correlated with the development of colon cancer; however, direct evidence for a role of VEGF in metastasis is lacking. This study was designed to more directly establish the role of VEGF in the growth and metastasis of human colon cancer using a genetically engineered cancer cell line. A stable VEGF gene-transfected human colon cancer cell line, LoVo, was made by genetic manipulation using eukaryotic expression constructs designed to express the complete VEGF121 cDNA in the sense orientation. Transfected clones were screened for VEGF mRNA expression by Northern blot analysis and for VEGF protein expression by Western blot analysis. Consequently, we obtained S17 cells that expressed a high level of both VEGF mRNA and VEGF protein. A vector-transfected clone (V7 cell) was used as a control. The experiment with the dorsal air sac method revealed that S17 cells elicited a stronger directional outgrowth of capillaries than V7 cells. S17 cells formed faster-growing tumors than did V7 cells when xenografted s.c. into nude mice, although there was no significant difference in their in vitro proliferation. Tumors derived from S17 cells had more vascularity, as assessed by counting capillary vessels after staining with factor VIII, than did tumors derived from V7 cells (P < 0.05). With regard to the metastatic potential, S17 cells exhibited a higher capacity for both hepatic metastasis after the splenic portal inoculation and peritoneal dissemination after i.p. injection than did V7 cells. However, S17 cells showed no apparent metastasis, despite their rapid growth after orthotopic implantation. In conclusion, the present study showed clearly that VEGF plays an important role in cancer growth due to stimulation of angiogenesis by accelerating cell growth after reaching the target organs.

INTRODUCTION

Neovascularization is a critical requirement for tumor growth and metastasis formation. Folkman et al. (1, 2) demonstrated in ground-breaking studies that solid tumors establish their own blood supplies by encouraging the growth of new blood vessels into the tumor tissue to extend beyond the small size of approximately 1 mm³. Once the tumor vasculature is established, solid tumors acquire the nutrients that enable them to expand exponentially and ultimately to metastasize (2–4). A commonly cited model of metastasis holds that once neoplastic cells leave a primary tumor and reach the circulation, a series of hurdles must be overcome to establish a metastatic deposit. Numerous angiogenic factors that regulate this process have been identified (5). Among these factors is VEGF,2 which has been implicated in the neovascularization of a wide variety of tumors (6–10). VEGF, also known as vascular permeability factor, is a Mᵋ 36,000–45,000 dimeric glycoprotein that has been identified in conditioned media from numerous cell lines and is expressed in various normal tissues (11) and most human and animal tumors (11, 12), including ascites tumors (6–19).

Recently, several studies suggested that VEGF is the angiogenic factor associated most closely with induction and maintenance of the neovasculature in human tumors (7, 17–19), and clinical studies have implicated VEGF expression in tumor progression and metastasis. Furthermore, using a murine model system for colon cancer, Warren et al. (20) demonstrated that a monoclonal antibody against VEGF inhibits s.c. tumor formation in a dose- and time-dependent manner and reduces the number and size of liver metastases. There have also been a few studies focusing on the suppression of tumor growth using VEGF antisense or c-src antisense expression vectors (20–23). These studies have thus been aimed at blocking VEGF, and they have resulted in the fairly general agreement that VEGF is one of the molecules that can be targeted to stop tumor growth and metastasis (24–28).

To our knowledge, however, there have been no studies that attempted to analyze the changes in malignant potential induced by high expression of VEGF. Considering this background, the current study was designed to investigate the effect of high constitutive expression of VEGF on tumor growth and metastasis, thereby establishing the role of VEGF in colon...
cancer, based on our previous reports that VEGF mRNA expression was closely correlated with highly malignant potential in the human colon cancer.

MATERIALS AND METHODS

Cell Line and Culture. LoVo, a human colon cancer cell line that expresses a low level of VEGF mRNA was obtained from the Health Science Research Bank and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Bio-Whittaker), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂. The crude wild-type LoVo cells were cloned by the limiting dilution method.

Subcloning of VEGF121 into pCAG-BSD and DNA Transfection. The full-length cDNA for VEGF121 was made from reverse transcription-PCR products of human colon cancer specimens. We used the following PCR primers, which were based on the human VEGF cDNA sequence: (a) sense primer, 5'-CCTCCGAAAACCATGAACTTT-3'; and (b) antisense primer, 5'-AGAGATCTGGTTCCCGAAAC-3'. The human VEGF121 cDNA was subcloned into pBluescript II SK(+) and we confirmed the sequence using an ALF red DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden). A eukaryotic expression vector, pCAG-BSD, was newly constructed with two parts of pCAGGS (a kind gift from Dr. J. Miyazaki, Osaka University, Osaka, Japan; Ref. 29) and pMAM2-BSD (Kaken, Tokyo, Japan). The human VEGF121 cDNA was cloned into the XhoI restriction enzyme site of pCAG-BSD to create the plasmid pCAG-BSD-VEGF, in which the transcription of VEGF was constitutively driven by the CAG enhancer promoter, and the drug-resistant selection gene BSD was present (30). The orientation of the VEGF insert was confirmed by restriction mapping.

The cloned wild-type LoVo cells were cultured in 6-well plates. DNA transfections were performed using 4 μl of LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) and 1 μg of pCAG-BSD-VEGF or 1 μg of pCAG-BSD vector alone (as a control) per well. The cells were cultured in 1 ml of serum-free OPTI-MEM (Life Technologies, Inc.) medium containing the DNA/LipofectAMINE complex for 4 h and then supplemented with 1 ml of RPMI 1640 containing 20% FBS. After 48 h, the cells in each well were trypsinized and replated 1:20 into two 10-cm culture dishes in complete culture medium containing 10 μg/ml blasticidin S (Kaken) to select transfecants. Some cell death was observed after 3–4 days in culture, and discrete colonies were apparent by 7 days after selection. Twenty-two individual colonies of sense VEGF transfecants and 10 individual colonies of vector transfecants were picked and pooled, and cells were maintained in complete culture medium with 10 μg/ml BSD to obtain stable transfecants. These transfecants were individual clones.

RNA Isolation and Northern Blot Analysis. All transfecants were grown to confluence in RPMI 1640 in 10-cm² Petri dishes, and total cellular RNA was extracted using Trizol regent (Life Technologies, Inc.). RNA samples were resolved by electrophoresis through 1% agarose-formaldehyde gels and transferred to Hybond-N⁺ nylon membrane filters (Amersham, Buckinghamshire, United Kingdom). The probes were labeled with [α-32P]deoxyctydine 5'-triphosphate using the Megaprime Random Primer DNA Labeling Kit (Amersham). Hybridization was performed at 65°C for 2 h in Rapid Hybridization Solution (Amersham) with a labeled probe. After hybridization, the filters were washed twice with 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at room temperature for 10 min, washed once with 1× SSC and 0.1% SDS at 65°C for 15 min, and then washed twice with 0.1× SSC and 0.1% SDS at 65°C for 15 min. Autoradiographs were made using...
**Detection of Neovascularization with Dorsal Air Sac Method.** The dorsal air sac method was used as described previously (28). Sense transfectant (S17) and vector transfectant (V7) cells were washed once with PBS and suspended in PBS at a concentration of 2 × 10⁶ cells/0.2 μl. A Millipore chamber (diameter, 10 mm; filter pore size, 0.45 μm) was filled with 0.2 ml of the cells and implanted s.c. into the dorsal side of the mice. At 5 days after implantation, the mice were anesthetized and fixed in the prone position. A wide, rectangular incision was made in the skin on the dorsal side, and the skin was carefully ablated. To locate the chamber-contacting region, a ring (Millipore) of the same shape as the chamber was placed onto the s.c. tissues adjacent to the chamber region, and the area was photographed. For histological analysis, all samples were taken from this skin area, embedded in 10% formaldehyde, and sliced into 4-μm-thick vertical sections, which were then stained with H&E.

**s.c. Tumors.** Confluent cultures of sense VEGF-transfected LoVo cells (S17) and vector-transfected LoVo cells (V7) grown in 10-cm² Petri dishes were harvested by a brief trypsinization (0.05% trypsin/0.02% EDTA in Ca²⁺/Mg²⁺-free PBS), washed several times in Ca²⁺/Mg²⁺-free PBS, and re-suspended at a final concentration of 3 × 10⁶ cells/0.2 ml in PBS. Athymic BALB/c nude mice (4-week-old males obtained from Charles River, Yokohama, Japan) were housed in sterilized cages and injected s.c. with 3 × 10⁶ viable tumor cells. Animals were observed daily for tumor growth, and s.c. tumors were measured every 7 days. Tumor volume was calculated as 1/2 × length × width² (length > width).

**Liver Metastases.** Sense VEGF transfectants (S17) and vector transfectants (V7) were grown to confluence and harvested as described above for s.c. injection and resuspended in PBS at a concentration of 2 × 10⁶ cells/0.2 ml. Athymic mice were anesthetized by ether inhalation and wiped with antiseptic solution, and the spleen was exteriorized through a left-flank incision. Two million cells were slowly injected into the splenic pulp through a 30-gauge needle. All animals were killed on day 28. The spleens and livers were excised, and the metastases were checked by H&E staining.

**i.p. Injection.** A single-cell suspension of 2 × 10⁶ cells with a viability of >95% was injected into the peritoneal cavity of the mice. The mice were monitored daily for evidence of disease (abdominal swelling, hunched posture, and listlessness) and were killed when moribund or approximately 42 days after the i.p. injection. All mice were necropsied, and the pattern (discrete solid lesions, carcinomatosis) and extent of abdominal disease (size and number of lesions, volume of ascites) were determined.

**Orthotopic Implantation to the Cecum.** Athymic BALB/c nude mice (4–5-week-old males) were anesthetized by...
ether inhalation, and the abdomen was prepared for sterile surgery. A small abdominal incision was made, and the cecum was identified. Tissue blocks of about 3 mm in diameter of each s.c. tumor were implanted to the serosal side. The abdomen was closed with continuous nylon sutures. The animals were killed 12 weeks later, and abdominal organs and the thorax were examined for the presence of macroscopic “primary” cecal tumors and metastases. Organs including the cecum, liver, mesenteric lymph nodes, and lungs were removed and examined histologically as described below.

**Histological Examination of Metastases.** Specimens for histological examination were fixed in 4% paraformaldehyde for 24 h. Representative sections of cecum, lymph nodes, lung, and liver were also cut and embedded in paraffin. Four-μm sections were then cut and stained with H&E.

**Microvessel Staining and Counting.** The streptavidin-biotin-peroxidase complex method was performed using a streptavidin-biotin kit. Anti-human von Willebrand factor (factor VIII) rabbit serum (DAKO, Copenhagen, Denmark) was used at a dilution of about 1:100 in BSA. As negative controls for immunohistochemical staining, tissue sections were treated with normal rabbit serum instead of primary antibodies. Microvessel density was assessed in tumor areas showing the highest density of staining, as determined by an initial scan with low magnification (×40). For vessel counting, one field magnified 200-fold (high-power field, i.e., ×20 objective and ×10 ocular, 0.739 per

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**Fig. 3** A and B, H&E staining of vertical skin sections 5 days after implantation of S17 cells (A) and V7 cells (B).
field) in each of three vascularized areas was counted, and the average counts were recorded (31).

**Statistical Analysis.** Microvessel densities in the s.c. tumors and cecal primary tumors were compared by the Wilcoxon-Mann-Whitney test. The statistical analysis was done using JMP 3.1 software (SAS Institute, Cary, NC), and a P value of less than 0.05 based on a two-tailed test was considered to indicate statistical significance.

**RESULTS**

**Selection of LoVo Transfected with VEGF.** We first constructed the eukaryotic expression vectors pCAG-BSD-VEGF for VEGF expression and pCAG-BSD as a control. Because wild-type LoVo human colon cancer cell lines were heterogeneous even in their morphology as seen by light microscopy, we cloned them to obtain homogeneous parental cells before transfection. Northern blot analysis (Fig. IA) revealed a high level of VEGF mRNA expression in sense VEGF-transfected LoVo (S17) cells and very low levels of expression in two cell lines, parent LoVo cells and vector-transfected LoVo (V7) cells. Two cell lines, S17 and V7, were chosen for further study. Western blot analysis revealed the presence of VEGF protein only in the conditioned medium of S17 cells (Fig. 1B).

**Proliferation Rate of the Transfectants in Vitro.** There was no significant difference between the proliferation rates of S17 cells and V7 cells (Table 1).

**Angiogenesis Evaluation by Dorsal Air Sac Method.** Representative images of the area adjacent to the s.c. implanted chamber are shown in Fig. 2. On the fifth day after implantation of the S17 cells, the blood vessels had expanded and branched out from the large blood vessels. In some of the mice injected with S17 cells, severe hemorrhaging from these vessels was observed. Fig. 3 shows representative views of H&E staining of skin sections 5 days after implantation. The surface with S17 cells attached showed the development of small vessels and large hemorrhages. On the other hand, neither markedly increased vessels nor hemorrhages were seen among the mice injected with V7 cells.

**Growth Rate of the Transfectants and VEGF mRNA Expression in Vivo.** s.c. injection of equal numbers of S17 cells and V7 cells into BALB/c nude mice gave rise to tumors that grew at the same rate until about 40 days, but thereafter, S17 cells grew faster than V7 cells (Figs. 4 and 5). The sizes of tumors at 90 days were 5215 ± 1612.04 (n = 10) and 528 ± 181.78 mm³ (n = 10) for S17 and V7 cells, respectively (P = 0.02; Table 1). VEGF mRNA expression in the s.c. tumors is shown in Fig. 1C. s.c. tumors derived from S17 cells had high levels of VEGF mRNA expression for as long as 90 days after the inoculation.

**Microvessel Density of s.c. Tumors.** The microvessel densities in S17 and V7 tumors were 6.63 ± 1.08 and 2.29 ± 1.08, respectively (P = 0.021).

**Hepatic Metastases after Splenic Portal Injection.** Among 10 mice injected with S17 cells, macroscopic metastasis and microscopic metastasis in the liver were seen in 5 mice (50%), and the percentage of mice with tumor growth in the spleen was 80%, whereas no mice injected with V7 cells had even microscopic metastasis in the liver or growth in the spleen (Fig. 6; Table 1).

**DISCUSSION**

We reported previously that expression of VEGF mRNA in colon cancer was correlated with tumor progression, including metastasis (32), and more recently, we obtained evidence that tumor angiogenesis occurred along with tumor development and was partially associated with VEGF expression in adenomas and the early stages of human colorectal cancer (33). Kumar et al. found increased serum levels of VEGF in patients with advanced (Dukes’ C) colorectal cancers compared with patients with early-stage disease (32, 34–38). These findings suggest a potential role of VEGF in the development and metastasis of colon cancer. However, no direct evidence has been provided indicating that VEGF is responsible for the acceleration of the appearance of malignant phenotypes in colon cancer. Therefore, we attempted to clarify the role of VEGF in tumor growth, neovascularization, and metastasis of human colon cancer by using cells transfected with the VEGF gene.
We found that sense VEGF transfectants have a growth advantage in vivo but not in vitro. The rapid in vivo growth of sense VEGF transfectants was due to vigorous tumor angiogenesis, which was confirmed using the dorsal air sac method. Namely, the constitutive expression of VEGF led to expansion of the blood vessels, the branching out of newly formed blood vessels from large blood vessels, and occasional severe hemorrhaging, possibly due to fragile neovascularity. In addition, we also evaluated the microvessel density of the s.c. tumors and concluded that the growth advantage of S17 cells appeared to arise from increased tumor vascularization induced by VEGF. With regard to the role of VEGF in metastasis, a number of clinical studies have shown a positive correlation between VEGF expression and metastatic potential. We also demonstrated that colon cancers with high levels of expression of VEGF mRNA metastasized to the liver and lymph nodes at significantly higher rates than those without high levels of VEGF mRNA expression (32).

However, those studies did not prove a causative role of VEGF in metastasis but rather showed an association of VEGF with metastasis. In the present study, we obtained direct evidence that VEGF plays a crucial role in hepatic metastasis in the intrasplenic portal inoculation model system, although it is true that the hepatic metastasis in this experimental model system occurs by the forced introduction of the cancer cells into the blood vessels and thus does not completely mimic spontaneous metastasis. We also checked whether these cells have the ability to complete all stages of the metastasis cascade after orthotopic inoculation, and we found that even S17 cells formed no apparent metastases, despite the rapid tumor growth. Taken together, these findings suggested that VEGF contributes predominantly to tumor growth, probably due to the induction of angiogenesis, and thereby leads to visible foci rather than dormant tumors in either the primary site or metastatic lesions. However, it appears that VEGF alone cannot overcome all of the barriers that must be surmounted for achieving metastasis.

Peritoneal dissemination is also a critical event affecting patient prognosis. Over the past few years, a considerable number of studies have been performed on the correlation between VEGF and peritoneal dissemination (39–41). The present study showed clearly that VEGF transfectants had a greater potential to induce peritoneal dissemination than did the controls. Considering that peritoneal dissemination seems to require lower neovascularization than solid tumor growth, we have not yet clarified why VEGF transfectants showed massive peritoneal dissemination. One putative mechanism is
that VEGF transfectants can be supplied more efficiently than the control transfectants with a large quantity of nutrients by diffusion because VEGF is also able to function as vascular permeability factor, which may, in turn, occasionally cause ascites. Indeed, Kraft et al. (42) reported that VEGF might play an important role in tumor progression and the formation of malignant effusions (43). Mesiano et al. (44) demonstrated that neutralization of VEGF activity may have clinical application in inhibiting malignant ascites formation in ovarian cancer.

In conclusion, the present study provided clear evidence that VEGF plays an important role in cancer growth due to

Fig. 6  Liver colonization by derivatives of S17 cells and V7 cells after splenic portal injection. Spleens and livers were harvested from athymic nude mice 4 weeks after splenic portal injection of 2 × 10⁶ cells. A and C, macroscopic views of S17 cells. B, macroscopic views of V7 cells. D, H&E staining of the liver metastases from S17 cells. (×400). Arrowheads, the metastasis lesion.
stimulation of neovascularization and subsequent metastasis, which is probably mediated by preventing tumors from entering a dormant state.

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