Advances in Brief

Angiogenesis and Tumor Proliferation/Metastasis of Human Colorectal Cancer Cell Line SW620 Transfected with Endocrine Glands-Derived-Vascular Endothelial Growth Factor, As a New Angiogenic Factor

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Abstract

Endocrine glands-derived-vascular endothelial growth factor (EG-VEGF) was recently cloned as a new angiogenic factor that selectively acts on the endothelium of endocrine gland cells. We evaluated the involvement of EG-VEGF in colorectal cancer. The expression of EG-VEGF was confirmed in all of the colorectal cancer cell lines. (On the other hand, the expression of EG-VEGF mRNA was not detected in colorectal normal mucosae.) Stable EG-VEGF infectors of colorectal cancer cell line SW620 were produced, EG-VEGF transfectants were implanted into cecum and s.c., and cell proliferation was evaluated. Angiogenesis was evaluated by dorsal air sac method. Liver metastasis was evaluated after the implantation of EG-VEGF transfectants into the mouse spleen. Tumor proliferation (cecum, s.c.) was significantly higher in the EG-VEGF transfectants than in the control cells. The small vessels were significantly increased in EG-VEGF transfectants as compared with those in control cells. Also, liver metastatic ratio was higher in the EG-VEGF transfectants than in the control cells. In this study, EG-VEGF, a new angiogenic factor, may lead to angiogenesis, promoting cell proliferation and liver metastasis in colorectal cancers. When the EG-VEGF gene-overexpressing colorectal cancer cell line that had been treated with phosphorothioate antisense EG-VEGF oligonucleotides was injected s.c. into mice, angiogenesis and tumor growth were inhibited. Although the novel angiogenesis factor EG-VEGF was not expressed in the normal colorectal mucosa, it was expressed in colorectal cancer cells, which indicates that it is a cancer-specific and possibly tissue-specific angiogenesis factor in the large intestines, and which suggests that it can be targeted by a novel antiangiogenesis therapy.

Introduction

Hematogenous metastasis occurs by the following processes: (a) cell proliferation and angiogenesis in the primary lesion; (b) infiltration to surrounding areas; (c) vascular invasion; (d) adhesion to vascular endothelial cells in the target organ; (e) extravascular escape and migration; and (f) proliferation and angiogenesis in metastatic lesions (1). Molecules that play an important role in each process have been clarified. In processes a and f, angiogenesis is controlled by the balance between its promoting and inhibiting factors (2). In the blood vessels of normal tissue, angiogenesis is maintained by the action of angiogenesis inhibitory factors, such as IFN and NK4/malignostatin (3, 4), that regulate the action of its promoting factors such as vascular endothelial growth factor, β-fibroblast growth factor, hepatocyte growth factor, and interleukin-8 (5, 6). In tumor tissue, the action of angiogenesis promoting factors exceeds that of inhibitory factors, resulting in angiogenesis (2, 7). Tumor proliferation and metastasis depend on tumor angiogenesis. Recently, attention has been directed to antiangiogenesis therapy in which tumor proliferation is inhibited by inhibiting the formation of new tumor vessels using angiogenesis inhibitory factors and drugs (8–11).

LeCouter et al. (12) recently cloned endocrine glands-derived-vascular endothelial growth factor (EG-VEGF), which selectively acts on the endothelium of endocrine gland cells. The coding region of EG-VEGF encodes 305 amino acids, and the M<sub>r</sub> was 8,600 daltons. Its mature protein was predicted to have 86 amino acids containing 10 cysteine residues. EG-VEGF has high homology (80%) with nontoxic protein purified from the venom of black mamba snakes but low homology with vascular endothelial growth factor (VEGF). Colorectal cancer cells have estrogen receptors β and are affected by endocrine hormones (13). We evaluated the involvement of EG-VEGF in colorectal cancer.

Materials and Methods

Cell Culture. The human colorectal cancer cell lines, SW620, HT29, DLD-1, and Colo320 were cultured at 37°C in 5% CO<sub>2</sub> in RPMI 1640 (HT29, DLD-1) or DMEM (SW620, Colo320) containing 10% fetal bovine serum.

Colorectal Normal Mucosae. Colorectal adjacent normal mucosae were obtained from 10 patients undergoing surgical resection of human advanced colorectal cancer at Fukui Medical University Hospital, Japan. Normal colorectal mucosae were dissociated from the muscle and connective tissues. All of the tissue specimens were then frozen in liquid nitrogen and stored at −80°C.

Reverse Transcription-PCR Analysis. Total RNA was extracted from colorectal tissues using guanidium-thiocyanate. Single-strand cDNA prepared from 3 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) with an oligo(dT) primer-14 was used as the template for the PCR. The primers for PCR to amplify EG-VEGF gene-coding regions were as follows: the 5′ primer, EG-VEGF-A, encompassed positions 1–27, ATGAGAGGTGCCACGCGAGTCTCAATC; and 3′ primer, EG-VEGF-B, encompassed positions 259–285, GCCGTACCTCGCTCCGGAACCTGGA. Glyceraldehyde-3-phosphate dehydrogenase amplification was used as internal PCR control with 5′-GGGGAGCCAAAAGGGTCATCATCT-3′ as the sense primer and 5′-GGGAGCCAAAAGGGTAGTTCTC-3′ as the antisense primer. Thirty cycles of denaturation (94°C, 1 min), annealing (50°C, 1.5 min), and extension (72°C, 2 min) were carried out in a thermal cycler (Program Temp Control System PC-700; Astec Inc.). Ten µl of the PCR product were resolved by electrophoresis in 1.2% agarose gel. The sequencing was performed on PCR products that showed the bands in reverse transcription-PCR analysis. Sequence analysis showed the presence of the EG-VEGF gene.

Construction of EG-VEGF Expression Plasmid. The human colon cancer cell line, HT29, was chosen to provide the reverse transcription-PCR template used to clone the full-length EG-VEGF cDNA. We amplified EG-VEGF cDNA using primers as follows: the 5′ primer, encompassed positions 1–27, ATGGAGGTGGCAAGGAGTATCTC; and EG-VEGF DX, the 3′ primer, encompassed positions 289–318, CTAAATTGTAAGCCTCAAGTCATGGA. Direct sequencing indicated that HT29 carried wild-type EG-VEGF. Using primers tagged with restriction enzyme sites (for 5′-BamHI and 3′-EcoRI), we amplified BamHI and EcoRI site-
tagged full-length EG-VEGF fragments and cloned them into a mammalian expression vector, (myc tag)-pcDNA3.1 (Invitrogen) between the EcoRI and BamHI sites. We confirmed the plasmid constructs by DNA sequencing and obtained the DNA for the constitutive expression vector of human EG-VEGF (pcDNA3.1-(myc)-EG-VEGF).

Establishment Stable Cell Lines. SW620 cells were cultured in six-well plates. DNA transfections were performed using LipofectAMINE (Invitrogen) and 1 µg of pcDNA3.1-(myc tag)-EG-VEGF or pcDNA3.1-(myc tag)-empty-vector alone (as a control) per well according to the manufacturer’s instructions. After transfection, cells were selected for neomycin resistance by treating them with G418 sulfate (Promega). Individual G418-resistant clones were picked up, expanded, and analyzed for myc-EG-VEGF expression by immunoblotting of total cellular protein.

Western Blot Analysis. The Western blot analysis was used as previously described (14). Total protein was extracted from the cultured cells using radioimmunoprecipitation assay buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were separated by SDS-polycrylamide. After electrophoresis, the proteins were transferred into nitrocellulose membrane. Protein bands were incubated with antibodies [α-myec monoclonal antibody (Invitrogen), glyceraldehyde-3-phosphate dehydrogenase (Biogenesis)] Signals were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham).

s.c. Tumors. EG-VEGF transfectant SW620 [SW620 cells transfected with the EG-VEGF gene of the human full sequence with pcDNA3.1-(myc tag)-empty-vector] and empty-vector transfectant SW620 [SW620 cells transfected with pcDNA3.1-(myc tag)-empty-vector] were harvested by trypsinization and were resuspended at final concentration of 5 × 106 cells/0.2 ml in PBS. Only single-cell suspensions of >90% viability determined by trypan blue dye exclusion were used for injection. Under sterile conditions, athymic BALB/c nude mice (Charles River) were anesthetized and 50,000 cells were injected s.c. into the back of the mice. The animals were observed daily for tumor growth. Tumor volume was calculated at 1/2 × length × width2. The abdominal organs, the thorax, and the brain were examined for the presence of macroscopic and microscopic metastasis.

Detection of Vascularization with Dorsal Air Sac Method. Colorectal cancer stable cell lines, EG-VEGF transfectant SW620, and empty-vector transfectant SW620, were suspended in PBS at a concentration of 0.5 × 106 cells/ml. A millipore chamber (Millipore; diameter, 10 mm; filter pore size, 0.45 μm) was placed onto the s.c. tissues adjacent to the chamber region, and the area was photographed.

Orthotopic Tumor Implantation into the Cecum. Athymic BALB/c nude mice (6 week-old) were anesthetized, and the abdomen was prepared for sterile surgery. An abdominal incision was made, and the cecum was identified. EG-VEGF transfectant SW620 and empty-vector transfectant SW620 (5 × 106 cells/0.2 ml in PBS) were orthotopically implanted into the subserosa. The mice were sacrificed 4 weeks later, and the abdominal organs, the thorax, and the brain were examined for the presence of macroscopic and microscopic metastasis. A left abdominal flank incision was made, and spleen was exteriorized. Using a 27-gauge needle, 0.1 ml (5 × 105 cells) of a tumor suspension was injected into the splenic subcapsule. The mice were sacrificed 4 weeks later, and the abdominal organs, the thorax, and brain were examined for the presence of macroscopic and microscopic metastasis.

Histological Examination. Specimens (s.c. tumors, abdominal organs, the thorax, and the brain) for histological examination were embedded in OCT compound (Sakura Finetechnical). Four-μm-thick sections were stained with H&E.

Microvessel Staining and Counting. Sections from the blocks were mounted on silanized glass slides. The labeled avidin-biotin method (LSAB kit, Dako) was performed for immunohistochemical staining. The sections were incubated overnight at 4°C with anti-CD31 monoclonal antibody (Novocastra). The sections were incubated with biotinylated antimouse immunoglobulin and streptavidin conjugated to horseradish peroxidase. The sections were incubated with diamobenzidine substrate. 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 in each of five vascularized areas was counted, and average counts were recorded.

Antisense DNA Assay. The EG-VEGF gene-overexpressing colorectal cancer cell line DLD-1 was lipofected with 1 μg phosphorothioate antisense EG-VEGF oligonucleotides, 5'-ACCTCTCATGGTCACTTG-3'; sense EG-VEGF oligonucleotides, 5'-CAAGTGACCATGAGAGGT-3'; or random oligonucleotides, 5'-CTCTGCTCCTCTCTCCA-3'. These lipofected cells or nonlipofected cells (5 × 105 cells/0.2 ml) were injected s.c. into mice, and tumor growth and vascular formation were measured on day 28.

Statistical Analysis. All of the data were evaluated by χ2 test or Student’s t test. Ps less than 5% were considered significant.

Results

Expression of EG-VEGF mRNA in Colorectal Cancer Cell Lines. The expression of EG-VEGF mRNA in four colorectal cancer cell lines was evaluated by reverse transcription-PCR (Fig. 1A). Its expression was confirmed in all of the cell lines. On the other hand, the expression of EG-VEGF mRNA was not detected in 10 colorectal normal mucosa. The association between known cell functions and EG-VEGF mRNA expression is unclear.

EG-VEGF Gene Transfection and Expression of EG-VEGF Protein. Colorectal cancer cell line SW620 was transfected with the EG-VEGF gene [pcDNA3-(myc tag)-EG-VEGF] of the human full sequence using a neomycin-resistant expression vector and treated with G418, and 10 clones each were obtained. Among these clones, those expressing exogenous EG-VEGF protein were selected by the Western blot method, and the clone with the highest expression was termed EG-VEGF transfectant SW620. The results of Western blotting are shown in Fig. 1B. The Mr 10,000 band was exogenous EG-VEGF protein expressed after transfection.

Evaluation of Tumor s.c. Proliferation (the Back of Mice). Empty-vector transfectant SW620 or EG-VEGF transfectant SW620 (5 × 105 cells) was implanted s.c. into. As shown in Fig. 2A and Table 1, the tumor volumes after 20 days were 418 ± 27 mm3 for empty-vector transfectant SW620 but 1115 ± 127 cm3 for EG-VEGF transfectant SW620 (P < 0.05). Tumor proliferation was significantly higher in EG-VEGF transfectant SW620 than in empty-vector transfectant SW620. After 20 days, the mice were sacrificed to examine for
metastatic lesions. No metastatic lesions were observed. Twenty days after implantation, a portion of cells was obtained, and the microvascular density in the tumor was measured using anti-CD31 antibody that identifies vascular endothelial cells. The microvascular density was 38/visual field in empty-vector transfectant SW620 but 102/visual field in EG-VEGF transfectant SW620, indicating a significant inhibition of vascular formation.

Liver Metastasis after Splenic Injection. Four weeks after splenic injection of empty-vector transfectant SW620 or EG-VEGF transfectant SW620, macroscopic and microscopic metastases to other organs were evaluated. Each group consisted of 10 mice. Four weeks after implantation, liver metastasis was observed in 9 (90%) of 10 mice in EG-VEGF transfectant SW620 and in 2 (20%) of 10 in empty-vector transfectant SW620 (P < 0.05; Fig. 3B, Table 1).

Evaluation of Tumor Growth and Vascular Formation in Antisense EG-VEGF Oligonucleotide-Lipofected Colorectal Cancer Cell Line in Mice. Colorectal cancer DLD-1 cells (5 × 10⁵ cells), lipofected with sense EG-VEGF oligonucleotides, random oligonucleotides, or antisense EG-VEGF oligonucleotides, were transplanted into the backs of mice; and posttransplant tumor size was measured. The results are shown in Table 2 (the panel at the bottom of the Table indicates the expression level of EG-VEGF mRNA after the introduction of each oligonucleotide). Three weeks after transplantation, the mean tumor size was 318–358 cm³ for cells lipofected with sense EG-VEGF oligonucleotides or random oligonucleotides and for nonlipofected cells, but it was 141 cm³ for cells lipofected with antisense EG-VEGF oligonucleotides, indicating a significant inhibition of cell proliferation. Furthermore, analysis of intratumoral vascular formation by immunohistological staining with anti-CD31 antibody detected 26–40 cells/field for nonlipofected colorectal cancer cells and those lipofected with sense EG-VEGF oligonucleotides or random oligonucleotides, but 18 cells/field for cells lipofected with antisense EG-VEGF oligonucleotides, indicating a significant inhibition of vascular formation.
Discussion

Many studies have shown a close relationship between angiogenic factors and the progression of malignant tumors (7). In addition, because of recent advances in the techniques of molecular biology, the mechanism of angiogenesis has been clarified, and many types of angiogenesis inhibitors have become clinically used.

In September 2001, LeCouter et al. (12) performed cloning of the EG-VEGF gene, a new angiogenic factor expressed mainly in normal endocrine cells. EG-VEGF is similar to vascular endothelial growth factor (VEGF), another angiogenic factor, in the promotion of angiogenesis, but has low homology with VEGF and differs from VEGF in various respects such as the area of expression. In human normal tissue, EG-VEGF is expressed only by hormone-producing cells such as the ovaries, testis, and placenta but is not observed in the normal mucosa of gastrointestinal organs such as the stomach and large intestine (12). There have been no reports on EG-VEGF expression in malignant tumors. Therefore, we considered that the evaluation of the expression and action of EG-VEGF on colorectal cancer is important in its clinical application in the future.

Arai et al. (13) observed estrogen receptor β expression in colorectal cancer cell lines, suggesting the association between colorectal cancer and hormones. In addition, tamoxifen and estradiol were shown to affect the cell proliferation and intracellular signal transmission system of colorectal cancer, which confirmed the association between colorectal cancer and endocrine hormones (15). Therefore, we evaluated the expression and action of the EG-VEGF gene, a new angiogenic factor in human colorectal cancer. Evaluation of EG-VEGF gene expression in colorectal cancer cell lines showed expression in five of the six cell lines, confirming the association between colorectal cancer and the EG-VEGF gene.

Concerning the association between tumors and angiogenesis, Folkman (16) reported an increase in the tumor volume due to vascular network formation by new blood vessels in the tumor tissue, whereas Kim et al. (17) reported increased angiogenesis in tumors showing more rapid proliferation. Thus, tumor growth is angiogenesis dependent. We transfected colon cancer cell lines with the EG-VEGF gene and established stable cell lines, and we evaluated in vivo proliferation. In the in vivo examination, the cell proliferation rate in s.c. implantations and cecum implanted with cells was significantly high in the EG-VEGF transfectants of colorectal cancer cell lines, which confirmed the action of the EG-VEGF gene on cell proliferation. The association between EG-VEGF gene expression and angiogenesis was evaluated by dorsal air sac analysis and immunohistostaining with anti-CD31 antibody in mouse s.c. tumors. The microvascular count significantly increased in the EG-VEGF transfectant, suggesting that angiogenesis by the EG-VEGF gene promotes cell proliferation. Metastasis was evaluated after implantation of EG-VEGF transfectants of colorectal cancer cell lines into the mouse spleen. After implantation

| Phosphorothioate-oligonucleotide | Vessel counts<sup>a</sup> (s.c. tumor) | s.c. tumor volume<sup>a</sup> (mean ± SD) |
|----------------------------------|--------------------------------------|---------------------------------------|
| (-)                              | 26 (22–48)                           | 318 ± 12                              |
| Sense, EG-VEGF                   | 40 (35–71)                           | 358 ± 41                              |
| Random                           | 33 (19–44)                           | 338 ± 36                              |
| Antisense EG-VEGF                | 18 (10–27)<sup>c</sup>              | 141 ± 66<sup>c</sup>                  |

Table 2 Angiogenesis and tumor growth inhibition of antisense endocrine gland-derived-vascular endothelial growth factor (EG-VEGF) oligonucleotide transfectant DLD-1 cells

<sup>a</sup> Units are means (with ranges given in parentheses) from 10 vascularized areas (0.25-mm<sup>2</sup>) at ×200.

<sup>b</sup> (-), no phosphorothioate oligonucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

<sup>c</sup> The vessel counts were significantly different from those in mice treated with antisense EG-VEGF oligonucleotide (P < 0.05, Student’s t test).
into the mouse spleen, liver metastases were observed. The normal colorectal mucosa that become colorectal cancer cells express the EG-VEGF gene. Its expression may lead to angiogenesis, promoting cell proliferation, and to liver metastasis.

In recent years, various angiogenic factors such as VEGF have been identified. These humoral factors and their receptors, adhesive molecules, and intracellular signal transmission molecules have been extensively analyzed by the gene-targeting method, which has gradually clarified the molecular mechanism of angiogenesis. Clinically, attention has been paid to antiangiogenesis therapy, which inhibits tumor proliferation by interrupting the formation of a new vascular network that supplies blood for tumor proliferation (18). In Western countries, clinical tests have already been carried out in various types of cancer such as gastroenterological cancer, as well as colorectal, gastric, esophageal, breast, brain, and prostatic cancers (19, 20).

In our study, when the EG-VEGF gene-overexpressing colorectal cancer cell line that had been treated with phosphorothioate antisense EG-VEGF oligonucleotides was injected s.c. into mice, angiogenesis and tumor growth were inhibited. Although the novel angiogenesis factor EG-VEGF studied was not expressed in the normal colorectal mucosa, it was expressed in colorectal cancer cells, which indicates that it is a cancer-specific, and possibly tissue-specific, angiogenesis factor in the large intestine and which suggests that it can be targeted by a novel antiangiogenesis therapy.

References
1. Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 1994;79:185–8.
2. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86:353–64.
3. Hong YK, Chung DS, Joe YA, et al. Efficient inhibition of in vivo human malignant glioma growth and angiogenesis by interferon-β treatment at early stage of tumor development. Clin. Cancer Res 2000;6:3354–66.
4. Kuba K, Matsumoto K, Date K, Shimura H, Tanaka M, Nakamura T. HGF/NK4, a four-knot ring antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumour growth and metastasis in mice. Cancer Res 2000;60:6737–43.
5. Roestad EK, Halsor EF. Vascular endothelial growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts. Cancer Res 2000;60:4932–8.
6. Bussolino F, Di Renzo MF, Ziche M, et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 1992;119:629–41.
7. Risau W. Mechanisms of angiogenesis. Nature (Lond.) 1997;386:671–4.
8. Hotz HG, Reber HA, Hotz B, et al. Angiogenesis inhibitor TNP-470 reduces human pancreatic cancer growth. J Gastrointest Surg 2001;5:131–18.
9. Beliveau R, Gingras D, Kruger EA, et al. The antiangiogenic agent neovastat (AE-941) inhibits vascular endothelial growth factor-mediated biological effects. Clin Cancer Res 2002;8:1242–50.
10. Laird AD, Vajkoczy P, Shawver LK, et al. SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. Cancer Res 2000;60:4152–60.
11. O’Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997;88:277–85.
12. LeCouter J, Kowalski J, Foster J, et al. Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature (Lond.) 2001;412:877–84.
13. Arai N, Strom A, Rather JH, Gustafsson JA. Estrogen receptor β mRNA in colon cancer cells: growth effects of estrogen and genistein. Biochem Biophys Res Commun 2000;270:425–31.
14. Guri T, Shishimiri M, Lu Z, Foster DA, Klimz SG, Feig LA. An EGF receptor/Ral-GTPase signaling cascade regulates c-Src activity and substrate specificity. EMBO J 2000;19:623–30.
15. Kurupp D, Christopf C, Bertram JF, O’Brien PE. Tamoxifen inhibits colorectal cancer metastases in the liver: a study in a murine model. J Gastroenterol Hepatol 2000;15:623–30.
16. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
17. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature (Lond.) 1993;362:841–4.
18. Folkman J. Clinical applications of research on angiogenesis. N Engl J Med 1995;333:1757–63.
19. Kudelka AP, Verschraegen CF, Loyer E. Complete remission of metastatic cervical cancer with the angiogenesis inhibitor TNP-470. N Engl J Med 1998;338:991–2.
20. Feng TA, Shawver LK, Sun L, et al. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res 1999;59:99–106.