P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wcjd@public.bta.net.cn www.wjgnet.com

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• GASTRIC CANCER •

Expression of vascular endothelial growth factor and its receptors KDR and Flt-1 in gastric cancer cells

Hua Zhang, Jian Wu, Lin Meng, Cheng-Chao Shou

Hua Zhang, Jian Wu, Lin Meng, Cheng-Chao Shou, Peking University School of Oncology, Beijing Institute for Cancer Research, Beijing 100034, China
Supported by National Nature Science Foundation for Outstanding Young Scientist of China (to S. CC., No. 39525021), National 863 program of China (2002 AA 216111) and Beijing Laboratory of Cancer Molecular Biology.
Correspondence to: Dr. Cheng-Chao Shou, Department of Biochemistry and Molecular Biology, Peking University School of Oncology, Beijing Institute for Cancer Research, No.1 Da-Hong-Luo-Chang Street, Western District, Beijing 100034, China. cshou_9@hotmail.com
Telephone: +86-10-66160960 **Fax:** +86-10-66175832
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Abstract

AIM: The expression of vascular endothelial growth factor (VEGF) and its receptors KDR and Flt-1 by gastric carcinoma tissues and different gastric carcinoma cell lines was detected to elucidate the molecular mechanism of this growth factor in promoting tumor growth.

METHODS: The expression of VEGF, Flt-1 and KDR was determined by reverse transcription-polymerase chain reaction (RT-PCR) in gastric cancer cell lines RF-1, RF-48, AGS-1, NCI-N87, NCI-SNU-1, NCI-SNU-5, NCI-SNU-16 and KATO-**III**. The expression of Flt-1 and KDR in paraffin-embedded specimens of gastric cancer was determined by immunohistochemistry. The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess the role of VEGF in tumor cell proliferation.

RESULTS: All 8 gastric cancer cell lines analyzed expressed $VEGF_{121}$ and $VEGF_{165}$ and six of them expressed both Flt-1 and KDR, while cell line NCI-SNU-5 expressed Flt-1 only and cell line KATO**III** expressed neither Flt-1 nor KDR. The gastric carcinoma tissues expressed Flt-1 and KDR widely, with the positive rate of expression of Flt-1 and KDR being 84.6 % and 70 % respectively. The exogenous VEGF stimulated the growth of KDR-positive cell lines NCI-N87 and AGS-1 in a dose-dependent manner but exhibited no effect on the growth of KDR-negative cell line NCI-N87.

CONCLUSION: VEGF and its receptors KDR and Flt-1 were expressed widely in gastric carcinoma cells and the VEGF stimulated KDR-positive tumor cell growth directly. These results suggest that VEGF may play a role in promoting tumor growth and metastasis by participating in both paracrine and autocrine pathways.

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INTRODUCTION

Angiogenesis is essential for the continued growth of solid tumors. Among the factors contributing to angiogenesis, vascular endothelial growth factor (VEGF, also called vascular permeability factor) is recognized as one of the most important molecules in the formation of new blood vessels[1-8]. A variety of malignant human tumors, including breast, lung and prostate carcinomas, are known to secrete VEGF. The level of VEGF expression correlates with tumor progression and metastasis. Moreover, over-expression of VEGF was suggested to participate in carcinogenic processes. Different investigators reported that VEGF might play an important role during the pre-malignant stages of tumorigenesis in colon, pancreas, and cervix.

VEGF binds with high affinity to its cognate VEGF receptors (VEGFRs) Flt-1/VEGFR-1, flk-1/KDR/VEGFR-2, and neuropilin-1[9,10]. KDR is responsible for mitogenic signaling, and plays an important role in vasculogenesis and blood island formations. However, Flt-1 does not mediate cell growth when introduced into NIH3T3 cells or into porcine aortic endothelial cells that do not express VEGFRs, but regulates the assembly of endothelial cells and tissue factor production in endothelial cells. Recently the third receptor of VEGF, neuropilin-1, was purified from tumor cells. It binds $VEGF_{165}$ but not $VEGF_{121}$, and modulates VEGF binding to VEGFR-2/Flk-1 and the subsequent bioactivity [9].

Recently a few studies have proved that Flt-1 and/or KDR were also expressed in tumor cells, such as hematopoietic malignancies[11-14], pancreatic cancer[15], breast cancer[16], neuroblastoma[17], Kaposi sarcoma[18], and lung carcinomas induced by N-nitrisobis (2-hydroxypropyl) amine in rats[19]. We previously demonstrated that the VEGF and KDR were co-expressed in gastric adenocarcinoma MGC803 cells, and exogenous recombinant human $VEGF_{165}$ stimulated growth of MGC803 cells directly[20]. The present study extended our previous work and detected the expression of VEGF, Flt-1 and KDR in eight gastric cancer cell lines by RT-PCR, and the expression of Flt-1 and KDR in gastric tumor specimens by immunohistochemistry. The results showed that VEGF and VEGFR were co-expressed in gastric tumor cells widely, and exogenous $VEGF_{165}$ stimulated the growth of KDR-positive gastric carcinoma cells, indicating that there is a possible autocrine pathway for VEGF in gastric cancer.

MATERIALS AND METHODS

Cell culture and reagents

Human gastric cancer cell lines from American Type Culture Collection (ATCC) were generously supplied by Dr. Ji JF. The cell lines RF-1 and RF-48 were cultured in Leibovitz' s L-15 medium containing 10 % fetal calf serum (FCS), AGS-1, NCI-N87, NCI-SNU-1 and NCI-SNU-16 were cultured in RPMI1640 medium containing 10 %FCS and NCI-SNU-5 and KATOIII were cultured in RPMI1640 medium containing 20 % FCS. Anti-Flt-1 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Anti-KDR mouse monoclonal antibody 6E2 was prepared in our laboratory. Recombinant human $VEGF_{165}$ was purchased from Sigma Inc.

RT-PCR

Total RNA was extracted from eight carcinoma cell lines of 2×10^6 cells each using TRIZOL following the manufacturer' s instructions. First-strand cDNA was synthesized from 10 μg of total RNA in a 50 μl reaction volume by reverse transcription (RT) using random hexamer and MMLV reverse transcriptase (GIBCO BRL) as described by the manufacturer. The cDNA of 2 μl was amplified by PCR in a 25 μl reaction volume with primers designed to span intron-exon boundaries to distinguish amplified cDNA from genomic DNA (Table 1). VEGF was amplified for 40 cycles at 94 ℃ for 45 sec, 55 ℃ for 40 sec and 72 ℃ for 1 min and its primers were chosen to recognize all the known VEGF splice variants according to the published sequences (GenBank: AF022375). GAPDH was amplified for 30 cycles using the same cycling conditions as for VEGF. Flt-1 and KDR were amplified by semi-nest PCR. The Flt-1 was first amplified with forward-1 (corresponding to nucleotides 1-15,GenBank:AF063657) and reverse primers (corresponding to nucleotides 1287-1270) for 30 cycles at 94 ℃ for 45sec, 64 ℃ for 1 min and 72 ℃ for 2 min, then amplified with forward-2 (corresponding to nucleotides 94-109) and reverse primers for 30 cycles. The KDR was first amplified with forward (corresponding to nucleotides 1316-1330, GenBank: AF063658) and reverse-2 (corresponding to nucleotides 2275-2260) primers for 30 cycles at 94 ℃ for 45 sec, 52 ℃ for 1min and 72 ℃ for 2 min, and then amplified with the forward and reverse-1 (corresponding to nucleotides 2225-2207) primers for 30 cycles at 94 ℃ for 45 sec, 64 ℃ for 1 min and 72 ℃ for 2 min.

The PCR products of Flt-1 were analyzed by restriction endonuclease digestion with *Sac*I and *Pst*I (New England Biolabs Inc.) and KDR were analyzed with *Hind*III(New England Biolabs Inc.). The amplified cDNA of Flt-1 and KDR from AGS-1 cells were cloned into pGEM-T Easy vector (Promega) and sequenced.

Table 1 Gene specific primers for PCR

gene	GenBank	Orientation	Accession Number	Sequence
VEGF	AF022375	Forward		5'-GGGGGATCCGCCTCCGAAACCATGAACTT-3'
		Reverse		5'-CCCGAATTCTCCTGGTGAGAGATCTGGTT-3'
Flt-1	AF063657	Forward-1		5'-TCTAGGATCCATGGTCAGCTACTGGGACACC-3'
		Forward-2		5'-AAGGGATCCCCTGAACTGAGTTTAAAA-3'
		Reverse		5'-GGCGAATTCTGGGGTTTCACATTGAC-3'
KDR	AF063658	Forward		5'-TAAGGATCCCACTCAAACGCTGAC-3'
				5'-GGAGAATTCTCAACTGCATGCCTGGCAG-3'
				5'-TCCTGGGCACCTTCTA-3'
GAPDH	AF261085	Forward		5'-ACCACAGTCCATGCCATCAC-3'
				5'-TCCACCACCCTGTTGCTGTA-3'

(Underlined sequences are restriction endonuclease recognition sites, which were added for further cloning).

Immunohistochemical analysis

Paraffin-embedded gastric carcinoma specimens were collected from the Department of Pathology, Beijing Institute for Cancer Research. The tissue sections were deparaffinized, treated with 3 % H_2O_2 to inhibit endogenous peroxidase and incubated in 0.1M sodium citrate buffer, pH6.0, at 92-98 ℃ for 10 min for antigen retrieval. The tissues were blocked with 10 % normal goat serum at 37 ℃ for 30 min and stained for Flt-1 with rabbit polyclonal antibody at 1:200 dilution, or for KDR with mouse monoclonal antibody 6E2 at 4 μg/ml, at 4 ℃ overnight. This was followed by sequential incubations in biotin-conjugated secondary antibody, streptavidin-peroxidase and 3, 3' -diaminobezidine (DAB) for visualization. Normal rabbit serum (1:10 000 dilution) or normal mouse IgG (4 μg/ml) was used as negative controls.

Cell proliferation assay

Cells AGS-1 and NCI-N87 were seeded into 96-well plates with 2×10^4 and 5×10^4 cells per well respectively and incubated in 10 % RPMI1640 medium for 24 hours. The culture medium was then replaced with serum-free medium and cells cultured for another 24 hours. KATO Ⅲ cells were seeded at 2×10^4/well and incubated in serum-free medium for 24 hours. All these cells were then treated with varying concentrations of $VEGF_{165}$ (0-10 ng/ml) for 72 hours followed by treatment with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) for 4 hours. The medium was gently aspirated, cells were lysed in 150 μl of dimethylsulfoxide and the cell lysates were measured for absorbance at 492 nm with a Model 550 microplate reader. (Bio-Rad Co.). The viability was expressed as mean percentage of untreated controls ±SE (n=4). Statistical analysis was performed by means of Student's t-test.

RESULTS

Expression of VEGF and its receptors in human gastric carcinoma cell lines

RT-PCR with primers designed to amplify all 5 known splicing variants of VEGF generated two products of 531bp and 663bp in size corresponding to VEGF isoforms $VEGF_{121}$ and $VEGF_{165}$ in all eight gastric carcinoma cell lines (Figure 1A). Flt-1 of the expected size (1212 bp) was amplified in all cell lines except KATOⅢ cells (Figure 1B) and the amplified products were successfully digested with *Sac*I and *Pst*I respectively (data not shown). KDR of the expected sized (927bp) was amplified in all cell lines except SNU-5 and KATOⅢ cells (Figure 1C) and the amplified products were successfully digested with *Hind*III (data not shown).

The fragments of Flt-1 and KDR amplified from AGS-1 were cloned into pGEM-T Easy vector and sequenced.

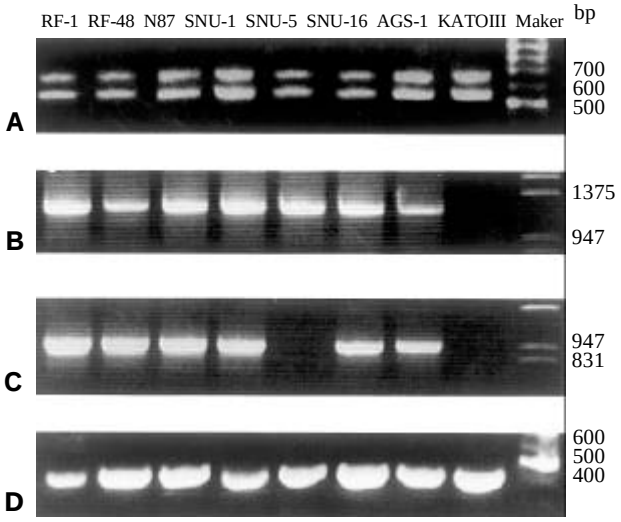

Figure 1 Detection of expression of VEGF and VEGFR in eight gastric carcinoma cell lines by RT-PCR. A. VEGF was amplified using primers designed to detect all known splicing variants and two isoforms 531bp and 663bp corresponding to $VEGF_{121}$ and $VEGF_{165}$ were obtained in all cell lines. B. Flt-1 of the expected size (1212bp) was amplified in all cell lines except KATOⅢ. C. KDR of the expected size (927bp) was amplified in all cell lines except SNU-5 and KATOⅢ. D. GAPDH was amplified in each cell line as a positive control for RT-PCR.

Flt-1 and KDR were widely expressed in gastric carcinoma

Specimens from 52 cases of gastric carcinoma were examined by immunohisto-chemistry to detect the expression of Flt-1. The results showed that Flt-1 was not only expressed in endothelial cells, but also in the tumor cells with a positivity of 84.6 % (44cases/52cases) (Figure 2A). The intensity of immunostaining was stronger in well-differentiated adenocarcinomas than in poorly differentiated adenocarcinomas. The vascular smooth muscles were also positive for Flt-1, consistent with published report[21], so were some normal cells in the bottom of gastric gland. Immunostaining was not observed on tumor tissues when normal rabbit serum was used as primary antibody.

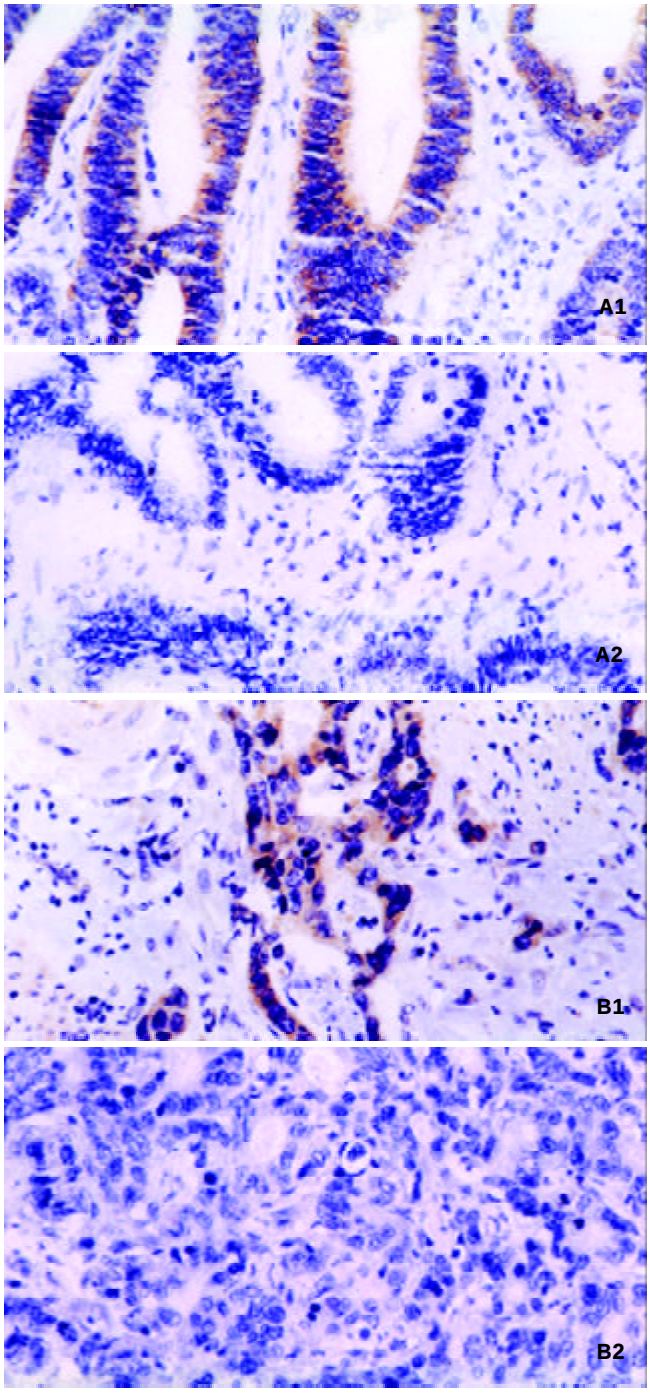

Figure 2 Immunohistochemical analysis of Flt-1 and KDR on gastric carcinoma specimens (x400). A. Expression of Flt-1. B. Expression of KDR. below sections are respectively negative controls of the left-hand side sections of the same specimens.

Specimens from 30 cases of gastric carcinoma were detected for expression of KDR and the results were similar to those of Flt-1 except that the positive rate was slightly lower (70 %) (Figure 2B). Among 24 cases detected for both receptors, Flt-1 was always positive when KDR was positive (Table 2).

Table 2 Expression of Flt-1 and KDR on gastric carcinoma specimens

Expression of Flt-1 and KDR	*n*	Percentage of 24 cases
Flt-1(+) KDR(+)	15	62.5
Flt-1(-) KDR(-)	5	20.8
Flt-1(+) KDR(-)	4	16.7
Flt-1(-) KDR(+)	0	0

VEGF stimulated growth of KDR-positive gastric carcinoma cell lines

To determine whether the exogenous recombinant human $VEGF_{165}$ was able to stimulate proliferation of gastric carcinoma cells through KDR receptor, AGS-1, NCI-N87 and KATOIII cells were incubated with varying concentrations of $VEGF_{165}$ and its effects were measured using MTT assay (Figure 3). The results showed dose-dependent effect of $VEGF_{165}$ on the growth of KDR-positive cells NCI-N87 and AGS-1 and the maximum dose of 10 ng/ml VEGF stimulated the growth of KDR-positive cells NCI-N87 and AGS-1 to 131.5 % (± 5.4 %) and 130.8 % (±11.3 %), of controls respectively (P<0.01). However, $VEGF_{165}$ had no effect on KDR-negative KATOIII cells (P>0.05).

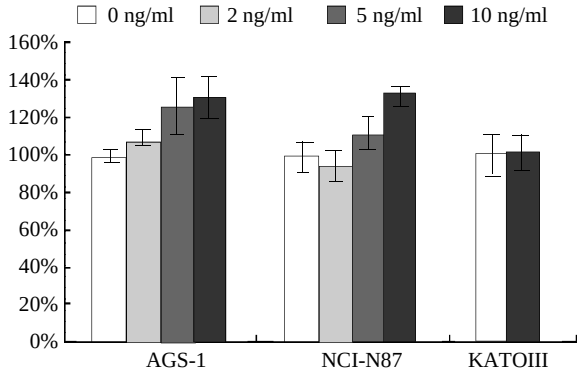

Figure 3 Effects of recombinant human $VEGF_{165}$ on proliferation of AGS-1, NCI-N87 and KATOIII cells. The cells were treated with concentrations of $VEGF_{165}$ indicated, and their viability was assessed using MTT and expressed as mean percentage of the untreated controls ±SE (n=4).

DISCUSSION

It is well known that tumor cells can secret VEGF, and its receptors KDR and Flt-1 are primarily expressed in endothelial cells. Therefore, it seems that the receptors of VEGF are endothelial cell-specific. However, recent emerging evidences have shown that VEGFRs are expressed in cell types other than endothelial cells, especially in tumor cells, indicating there is an autocrine pathway of VEGF on tumor cells. The presence of VEGF autocrine growth factor activity has been demonstrated in 5 different human tumor types, including melanoma, ovarian and pancreatic carcinoma, Kaposi sarcoma, and leukemia[11-19]. Gastric cancer is common in China and aboard[22-36]. Our previous study by Tian *et al*[20] demonstrated that VEGF acted as an

autocrine growth factor for human gastric adenocarcinoma cell MGC803. To investigate whether this is a common mechanism in gastric carcinoma, we determined the expression of VEGF and VEGFRs in eight gastric carcinoma cell lines at mRNA level. It was found that all the cell lines examined expressed $VEGF_{121}$ and $VEGF_{165}$. Meanwhile, both Flt-1 and KDR were expressed in these tumor cells, except that NCI-SNU-5 cells expressed Flt-1 only, and KATOIII expressed neither of the receptors. It seems that the co-expression of VEGF and VEGFR is common in gastric carcinoma cell lines. To investigate this phenomenon further, we detected the expression of VEGFRs in gastric carcinoma specimens. The immunohistochemical analysis showed similar results to that from cell lines, e.g. VEGFRs were expressed in endothelial cells of tumor tissue as well as in tumor cells. The positive rate of Flt-1 expression was slightly higher than that of KDR expression and the KDR-positive specimens were always Flt-1 positive. These results suggest that VEGF acted not only as a paracrine factor on endothelial cells, but also as an autocrine factor on tumor cells.

It has been reported that the expression of KDR in non-endothelial cells is associated with increase in DNA synthesis in response to VEGF stimulation. We therefore investigated whether the exogenous VEGF could stimulate the growth of KDR- positive tumor cells. $VEGF_{165}$ of 10 ng/ml increased the growth of AGS-1 and NCI-N87 cells, which were KDR positive, to 130.8 % and 131.5 % of the control unstimulated cells respectively, but showed no effect on KDR-negative KATOIII cells, indicating that the KDR receptor on gastric carcinoma is functional. Although the concentrations of VEGF in different cell cultures reported were lower (from 0.1 ng/ml to 2.8 ng/ml[11,12,14,17]) than what we used in this experiments, it is believed that the VEGF is actively secreted by tumor cells and its local concentration might be much higher, so that an autocrine pathway through KDR receptor for the growth factor is possible in gastric carcinoma. The expression of VEGF is regulated by several factors, including hypoxia, cytokines such as interleukin (IL)-1, activation of certain oncogenes (Ras, Raf, Src), and loss-of-function mutations of p53 and the von Hippel-Lindau genes. Mutation of ras and p53 is usually seen in early stage of gastric carcinogenesis[37], which can result in significant up-regulation of VEGF. Therefore, autocrine pathway of VEGF may play an important role in the progression of early stage gastric carcinoma and the antagonist of VEGF/VEGFR may inhibit tumor progression by direcly inhibiting angiogenesis. In fact, this has been confirmed in animal model of human leukemia[13]. Dias *et al* used neutralizing antibodies specific for murine endothelial cell or human endothelial cell VEGFR-2 to inhibit the paracrine or autocrine VEGF/VEGFR pathway. They showed that blocking either the paracrine pathway or the autocrine VEGF/VEGFR-2 pathway delayed leukemic growth and engraftment *in vivo* but failed to cure inoculated mice, and long-term remission with no evidence of disease was achieved only if mice were treated with antibodies against both murine and human VEGFR-2.

In conclusion,we first demonstrated that VEGF and VEGFR were co-expressed in gastric cancer, and the exogenous VEGF could stimulate the growth of KDR-positive tumor cells. These results suggest that there might exist an autocrine mechanism of VEGF in gastric carcinoma, and VEGF could promote tumor growth and metastasis by both direct and indirect pathways.

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