ORIGINAL

Vascular endothelial growth factors and their receptors in the novel human cell line, HN-Eso-1, established from esophageal spindle cell carcinoma

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Abstract : Human carcinosarcomas of esophagus are uncommon malignant neoplasms that are composed both carcinomatous and sarcomatous components. We established a novel cell line, HN-Eso-1, from the metastatic esophageal spindle cell carcinoma (so-called carcinosarcoma). In this study, we estimated the vascular endothelial growth factors (VEGFs) and VEGF receptors (VEGFRs). Reverse transcription polymerase chain reaction (RT-PCR) studies revealed that VEGF-A,-C,-D and VEGFR-1,-2 were upregulated. Cisplatin reduced the cell viability of HN-Eso-1 cells and VEGF attenuated its effect. These results suggest that expression of VEGF-A, VEGF-C, VEGF-D, VEGFR-1, and VEGFR-2 are involved in the cell's autocrine system and that VEGF protected these cells from the anti-tumor agent. J. Med. Invest. 57 : 232-236, August, 2010

Keywords **:** *esophagus, so-called carcinosarcoma, vascular endothelial growth factor, vascular endothelial growth factor receptor*

INTRODUCTION

Human esophageal carcinosarcomas are uncommon malignant tumors that have a characteristic gross polypoid configuration and contain carcinomatous and sarcomatous component. Spindle cell carcinoma (so-called carcinosarcoma) is composed of squamous cell carcinoma and sarcomatous spindle cells that are derived from metaplasia of the carcinoma cells (1).

The vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells, its expression is induced by hypoxia, oncogene activation, and various cytokines. VEGF is important in angiogenesis and the neovascularization of solid tumor growth (2). Expression of VEGFs and VEGFRs in the esophageal so-called carcinosarcoma has not been fully undestood, although Kato et. al. reported that VEGF expression in the esophageal cancers is correlated with a poor prognosis (3). It is very important to investigate the expression of VEGFs and their receptors in clinically. The VEGF family members VEGF-A,-B,-C, and -D have been identified (4) of which VEGF-A is the most potent promoter of angiogenesis. Six VEGF-A isoforms, consisting of 121, 145, 165, 183, 189, or 206 amino acids, are generated as a result of alternative splicing from a single gene (5). The 121 and 165 isoforms are usually the predominant forms. VEGF121 is more angiogenic and tumorigenic than the other isoforms (5). VEGFs bind to three receptor protein tyrosine kinases, VEGF-R1 (Flt-1), VEGF-R2

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(KDR/Flk-1), and VEGF-R3 (Flt-4) (4). VEGF-R2 which is a VEGF-A receptor plays an important role in pathological neovascularization via its tyrosine kinase activity and growth promoting signals to blood vessels (4). VEGF-R3 interacts with VEGF-C and is involved in lymphangiogenesis (4).

We have established a novel human cell line from a metastatic esophageal so-called carcinosarcoma. HN-Eso-1 is, to our knowledge, the only such cell line in existence. It is very important to estimate the biological aspects in the HN-Eso-1. We have demonstrated that VEGF plays an important role in protecting HN-Eso-1 cells from the anti-tumor agent cisplatin.

MATERIALS AND METHODS

Establishment of the HN-Eso-1 cell line

Tumor tissue was obtained from the metastatic adrenal gland of the esophageal so-called carcinosarcoma in 2002, and the informed consent was obtained from the patient. Tumor tissue was cut thoroughly by the scalpel and cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 8% fetal bovine serum (FBS) with penisilin and streptomycin on the dish. After 4 weeks, each colony was moved to a 96-well plate. HN-Eso-1 cells were established from one of the colonies and have been cultured for 2 years.

Cell culture

DLD-1 (colon adenocarcinoma), SW480 (colon adenocarcinoma), and Lovo (colon adenocarcinoma) cells were a kind gift from Dr. Ryoko Suzuki (Kochi Medical School). They were cultured in DMEM containing 8% FBS as described above.

Reagents

Cisplatin was purchased from Kyowa Hakko (Tokyo Japan). VEGF165 was purchased from PeproTech EC (London UK). Anti-VEGF antibody (A-20) was obtained from Santa Cruz Biotechnology (Los Angeles, CA, USA).

Isolation and detection of VEGF and VEGFR mRNA

1×10^6 cells were seeded on 6-cm dishes and incubated for 24 hours. Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and the total cellular RNA isolated using the TRIZOL reagent (Gibco-BRL) according to the manufacturer's protocol. Specific mRNA was assayed using reverse transcription polymerase chain reaction (RT-PCR). PCR conditions : VEGF-A : denaturing, 94°C for 1 min, annealing, 60°C for 1 min and elongation, 72°C for 1 min, for 30 cycles ; VEGF-B : denaturing, 94°C for 1 min, annealing, 60°C for 1 min and elongation, 72°C for 1 min, for 30 cycles ; VEGF-C : denaturing, 94°C for 1 min, annealing, 60°C for 1 min and elongation, 72°C for 1 min, for 30 cycles ; VEGF-D denaturing, 94°C for 1 min, annealing, 60°C for 1 min and elongation, 72°C for 1 min, for 30 cycles ; VEGFR-1 : denaturing, 94°C for 1 min, annealing, 60°C for 1 min and elongation, 72°C for 1 min, for 30 cycles ; VEGFR-2 : denaturing, 94°C for 1 min, annealing, 60°C for 1 min and elongation, 72°C for 1 min, for 30 cycles ; VEGFR-3 : denaturing, 94°C for 1 min, annealing, 60°C for 1 min and elongation, 72°C for 1 min, for 30 cycles ; Beta-actin : denaturing, 94°C for 30 s, annealing, 50°C for 40 s and elongation, 72°C for 1 min, for 30 cycles ; and final elongation at 72°C for 10 min-1 cycle. PCR products were subjected electrophoresis in 2% agarose gel. The primer sequences were VEGF-A sense : 5'-CGAAGTGGTGAAGTTCATGGATG-3' ; VEGF-A antisense : 5'-TTCTGTATCAGTCTTTCCTGGTGA-3' ; VEGF-B sense : ACATCACCCATCCCACTCC-AGGCTCCTTTGTTCCCCCACT-3', VEGF-B antisense : 5'-GCTCCTTTGTTCCCCCACT-3', VEGF-C sense : 5'-TGTTTTCCTCGGATGCTGGAG-3', VEGF-C antisense : 5'-TGGGGCAGGTTCTTTTA-CATACAC-3', VEGF-D sense : 5'-CCACTTGCTG-GAACAGAAGACCAC-3', VEGF-D antisense : 5'-ATGACAGGGATGGGGAACTTGG-3', VEGFR-1 sense : 5'-GCACCTTGGTTGCTG-3', VEGFR-1 antisense : CGTGCTGCTTCCTGGTCC-3', VEGFR-2 sense : 5'-CTGGCATGGTCTTCTGTGAAGCA-3', VEGFR-2 antisense : 5'-AATACCAGTGGATGTG-ATGCGG-3', VEGFR-3 sense : 5'-CAGGTGCCT-TCCCAGACACTGGCGTTACT-3', VEGFR-3 antisense : 5'-ACTCATATTACCAAGGAATAACTGG-CGGGCA-3', beta-actin sense : 5'-ATTGGCAATG-AGCGGTTCCGC-3' ; beta-actin antisense : 5'-CT-CCTGCTTGCTGATCCACATC-3', as previously described (4, 6).

Western blotting

We performed Western blotting as previously described (6-9). 4×10^6 cells were seeded on a 10-cm dish for 24 hours. Cells were washed with PBS and lysed in RIPA buffer (Upstate Biotechnology Inc., NY, USA) containing 20 mM sodiumpyrophosphate, 20 mM NaF, 1 mM orthovanadate, 2 mM pyrophosphate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/

ml leupeptin. Cell lysates containing comparable amounts of proteins, estimated by a Bradford assay (Bio-Rad, Munchen, Germany) were separated by SDS-PAGE and subjected to Western blotting.

3,-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay to determine cell viability

The MTT assay was performed as previously described (6, 9). In a 96-well plate, 1×10^4 cells/100 μl of cell suspension were used to seed each well. After 24 h, reagents (cisplatin and/or VEGF) were added to each well and the cells were incubated for 24 h. After 24 h incubation with/without reagents, 10 μl of a 2.5 mg/ml solution of MTT in PBS was added to each well and incubated for 2 hours at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 100 μl of a 50% N,N,-dimethyl formamide/10% SDS solution, and incubated for 6 hours at room temperature. The plate was then analyzed on a plate reader at 570 nm to measure sample absorbances.

Microscopic analysis

Cells were seeded and cultured on the covered glass in the dish for 24 h and then covered glass were washed twice with PBS and fixed with 100% ethanol. Hematoxylin and eosine (H&E) staining was done and electron microscopic analysis was performed as previously described.

RESULT

RT-PCR

RT-PCR was performed as described in the materials and methods section and the results are shown in Fig. 1. VEGF-A was expressed more in HN-Eso-1 than in the SW480, DLD-1, or Lovo cell lines. We previously revealed that VEGF in DLD-1 was detected in 35 cycles of PCR (6) . VEGF-C was detected only in HN-Eso-1, while VEGF-D was detected in SW480, Lovo, and HN-Eso-1 cells. VEGFR-1 was detected in all four cell lines. VEGFR-2 was detected in SW480 and HN-Eso-1 only. We could not detect the transcription of VEGF-B or VEGFR-3 in any cell line.

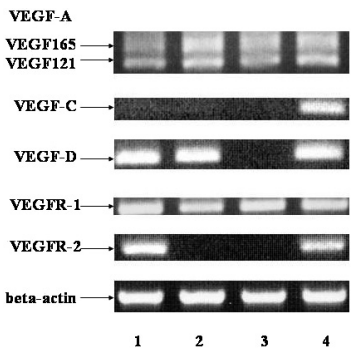

Figure 1. RT-PCR
VEGF-A (VEGF121, VEGF165), VEGF-C, VEGF-D, VEGFR-1, and VEGFR-2 were detected by RT-PCR. Lane 1, SW480 ; 2, Lovo ; 3, DLD-1 ; 4, HN-Eso-1 cells

Expression of VEGF in the HN-Eso-1

Expression of VEGF was estimated by Western blotting (Fig. 2). VEGF was expressed in all cell lines, but most strongly in HN-Eso-1 and Lovo cells.

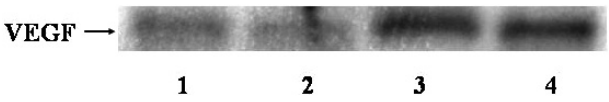

Figure 2. Expression of VEGF
30 μg protein was loaded in each lane. Lane 1, SW480 ; 2, DLD-1 ; 3, HN-Eso-1 ; 4, Lovo cells

Cell viability

We tested whether VEGF could protect HN-Eso-1 cells from the anti-tumor agent, cisplatin. Cisplatin reduced the viability of HN-Eso-1 cells in a dose-dependent fashion as shown in Fig. 3 (lane2-5). The percent of cell viability± standard deviation (S.D.) was described as follows ; 68± 6.44% (0.5 μg/ml), 65.2± 2.86% (1 μg/ml), 63.2± 3.45% (2 μg/ml),

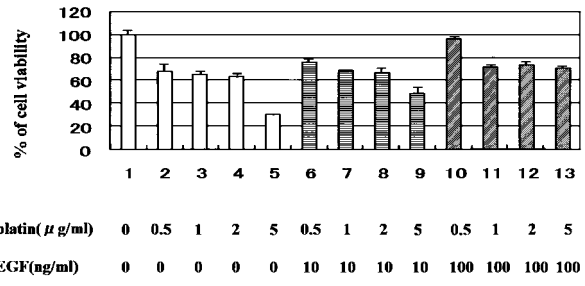

Figure 3. Assessment of cell viability
Cell viability was determined by MTT assay. Results were inducated as % of cell viability± standard deviation (S.D.). Triplicate experiments were performed. Treatment : 1 ; none, 2 ; cisplatin 0.5 μg/ml, 3 ; Cisplatin 1 μg/ml, 4 ; Cisplatin 2 μg/ml, 5 ; Cisplatin 5 μg/ml, 6 ; VEGF (10 ng/ml)+Cisplatin 0.5 μg/ml, 7 ; VEGF (10 ng/ml)+Cisplatin 1 μg/ml, 8 ; VEGF (10 ng/ml)+Cisplatin 2 μg/ml, 9 ; VEGF (10 ng/ml)+Cisplatin 5 μg/ml, 10 ; VEGF (100 ng/ml)+Cisplatin 0.5 μg/ml, 11 ; VEGF (100 ng/ml)+Cisplatin 1 μg/ml, 12 ; VEGF (100 ng/ml)+Cisplatin 2 μg/ml, 13 ; VEGF (100 ng/ml)+Cisplatin 5 μg/ml

30± 1.23% (5 μg/ml). In the case of combination with VEGF (10 or 100 ng/ml), effect of cisplatin was attenuated. Results were described as follows ; 76.4± 2.91% (cisplatin 0.5 μg/ml and VEGF 10 ng/ml), 68.8± 0.61% (cisplatin 1 μg/ml and VEGF 10 ng/ml), 67.8± 2.88% (cisplatin 2 μg/ml and VEGF 10 ng/ml), 49.1± 5.57% (cisplatin 5 μg/ml and VEGF 10 ng/ml), 96.6 ± 2.2% (cisplatin 0.5 μg/ml and VEGF 100 ng/ml), 72.2± 1.28% (cisplatin 1 μg/ml and VEGF 100 ng/ml), 73.7± 2.56% (cisplatin 2 μg/ml and VEGF 100 ng/ml), 71± 1.69% (cisplatin 5 μg/ml and VEGF 100 ng/ml).

Microscopic analysis

HN-Eso-1 cells are epithelial cell shape and they have a large nuclei and were developed mitochondria (Fig. 4). The mitotic cells were shown in Fig. 4 A.

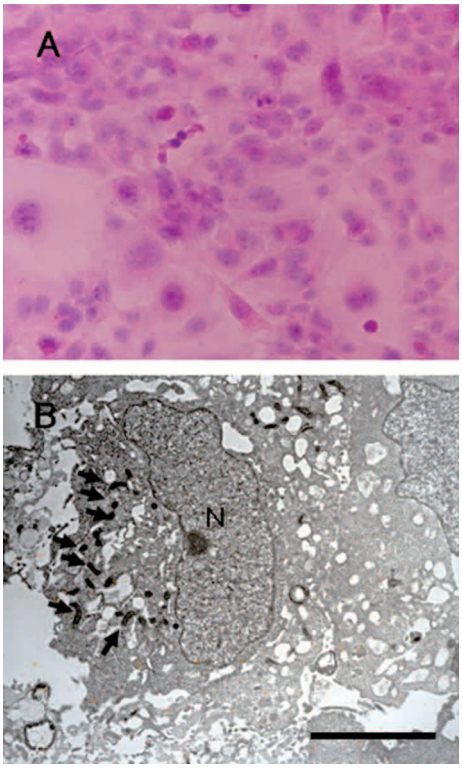

Figure 4. Microscopic analysis of HN-Eso-1 cells
A ; Hematoxylin and eosin staining. B ; Electron microscopic analysis. Arrow : mitochondria, N : nucleus.

DISCUSSION

We have established the novel HN-Eso-1 cell line from the esophageal so-called carcinosarcoma and used RT-PCR analysis to examine the VEGFs and VEGFRs in cells.

VEGF is a highly specific mitogen for vascular endothelial cells that is induced by hypoxia, oncogene activation, and variety of cytokines. VEGF is important in angiogenesis and neovascularization of solid tumor growth. Expression of VEGF and VEGFRs in the esophageal so-called carcinosarcoma has not been fully understood. It is very important to investigate the role of expression of VEGF and VEGFRs in the esophageal so-called carcinosarcoma. In these cells, transcription of VEGF-A, C, D, VEGFR-1, and VEGFR-2 were detected (Fig. 1). Expression of VEGF (VEGF-A) was detected in HN-Eso-1 cells (Fig. 2) and the other cell lines, Lovo, SW480, and DLD-1 also expressed VEGF (Fig. 2). VEGF-R2, which is a VEGF-A receptor plays an important role in pathological neovascularization via its tyrosine kinase activity and growth promoting signals to blood vessels (4). These results suggest that expression of VEGFs and VEGFRs might be implicated not only in the HN-Eso-1 autocrine system but also in promotion of tumor environment. Indeed, the pathological analysis in a sample tissue suggests that both angiogenesis and lymphangiogenesis was well developed (data not shown).

Next we evaluated the effect of VEGF on HN-Eso-1 cells, although its direct effect on cancer cells has not been fully understood. Recombinant VEGF attenuated the cytotoxic effect of cisplatin on HN-Eso-1 cells (Fig. 3). However VEGF could not rescue the cell death of SW480, DLD-1, or Lovo treated with cisplatin. (data not shown) VEGF appears to play an important role in the cytoprotective effect on HN-Eso-1 cells against anti-cancer agents.

SU11248, a VEGFR-2 inhibitor, has been approved for cancer therapy, and undergone clinical setting (10). Our results suggest that SU11248 could be useful of esophageal so-called carcinosarcoma, in combination with other anti-cancer agents. Further studies are required to investigate in detail the cell survival signals of HN-Eso-1.

REFERENCE

1 Hemmi A, Terada N, Mizutani G, Fujii Y, Ohno S, Nemoto N : Squamous cell carcinoma with sarcomatous feature (so-called carcinosarcoma) of stomach probably metastasized from esophageal tumor : a case report with quick-freezing and deep-etching method. Med Electron microsc 37 : 119-29, 2004

2 Stimpfl M, Tong D, Fasching B, Schuster E,

bermair A, Leodolter S, Robert Zeillinger R : Vascular endothelial growth factor splice variants and their prognostic value in breast and ovarian cancer. Clin Caner Res 8 : 2253-9, 2002

3 Kato H, Yoshikawa M, Miyazaki T, Nakajima M, fukai Y, Masuda N, Fukuchi M, Manda R, Tsukada K, Kuwano H : Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) in esophageal squamous cell carcinoma. Anticancer Res 6C : 3977-84, 2002

4 Aparicio S, Sawant S, Lara N, Barnstable CJ, Tombran-Tink J : Expression of angiogenesis factors in human umbilical vein endothelial cells and their regulation by PEDF. Biochem Biophis Res Com 326 : 387-94, 2005

5 Margit Stimpfl, Dan Tong, Barbara Fasching, Eva Schuster, Andreas Obermair, Sepp Leodolter, and Robert Zeillinger : Vascular Endothelial Growth Factor Splice Variants and Their Prognostic Value in Breast and Ovarian Cancer. Clin Cancer Res 8 : 2253-2259, 2002

6 Jin T, Nakatani H, Taguchi T, Nakano T, Okabayashi T, Sugimoto T, Kobayashi M, Araki K : STI571 (Glivec) suppresses the expression of vascular endothelial growth factor in the gastrointestinal stromal tumor cell line, GIST-T1 World. J Gastroenterol 12 : 703-8, 2006

7 Nakatani H, Kobayashi M, Jin T, Taguchi T, Sugimoto T, Nakano T, Hamada S, Araki K : STI571 (Glivec) inhibits the interaction between c-KIT and Heat shock protein90 of the gastrointestinal stromal tumor, GIST-T1 Cancer Sci 96 : 116-9, 2005

8 Nakatani H, Araki K, Jin T, Kobayashi M, Sugimoto T, Akimori T, Namikawa T, Okamoto K, Nakano T, Okabayashi T, Hokimoto N, Kitagawa H, Taguchi T : STI571 (Glivec) induces cell death in the gastrointestinal stromal tumor cell line, GIST-T1, via endoplasmic reticulum stress response. Int J Mol Med 17 : 893-7, 2006

9 Jin T, Nakatani H, Taguchi T, Sonobe H, Morimoto N, Sugimoto T, Akimori T, Nakano T, Namikawa T, Okabayashi T, Kobayashi M, Araki K : Thapsigargin enhances cell death in the gastrointestinal stromal tumor cell line, GIST-T1, by treatment with Imatinib (Glivec). J Health Sci 52 : 110-7, 2006

10 Faivre S, Delbaldo C, Vera K, Robert C, Lozahic S, Lassau N, Bello C, Deprimo S, Brega N, Massimini G, Armand JP, Scigalla P, Raymond E : Safety, Pharmacokinetic, and Antitumor Activity of SU11248, a novel Oral Multitarget Tyrosine Kinase Inhibitor, in Patients with Cancer. J Clin Oncol 24 : 25-35, 2006