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VEGF receptor antisense therapy inhibits angiogenesis and peritoneal dissemination of human gastric cancer in nude mice

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The efficacy of a phosphorothioate antisense oligonucleotide (ASO) for KDR/Flk-1 (KDR/Flk-1-ASO), an endothelial cell–specific vascular endothelial growth factor (VEGF) receptor, was investigated on the peritoneal dissemination and angiogenesis of a human gastric cancer cell line in nude mice. Green fluorescent protein (GFP)–transduced NUGC-4 (NUGC-4-GFP) human gastric cancer cells were implanted into the peritoneal cavity of nude mice. KDR/Flk-1-ASO, -SO, or phosphate-buffered saline was administrated from days 7 to 14, 200 μg/mouse, once a day. The mice were sacrificed on day 28. Disseminated peritoneal tumor nodules expressing GFP were visualized by fluorescence microscopy. KDR/Flk-1-ASO significantly decreased the extent of peritoneal dissemination of the tumors. The number of cells undergoing apoptosis was significantly increased in the KDR/Flk-1-ASO–treated tumors. Microvessel density was significantly reduced in the KDR/Flk-1-ASO–treated tumor nodules. The KDR/Flk-1 antisense strategy, therefore, decreases tumor dissemination apparently by inhibiting angiogenesis.
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A large number of growth factors have been reported to have angiogenic activity *in vitro* and *in vivo*, e.g., FGF, EGF, and TGF-α. However, these ligands have a broad spectrum of target cells and, therefore, are not specific to vascular endothelial cells (ECs).[1,2] Vascular endothelial growth factor, VEGF, is one of major factors mediating tumor angiogenesis. Two tyrosine kinase receptors, Flt-1 and KDR/Flk-1, have been cloned and shown to be high-affinity receptors for VEGF localized predominantly on EC.[1,3,4]

Recently, it has been reported that inhibition of Flt-1 protein expression did not significantly affect VEGF activity, suggesting that activation of KDR/Flk-1 was sufficient to mediate VEGF effects on EC.[5] Therefore, we have selected KDR/Flk-1 as a target for inhibition of tumor angiogenesis and metastasis.

KDR/Flk-1 was shown to be a regulator of VEGF-induced tumor development and angiogenesis in a murine hepatocellular carcinoma (HCC) model.[6] VEGF-neutralizing antibody, MV833, inhibited angiogenesis and induced apoptosis in an orthotopically-transplanted human gastric cancer model.[7]

In the present study, we have designed an antisense oligonucleotide (ASO) that specifically inhibits KDR/Flk-1 synthesis, which was effective in inhibiting peritoneal dissemination of gastric cancer cell line NUGC-4 in nude mice. The NUGC-4 cells were engineered to express green fluorescent protein (GFP), which enabled high-resolution visualization of tumor growth, spread, and angiogenesis.[9–11]

Materials and methods

Antisense phosphorothioate oligonucleotide

ASO sequence and scrambled control oligonucleotide (CO) were designed by a computer program (Advanced Gene Computing Technologies, Irvine, CA). The following phosphorothioate oligodeoxyribonucleotides sequences were synthesized: KDR/Flk-1-ASO, 5′-GCCCGCTTAA-CGGTC-3′; KDR/Flk-1-CO, 5′-AGGCTGCCGCCA-TTC-3′. The sequence of ASO or CO was designed for both human and mouse. These oligonucleotides were dissolved in phosphate-buffered saline (PBS) to make a working solution.

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Cell lines, culture, and administration of ASO in vitro

HUVEC isolated from human umbilical vein was grown on 0.003% type I collagen-coated tissue culture plates in MCDB-131 (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (FBS), 50 μg/mL heparin (Wako, Osaka, Japan), 20 μg/mL bovine pituitary extract (BPE), 10 ng/mL human recombinant EGF (Life Technologies), and 100 U/mL penicillin and streptomycin. PC-3 was obtained from Health Science Research Resources Bank (Osaka, Japan). The human prostate cancer cell line PC-3, was grown in Kaighn's Modification F-12K Nutrient Mixture medium (Life Technologies) containing 10% FBS and 100 U/mL penicillin and streptomycin. Human gastric cancer cell line, NUGC-4, was provided by the Japanese Collection of Research Bioresources (Osaka, Japan). NUGC-4 was maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS and 100 U/mL penicillin and streptomycin. Administration of ASO to cultured cells was as described previously.[5] In brief, KDR/Flk-1-ASO or KDR/Flk-1-CO at a final concentration of 10 μM or PBS was added once a day to HUVEC, PC-3, and NUGC-4 cell cultures with a medium containing low serum. The cell number was counted and protein expression of KDR/Flk-1 examined by Western blotting on culture day 5.

Immunoprecipitation and Western blotting

After treatment with ASO, HUVEC, PC-3, and NUGC-4 were washed with ice-cold PBS and dissolved with lysis buffer [50 mM HEPES (pH 7.0), 150 mM NaCl, 10% glycerol, 2% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, aprotinin, leupeptin, PMSF, and 1 mM Na_3VO_4]. The protein lysates were centrifuged at 12,000 rpm for 10 min at 4°C. For KDR/Flk-1 Western blots, the protein lysates were enriched with KDR/Flk-1 by immunoprecipitation with two human KDR/Flk-1–specific antibodies directed against different epitopes (Ab: directed against residues 1158–1345 and 931–997; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies and the protein A Sepharose CL-4B resin (Pharmacia LKB Biotechnology, Uppsala, Sweden) were added to the protein lysates and incubated overnight at 4°C. The sample was centrifuged and was rinsed with lysis buffer.[8] The sample was mixed with 2× sample buffer [125 mM Tris–HCl (pH 6.7), 4% SDS, 20% glycerol, 0.002% BPB, 10% 2-mercaptoethanol], boiled for 5 minutes, and analyzed by 7.5% SDS-PAGE. After SDS-PAGE, the gel was transferred to a 0.45-μm pure nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 0.5% skim milk in TBS–Tween 20 [20 mM Tris–HCl (pH 7.4), 136 mM NaCl, 2 mM KCl, and 0.2% Tween 20], for 1 hour at room temperature, and reacted with anti-KDR/Flk-1 antibody (Santa Cruz; 1:100) at 4°C overnight. The membrane was then incubated with an anti–rabbit Ig horseradish peroxidase–linked whole antibody (Amersham LIFE Science, Buckinghamshire, England; 1:1000) for 30 minutes at room temperature. Protein bands were visualized by an ECL detection system (Amersham LIFE Science).

GFP expression vector, production, transfection, and subcloning

The method of transduction of tumor cells with the *Aequorea victoria* GFP gene cells was previously described.[9–11] In brief, GFP-containing retrovirus pLEIN was purchased from Clontech Laboratories (Palo Alto, CA). The pLEIN vector expressed enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contained an internal ribosome entry site (IRES). PT67, an NIH3T3-derived packaging cell line expressing the 10Al viral envelope, was purchased from Clontech Laboratories. PT67 cells were cultured in 10% FBS DMEM. For vector production, packaging cells (PT67), at 70% confluence, were incubated with the precipitated mixture of DOTAP reagent (Boehringer Mannheim) and a saturating amount of pLEIN plasmid for 18 hours. The medium was replenished at this time. The cells were examined by fluorescence microscopy 48 hours post-transfection. For selection of GFP transductants, the cells were cultured in the presence of stepwise increases of 200–1000 μg/mL G418 (Life Technologies) for 7 days.

GFP gene transduction of NUGC-4 cells

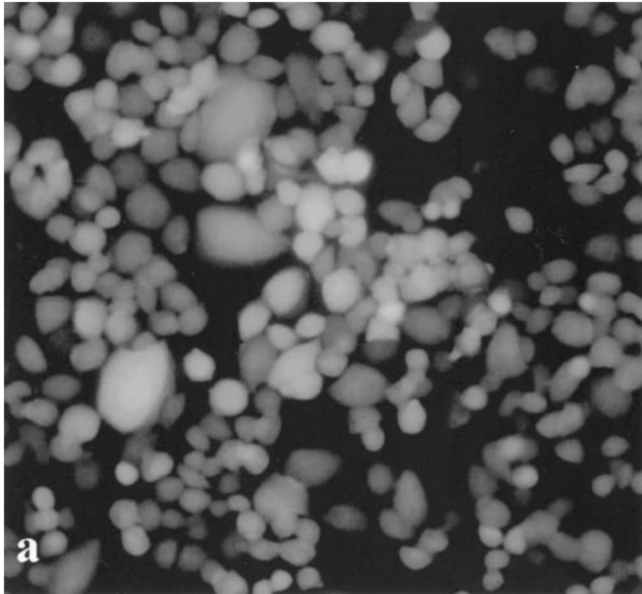
For GFP gene transduction, 60% confluent NUGC-4 cells were incubated with a 1:1 precipitated mixture of retroviral supernatant of PT67 cells and 10% FBS-DMEM for 72 hours. The medium was replenished at this time. NUGC-4 cells were harvested with 0.25% trypsin–EDTA 72 hours post-transduction and subcultured at a ratio of 1:15 in the selective medium that contained 200 μg/mL G418. The concentration of G418 was increased to 750 μg/mL stepwise.[9] The brightest NUGC-4 clones expressing GFP (NUGC-4-GFP) were selected (Fig 1a), combined, and then amplified and transferred by conventional culture methods.

Peritoneal implantation of NUGC-4-GFP in nude mice

Six to 8-week-old BALB/c *nu/nu* male mice (Nihon CLEA, Tokyo, Japan) were used. The mice were anesthesized with 2.5% Avertin solution, 0.4 mL/mouse. The cecum and mesocolon were exteriorized under sterile conditions. Two hundred microliters of the NUGC-4-GFP cell suspension containing 2×10^6 cells was slowly layered on the mesocolon with a 1-mL syringe and a 27-gauge needle. Thirty minutes later, the mesocolon was rinsed with saline and then returned in the peritoneum. The seeded NUGC-4-GFP cells formed small nodules less than 1 mm in diameter by 1 week.

Administration and efficacy determination of ASO in nude mice

Administration of KDR/Flk-1-ASO and/or KDR/Flk-1-CO was by intraperitoneal injection. KDR/Flk-1-ASO, -SO, or PBS was administrated from days 7 to 14, 200 μg/mouse, once a day. The mice were sacrificed on day 28, at which time the stomach and colon were excised. Disseminated nodules of NUGC-4-GFP cells in the stomach and



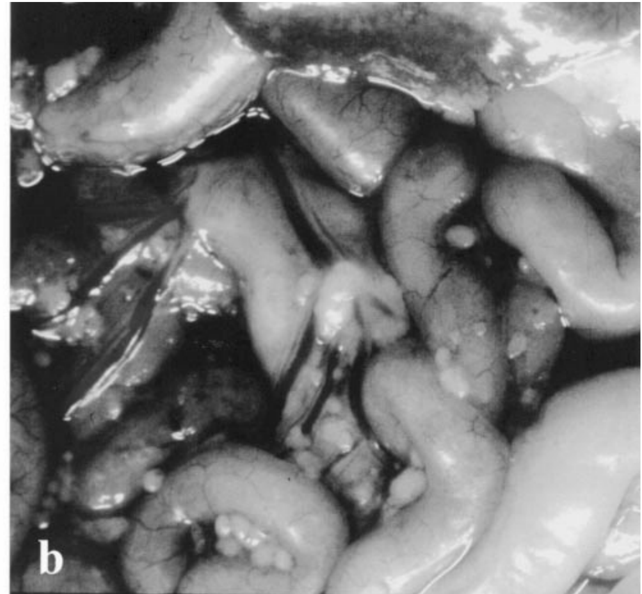


Figure 1 a: Stable high-level GFP expression of human gastric cancer NUGC-4 transductants *in vitro*. The human gastric cancer cell line NUGC-4 was transduced with the pLEIN vector that expressed enhanced GFP and the neomycin resistance gene on the same bicistronic message. A stable high-expression clone was selected in 750 μg/mL G418. **b:** Peritoneal disseminated NUGC-4-GFP in abdomen of nude mice.

colon were visualized, counted, and measured under fluorescence microscopy (Fig 1b).

Immunohistochemistry

To determine whether tumor angiogenesis was inhibited by KDR/Flk-1-ASO, we carried out immunohistochemical analysis of the EC marker, Factor VIII. Immunohistochemistry was performed using a commercial kit according to the manufacturer's instruction (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) with the following modifications: paraffin-embedded sections (3–5 μm) were deparaffinized in xylene, followed by treatment with a graded series of alcohol [100%, 90%, 80%, and 70% ethanol/double-distilled water (vol/vol)] and rehydrated in PBS. Antigen unmasking sections were treated for 6 minutes in a microwave oven in 10 mM citrate buffer (pH 6.0), then incubated with 0.03% hydrogen peroxidase in methanol (vol/vol) for 30 minutes at room temperature to block endogenous peroxidase, and washed with PBS. The sections were incubated with the primary anti–Factor VIII–related antigen polyclonal antibody (Nichirei; 1:100) overnight at 4°C. Reactions were visualized by incubating the slides with stable 3,3′-diaminobenzidine for 5–10 minutes (Wako) and counterstained with hematoxylin. To examine whether KDR/Flk-1-ASO caused tumor cell apoptosis, we carried out the immunohistochemical TUNEL assay using a commercial kit according to the manufacturer's instruction (Roche Molecular Biochemicals, Mannheim, Germany).

Fluorescence microscopy

Light and fluorescence microscopy was carried out using an Olympus fluorescence stereomicroscope, model SZX-RFL2 (Olympus Optical, Tokyo, Japan).

Results and discussion

Effect of KDR/Flk-1 ASO on in vitro *cell growth*

Proliferation of HUVEC and PC-3 was significantly inhibited by KDR/Flk-1-ASO. The number of untreated HUVEC and PC-3 was increased from 1×10^5 to $2.2\times10^5\pm0.11$ and $1.83\times10^5\pm0.11$ cells in 5 days culture, respectively. The administration of ASO reduced this number to $0.96\times10^5\pm0.88$ and $0.90\times10^5\pm0.17$ cells, respectively after 5 days culture. Thus, treated cells grew to 43.6% and 49.1% of control, respectively. In contrast, KDR/Flk-1-CO did not inhibit growth of either cell line. KDR/Flk-1-ASO did not suppress NUGC-4-GFP cell proliferation *in vitro*. KDR/Flk-1-ASO inhibited expression of the KDR/Flk-1 protein by 42% in PC-3 determined by Western blot analysis (data not shown).

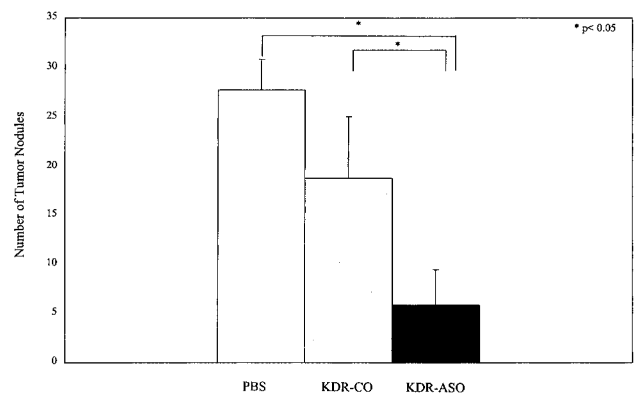
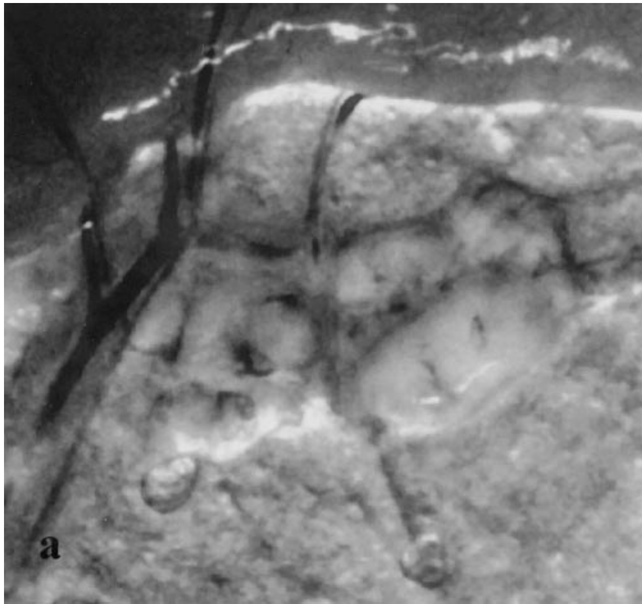


Figure 2 Inhibition of peritoneal dissemination by KDR/Flk-1-ASO. The KDR/Flk-1-ASO and KDR/Flk-1-CO were injected intraperitoneally at a dose of 200 μg/200 μL PBS every day beginning 1 week after tumor implantation. The number of nodules was significantly reduced in the KDR/Flk-1-ASO treated animals.



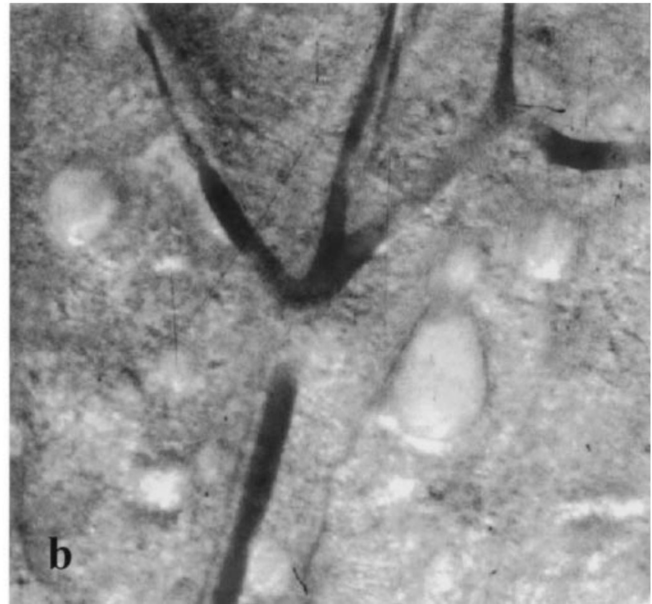


Figure 3 Newly formed vessels in disseminated nodule were clearly visualized by contrast to the fluorescent tumors. **a:** Control. **b:** KDR/Flk-1-ASO–treated.

Inhibition of peritoneal tumor dissemination by KDR/Flk-1-ASO

The number of tumor nodules visualized by GFP expression in KDR/Flk-1-ASO–treated animals (Fig 1b) was significantly fewer than in KDR/Flk-1-CO–treated animals or control on day 28 (control, 27.7±3 nodules; CO, 18.8±6.2 nodules; ASO, 5.8±3.7; $P<0.05$) (Fig 2). The mean nodule size, however, was not significantly different among the three groups (control: 5.45±1.19 mm^2; CO: 5.08±0.97 mm^2; ASO: 4.33±0.81 mm^2; data not shown).

Inhibition of tumor angiogenesis by KDR/Flk-1-ASO

Newly formed vessels in disseminated nodules were clearly visualized by contrast to the brightly fluorescing tumors. In the KDR/Flk-1-CO and control group, vessels were thicker than in the KDR/Flk-1-ASO group (Fig 3). Immunohistochemical studies with anti–Factor VIII antibody demonstrated that Factor VIII–positive cells in the tumor nodules were significantly fewer in the ASO group than in CO and control groups (control: 4.84±0.50; CO: 3.80±0.29; ASO: 0.9±0.17; $P<0.05$). Figure 4 shows the significant decrease in Factor VIII–positive cells in the KDR/Flk-1-ASO–treated cells compared to both PBS- and KDR-CO–treated cells in the NUGC-4-GFP tumors. There is only a slight decrease due to KDR-CO treatment compared with PBS treatment.

Induction of tumor apoptosis by KDR/Flk-1-ASO

KDR/Flk-1-ASO treatment significantly increased the TUNEL-positive cells in the tumor nodules compared with CO treatment or PBS control. The apoptotic index was 27.5±0.70 for PBS treatment, 24.5±0.70 for CO-treated tumors, and 41±4.24 for ASO-treated tumors, ($P<0.05$) (Fig 5).

In conclusion, we developed a stable high-expression GFP transductant of human gastric cancer cell line NUGC-4 (NUGC-4-GFP) to establish a peritoneal tumor dissem-

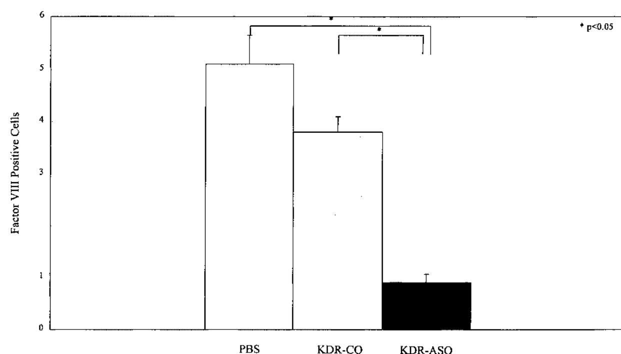


Figure 4 Effect of KDR/Flk-1-ASO on Factor VIII–positive disseminated tumors in nude mice. Factor VIII–related antigen-positive cells were counted in 20 high-power fields at ×200 magnification. See text for immunohistochemical methods.

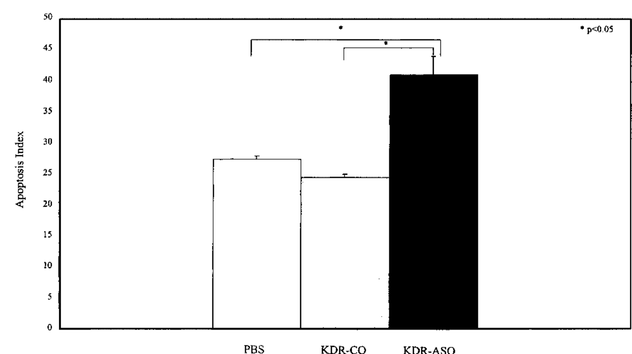


Figure 5 Effect of KDP/Flk-1-ASO on TUNEL-positive cells in disseminated tumors in nude mice. Apoptosis was evaluated by the number of TUNEL-positive cells in 1000 cells counted at ×200 magnification.

ination model. This fluorescent peritoneal dissemination model is a useful tool for observation and understanding of the early stage of cancer dissemination. No nodule that did not express GFP was noted. With this gastric cancer model, we have demonstrated that KDR/Flk-1-ASO reduced peritoneal dissemination. This effect appears to be due to the reduction of tumor angiogenesis, as formation of vessels was inhibited in KDR/Flk-1-ASO–treated mice compared with KDR/Flk-1-CO–treated mice (Figs 3 and 4). Our results are consistent with previously reported demonstration that an antisense oligomer to KDR/Flk-1 completely impaired VEGF-induced EC proliferation, migration, and PAF synthesis.[5] Our results are also consistent with previously reported efficacy of KDR/Flk-1 monoclonal antibody on HCC *in vivo*.[6] The results in this report are also consistent with efficacy of monoclonal anti-VEGF antibody on gastric cancer metastasis in nude mice and inhibition of both NCI-H358 non–small cell lung carcinoma and A673 rhabdomyosarcoma in nude mice.[12]

Thus, KDR/Flk-1 is a target for inhibition of various cancer types including peritoneal dissemination of gastric cancer. The ASO for KDR/Flk-1 strategy to inhibit tumor angiogenesis and metastasis is thus promising for further development. Future studies will investigate efficacy on survival of tumor-bearing animals.

Acknowledgment

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