A Novel Tumor-Promoting Function Residing in the 5′ Non-coding Region of vascular endothelial growth factor mRNA

Kiyoshi Masuda, Shigetada Teshima-Kondo*, Mina Mukaijo, Naoko Yamagishi, Yoshiko Nishikawa, Kensei Nishida, Tomoko Kawai, Kazuhito Rokutan

Department of Stress Science, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan

Funding: This work was supported in part by grants from Grants-in-Aid for Scientific Research C (16590598) and Grants-in-Aid for Exploratory (19659187) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to ST-K), and from the Japan Society for the Promotion of Science (to ST-K). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Academic Editor: Pam Jones, University of Leeds, United Kingdom

Citation: Masuda K, Teshima-Kondo S, Mukaijo M, Yamagishi N, Nishikawa Y, et al. (2008) A Novel Tumor-Promoting Function Residing in the 5′ Non-coding Region of vascular endothelial growth factor mRNA. PLoS Med 5(5): e94. doi:10.1371/journal.pmed.0050094

Received: October 5, 2007
Accepted: March 13, 2008
Published: May 20, 2008

Copyright: © 2008 Masuda et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; 5-FU, 5-fluorouracil; GAS, IFN-γ-activated sequence; IFN, interferon; ISRE, IFN-stimulated regulatory element; nt, nucleotide; PKR, RNA-activated protein kinase; rh, recombinant human; RT-PCR, real-time PCR; SEM, standard error of the mean; siRNA, small-interfering RNA; STAT, signal transducers and activators of transcription; UTR, untranslated region; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; YFP, yellow fluorescent protein; Ab, antibody

* To whom correspondence should be addressed. E-mail: kondoshi@med.tokushima-u.ac.jp

ABSTRACT

Background

Vascular endothelial growth factor-A (VEGF) is one of the key regulators of tumor development, hence it is considered to be an important therapeutic target for cancer treatment. However, clinical trials have suggested that anti-VEGF monotherapy was less effective than standard chemotherapy. On the basis of the evidence, we hypothesized that vegf mRNA may have unrecognized function(s) in cancer cells.

Methods and Findings

Knockdown of VEGF with vegf-targeting small-interfering (si) RNAs increased susceptibility of human colon cancer cell line (HCT116) to apoptosis caused with 5-fluorouracil, etoposide, or doxorubicin. Recombinant human VEGF165 did not completely inhibit this apoptosis. Conversely, overexpression of VEGF165 increased resistance to anti-cancer drug-induced apoptosis, while an anti-VEGF165-neutralizing antibody did not completely block the resistance. We prepared plasmids encoding full-length vegf mRNA with mutation of signal sequence, vegf mRNAs lacking untranslated regions (UTRs), or mutated 5′UTRs. Using these plasmids, we revealed that the 5′UTR of vegf mRNA possessed anti-apoptotic activity. The 5′UTR-mediated activity was not affected by a protein synthesis inhibitor, cycloheximide. We established HCT116 clones stably expressing either the vegf 5′UTR or the mutated 5′UTR. The clones expressing the 5′UTR, but not the mutated one, showed increased anchorage-independent growth in vitro and formed progressive tumors when implanted in athymic nude mice. Microarray and quantitative real-time PCR analyses indicated that the vegf 5′UTR-expressing tumors had up-regulated anti-apoptotic genes, multidrug-resistant genes, and growth-promoting genes, while pro-apoptotic genes were down-regulated. Notably, expression of signal transducers and activators of transcription 1 (STAT1) was markedly repressed in the 5′UTR-expressing tumors, resulting in down-regulation of a STAT1-responsive cluster of genes (43 genes). As a result, the tumors did not respond to interferon (IFN)α therapy at all. We showed that stable silencing of endogenous vegf mRNA in HCT116 cells enhanced both STAT1 expression and IFNα responses.

Conclusions

These findings suggest that cancer cells have a survival system that is regulated by vegf mRNA and imply that both vegf mRNA and its protein may synergistically promote the malignancy of tumor cells. Therefore, combination of anti-vegf transcript strategies, such as siRNA-based gene silencing, with anti-VEGF antibody treatment may improve anti-cancer therapies that target VEGF.

The Editors’ Summary of this article follows the references.
Introduction

Vascular endothelial growth factor-A (VEGF) is one of the key regulators in tumor formation and progression [1–4]. The clinical significance of VEGF in tumors has been demonstrated by many studies showing that the VEGF expression level is correlated with tumor grade, depth of invasion, status of nodal and distant metastasis, and clinical stage [5–10]. In addition, high levels of circulating VEGF are associated with resistance to chemotherapy in patients with metastatic solid tumors, including colorectal cancer [11,12]. The stimulatory action of VEGF on tumor angiogenesis is believed to play a central role in promotion of tumor development. At the same time, VEGF is known to act as an autocrine survival and growth factor for tumor cells [13]. Based on the evidence outlined above, a number of strategies to target VEGF or VEGF receptors (VEGFR) have been developed and subjected to clinical evaluation [14]. In contrast to preclinical studies in animal models, clinical trials suggest that anti-VEGF monotherapy was less effective than standard chemotherapy [15], raising the possibility that VEGF or possibly vegf mRNA might possess unrecognized function(s).

A recent report has shown that VEGF protein functions as an internal autocrine survival factor in human breast cancer cells through internally expressed VEGFR [16]. In addition, non-coding RNAs have now been recognized to play critical roles in tumorigenesis [17]. One class of the tumor-associated non-coding RNAs is the untranslated region (UTR) of certain mRNAs, including the 3'UTR of α-tropomyosin mRNA [18], the 3'UTR of prohibitin mRNA [19], the 3'UTR of ribonucleotide reductase mRNA [20], and the 5'UTR of c-myc mRNA [21]. All of these UTR RNAs function as tumor suppressors. From this point of view, we directed our attention to a unique structure of vegf mRNA characterized by its unusually long 5'UTR (1,038 nucleotide [nt]) containing two internal ribosome entry sites (IRES) [22,23].

Methods

Reagents

Recombinant human (rh) interferon alpha (IFNα) was purchased from PBL Biomedical Laboratories. rhIFNγ, rhVEGF165, and anti-VEGF165-neutralizing monoclonal antibody (Ab) were from R&D Systems.

Cell Culture and Transfection

The human colon carcinoma cell lines (HCT116 and RKO) were maintained in McCoy’s 5A medium, supplemented with 10% (v/v) FCS and antibiotics. The human embryonic kidney cell line (HEK293) was maintained in DMEM medium, supplemented with 10% (v/v) FCS and antibiotics. The human gastric carcinoma cell line (AGS) was maintained in McCoy’s 5A medium, supplemented with 10% (v/v) FCS and antibiotics. Amounts of VEGF165 secreted into the culture medium were measured by ELISA assay (Quantikine, R&D Systems), according to the manufacturer’s instructions.

Transfection of cells was performed using the jetPEI (Polyplus-transfection) or FuGENE HD (Roche Diagnostics) transfection reagent, according to the manufacturer’s instructions. We checked the transfection efficiency by monitoring expression of yellow fluorescent protein (YFP)-expressing plasmid and determined the efficiency to be 60%–65% in HCT116 cells, 80%–85% in HEK293 cells, 60%–65% in RKO cells, and 60%–65% in AGS cells.

Cell Growth

Cell growth was assessed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer’s instructions. Cells were plated in a 96-well flat-bottom plate and cultured in 100 μl of McCoy’s 5A medium containing 10% (v/v) FCS and antibiotics, and the MTT reagent (20 μl/well) was added to each well and incubated for 1 h. The levels of blue formazan were measured spectrophotometrically at 490 nm at each time point using a microplate reader (Wallac 1420 ARVO MX; Perkin Elmer).

To assess anchorage-independent growth, cells (1 × 10²) were plated in 2 ml of McCoy’s 5A medium containing 10% (v/v) FCS and 0.33% agar in 60-mm diameter culture dishes. Following a 28-d incubation at 37 °C in 5% CO₂, the number of colonies (approximately >0.1 mm in diameter) with dense centers was scored for each plate.

Assessment of Apoptosis

Apoptotic cells were assessed by the TUNEL-staining method using an in situ Cell Death Detection kit (Roche Diagnostics). TUNEL-positive cells were viewed using a microscope, and percentages of the positive cells were calculated. A minimum of 300 cells was counted for each determination. Apoptosis was also detected using the APOPercentage apoptosis assay kit (BioColor), according to the manufacturer’s instructions. The APOPercentage dye stains cells undergoing the membrane “flip-flop” event when phosphatidylserine is translocated to the outer leaflet. This event is specific for apoptosis, but not for necrosis.

The caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay (Promega), according to the manufacturer’s instructions. After cells were treated with 80 μM of 5-fluorouracil (5-FU) for 24 h, the Caspase-Glo 3/7 reagent was added to each well and incubated for 1 h. The luminescence was measured using a microplate-reading luminometer (Wallac 1420 ARVO MX; Perkin Elmer).

Plasmid Constructions and Establishment of Stable Transfectants

The plasmids expressing vegf small-interfering RNAs (siRNAs) were constructed using a BLOCK-iT Pol II miRNA expression vector kit (Invitrogen), in accordance with the manufacturer's protocol. A pcDNA6.2-GW/EmGF-miRNA vector that contains the GFP-coding region within the pre-miRNA expression cassette was inserted with the following pairs of sense and antisense DNA: vegf siRNA number 1, 5'-TGC TGA GCA AGGCAA GCC TCC AAT GCC TTT TGG CCA CTG ACT GAC GCA TTG GTG TTG CTC CT G TGCA CTA A-3' (sense) and 5'-AGC AAG GAG ATG CCA ATG CTG TCA GTG GCC AAA AGC CAT TTG AGC CTG GCC TCT AGC A-3' (antisense); vegf siRNA number 2, 5'-TGC TGA GAG CAG CAA GCC TCC TTG TGG CCA CTG ACT GAC GCA TTG GTG TTG CTC CTG A-3' (sense) and 5'-AGC AAG GAG ATG CCA ATG CTG TCA GTG GCC AAA AGC GAG CTG TCT GCT GCT GCT CTC AGC A-3' (antisense). These siRNAs were cloned into pcDNA6.2-GW/EmGF-miRNA vector (siVEGF#1 and siVEGF#2 in Figure 1).

Tumor Progression by vegf mRNA
As a control siRNA-expressing plasmid, we used the pcDNA6.2-GW/EmGFP-miR-negative control plasmid expressing mature siRNA that is predicted not to target any known vertebrate genes (Invitrogen).

The following plasmid constructs were kindly provided by Drs. Hervé Prats and Anne-Catherine Prats [24–27]: pAUG165mSPHA (construct I in Figure 2A), p165mSPHA 3′ (construct II), pVC (construct V), pSCT-chloramphenicol acetyltransferase (CAT) (construct IX), pVCTTT (construct XI), the plasmids expressing a chimeric mRNA fused to CAT coding sequence comprising one of the constructs (the human bip 5′UTR, the human c-myc 5′UTR, the human fgf-2 5′UTR, and the human pdgfb 5′UTR), and the bicistronic plasmid contains two luciferase genes, Renilla luciferase (LucR) and firefly luciferase (LucF), which are separated by a hairpin (hairpin control) or an IRES of human bip (pRBL), human c-myc (pRMp2L), and human fgf-2 (pRFL).

p165mSPHA 3′ and p165AUGmSPHA have H9D and L14E mutations in the signal sequence that prevent secretion of the VEGF165 protein. p165mSPHA 3′ was digested with Clal and Apal, blunted by Klenow treatment, and religated. The resulting plasmid (p165mSPHA, construct III in Figure 2A) did not contain the 3′UTR sequence. p165mSPHA 3′ was digested with Xbal and NgoMIV, blunted by Klenow treatment, and religated. The resulting plasmid (pAUG165mSPHA 3′, construct IV) did not encode the 5′UTR sequence. Several deletion constructs of the vegf 5′UTR were also made from the pVC plasmid by digestion with NcotIV (construct VI), Xbal and Nhel (construct VII), or BamHI (construct VIII). We also prepared a plasmid encoding full-length vegf mRNA with an intact signal peptide sequence. The construct pVC, which has an intact signal sequence, was digested with Narl and BsaBI, and the resultant Narl-BsaBI fragment was ligated into p165mSPHA 3′ to reconstruct full-length vegf mRNA with an intact signal peptide sequence (p165HA 3′). A construct mutated between nt 591 and nt 746 of the vegf 5′UTR was synthesized using two serial double-stranded DNA fragments (mutations are indicated by lower case letters): nt 576-GCG AGC CGC GGG Caa GaG Cca GaA Cca Cca Cca Cca Gag Gga Gga Gta Gaa Gaa Gta GgG GCT C- nt 636 and nt 637-GGg CCT Cga GgA Gta Gca Gta Cta TtT Gta CaG tgt tga Cga Tgg Tgc Tca Cga Ttt Ggg Ggg Gga Gta Gta Gta aga Gca Gga Gag Gca GaA Cca AGT GGG Gca Cga AGG AGT AGT AGT AGC- nt 752. The two synthesized fragments were ligated and inserted into pVC digested with SacII and Nhel to generate plasmid pVCmut (construct X in Figure 2A). All the constructs were confirmed by DNA sequencing. The secondary structures of vegf 5′UTR and its mutant were analyzed using the mfold 3.2 algorithm of Zuker [28].

We also constructed a plasmid expressing a chimeric mRNA comprising the vegf 5′UTR fused to YFP. The constructs pVC and pVCmut were digested with EcoRI and Ncol, and the resulting EcoRI-Ncol fragments were ligated into pd2EYP-N1 (Clontech) to generate pVY and pVYmut, respectively.

Stable transfectants were constructed using early passages of HCT116 cells that had been plated at approximately 1 × 10^5 in a 60-mm diameter culture dish and cultured overnight. The cells were transfected with 5 μg of pYFP, pYV, pVYmut, siVEGF#1, or pcDNA6.2-GW/EmGFP-miRNA-neg control plasmids. Clones were selected and maintained in McCoy’s 5A medium supplemented with 450 μg/ml G418 (Invitrogen), or with 5 μg/ml Blasticidin (Invitrogen). Three stably transfected cell lines (HCT116/YFP, HCT116/vegf 5′, HCT116/lvegf

**Figure 1.** Effect of vegf Transcript on Susceptibility to Anti-Cancer Drugs

(A) Knockdown efficiency of vegf mRNA. Expression levels of vegf mRNA were measured using quantitative RT-PCR. Values were normalized for the amount of gapdh mRNA (means ± SEM, n = 4).

(B) Knockdown of vegf mRNA increases 5-FU induced apoptosis. Cells untransfected (−) or transfected with the indicated plasmids for 24 h were treated with 20 μM 5-FU or rhVEGF protein (1, 2, 4, or 6 ng/ml) combined with 5-FU (20 μM) for 48 h. Apoptosis was evaluated by calculating the percentages of TUNEL-positive cells. Values are means ± SEM from four independent experiments. Means with different superscripts are significantly different by ANOVA followed by Scheffé’s test (p < 0.05).

(C) Overexpression of vegf transcript induces resistance to 5-FU. Cells were transfected with a plasmid encoding full-length vegf mRNA or an empty vector (mock) for 24 h. The cells were treated without or with 80 μM 5-FU in the presence of a monoclonal anti-human VEGF165 9 neutralizing Ab at the indicated concentrations for 48 h, and then apoptotic cells were identified and quantitated by the TUNEL-staining method. Values are means ± SEM from three independent experiments. Means with different superscripts are significantly different by ANOVA followed by Scheffé’s test (p < 0.05).

doi:10.1371/journal.pmed.0050094.g001
mut, HCT116/siVEGF, or HCT116/siControl) were isolated after 28 d of selection.

Western and Northern Blot Analyses

Cell lysates were prepared using a lysis buffer containing 100 mM Tris-HCl (pH 6.8), 300 mM NaCl, 2 mM EDTA, and 4% (v/v) SDS. Western immunoblotting was performed as described previously [29] using a rabbit polyclonal anti-human influenza virus hemagglutinin (HA) Ab (Santa Cruz Biotechnology) at a 1/10,000 dilution, a mouse monoclonal Ab against human signal transducers and activators of transcription 1 (STAT1) (Cell Signaling) at a 1/1,000 dilution, a
rabbit polyclonal anti-L-VEGF Ab at a 1/500 dilution (a gift from Hervé Prats) [26], or a mouse anti-β-actin monoclonal Ab (Sigma). The specificity of individual antibody reactivity was confirmed by the absorption test with the corresponding antigen peptide (Figure S10). Blocking antigen peptide for human STAT1 and HA were obtained from Cell Signaling and Santa Cruz Biotechnology, respectively. Antigen peptides for L-VEGF were synthesized using an Applied Biosystems 432A peptide synthesizer.

Total RNA was prepared by the guanidinium thiocyanate method. Northern hybridization was performed as described previously [29] using following cDNA probes. cDNA probes for vegf 5’UTR RNA and vegf ORF RNA were prepared by digestion of the plasmid p165mSPHA3’ with NgoMIV and ClaI, and SacII and NheI, respectively. A cDNA probe for CAT mRNA was prepared as a digestion fragment of the plasmid pVC using EcoRI and BglII sites. These probes were labeled with [α-32P]dCTP using Klenow enzyme (New England BioLabs), according to the manufacturer’s instructions.

Animal Studies and Immunohistochemistry

The use of animals in the experiments described here was approved by the Animal Care Committee of University of Tokushima. Seven-wk-old male athymic nude mice (Nippon SLC) were caged in groups of five and acclimated for 1 wk. A cell suspension (5 × 10^6 cells) in serum-free McCoy’s 5A medium, prepared from each stably transfected clone, was injected subcutaneously into the flanks of nude mice. The sizes of tumors that developed in the injected mice were measured in two dimensions with a caliper and their volumes calculated using the formula (L × W^2) × 0.5, where L is length and W is width. We confirmed that parental HCT116 cells (5 × 10^6 cells) injected subcutaneously into nude mouse formed tumors whose volumes were around 500 mm^3 day 28 after transplantation. Therefore, HCT116/YFP clones appeared to form xenograft tumors less efficiently than parental HCT116 cells, probably because expression of YFP and/or procedures during the establishment of permanent cell cloning was likely to nonspecifically reduce the tumor-forming capability.

Some mice were injected intraperitoneally with BrdU (1.5 g/kg body weight, Sigma-Aldrich) 2 h before they were humanely killed. The mice were killed on the indicated days, and growing tumors were removed and fixed with formalin in PBS. The fixed xenografts were sectioned and stained with hematoxylin and eosin (HE), or by immunohistochemistry using a mouse monoclonal anti-BrdU Ab (Sigma-Aldrich), or by a DeadEnd colorimetric TUNEL System (Promega). Vasculature was stained using a rat anti-mouse CD31 Ab (BD Pharmingen).

We also examined the effects of 5-FU on implanted tumors. In this experiment, HCT116/voegf 5’ transfectants (5 × 10^6 cells) were injected subcutaneously into the left flanks of nude mice, and HCT116/YFP (1 × 10^7 cells) or HCT116/voegf 5’mut transfectants (1 × 10^6 cells) were injected into the right flanks of the same mice. When the tumors reached a size of approximately 40 mm^3, usually by day 7, the mice were treated daily with an intraperitoneal injection of 5-FU (30 mg/kg weight, Sigma-Aldrich), or with 5-FU (30 mg/kg weight) plus IFNα (50,000 U/mouse; PBL Biomedical Laboratories). The tumor sizes were measured daily using a caliper, and their volumes were calculated as described above.

Microarray Analysis

We used Agilent Human 1A oligomicroarrays (Agilent Technologies) containing 60-mer DNA probes synthesized in situ in a 22-k format. Total RNA was prepared from three independent vegf 5’-G5 tumors on day 14, and an equal amount of RNA from each tumor was mixed. After contaminating DNA had been removed using a DNase kit (Qiagen), the resultant RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was also prepared from three vegf 5’-mut-D5 tumors on day 14 in the same manner. Synthesis, amplification, and labeling of cRNA were carried out in accordance with the manufacturer’s protocol. Cy5-cRNA (0.75 µg) prepared from vegf 5’-G5 tumors was mixed with the same amount of Cy3-cRNA from vegf 5’-mut-D5 tumors. Hybridization was performed on the Agilent Human 1A oligomicroarray (Agilent Technologies), according to the manufacturer’s protocol. Fluorescence images of the hybridized microarrays were obtained using an Agilent microarray scanner (G2565BA). Signal intensities of Cy5 and Cy3 were quantified and analyzed by subtracting the backgrounds using feature extraction software (Agilent, version 9.5). The Cy5/Cy3 ratios of 12,178 genes that had a higher fluorescence value than 100 for both Cy5 and Cy3 signals were transformed to logarithms for global normalization. We selected genes whose mRNA levels differed by >2-fold between vegf 5’-G5 and mut-D5 tumors.

Dual-Luciferase Assay

To measure IRES activity, we used the bicistronic plasmid. The bicistronic cassette expresses the LucR in a cap-dependent manner and LucF in an IRES-dependent manner. Each IRES or hairpin control is located between the two cistrons.

To measure IFN-dependent transcriptional activity, we used plasmids containing the motifs IFN-stimulated regulatory element (ISRE) and IFN-γ-activated sequence (GAS) upstream of the LucF reporter gene (Pathway Profiling Luciferase System).

LucF and LucR activities were measured using the Dual-Luciferase reporter assay system (Promega), according to the manufacturer’s instructions. Transfected cells were rinsed twice with PBS, scraped, and homogenized in 50 µl of lysis reagent provided with the kit. The lysate was cleared by a 2-min centrifugation at 4 °C. Chemiluminescent signals were measured in a luminometer (Wallac 1420 ARVO MX; Perkin Elmer) equipped with automatic injectors.

Quantitative Real-Time PCR

SuperScript II RNase H-reverse transcriptase (Invitrogen) was used to synthesize cDNAs from 1 µg aliquots of total RNA prepared from three independent tumor tissues. Taqman Gene Expression assays (Applied Biosystems) were used with the following cDNA-specific primers and probes: mia (Hs00197954_m1); bcl6 (Hs00153368_m1); egfr (Hs00193306_m1); vegf (Hs00247624_m1); pcd1 (Hs00169472_m1); fas (Hs00531110_m1); bax (Hs00180269_m1); srlf (Hs00138822_m1); trailapo2 (Hs00234356_m1); nmi (Hs00190768_m1); isg20 (Hs00158122_m1); oas1 (Hs00243943_m1); oas2 (Hs00213443_m1); irf1 (Hs00235698_m1); pml (Hs00231241_m1); and gapdh (Hs99999905_m1). The levels of transcripts for IFN receptors (ifnar1, ifnar2, ifngr1, and ifngr2), VEGF receptors (vegfr1, vegfr2, fps1 and fps2), stat1,
vegf, and β-actin were measured by real time (RT)-PCR using the following specific primer sets: ifnar1, 5′-CCC AGT GTG TCT TTC TCT ACA A-3′ (forward) and 5′-AAG GGA GGC AGG AAA GG-3′ (reverse); ifnar2, 5′-AGT GAG TTC GGG AGC CAT CC-3′ (forward) and 5′-CTG GTC TCT ATG GAG TT-3′ (reverse); ifngr1, 5′-ACT TTA GTT GGT GTA GGC ACT G-3′ (forward) and 5′-GAA CAT TTG GGA AAT CTC TTG C-3′ (reverse); ifngr2, 5′-GGG CTG AGT TGG GTC TTT TA-3′ (forward) and 5′-GGA CAC CAA CAT TTG GCC ATC TT-3′ (reverse); stat1, 5′-CTG CTC CTT TGG TTG-3′ (forward) and 5′-AGC ACC CCG ATT ATG TGA GAA A-3′ (reverse); vegfr2, 5′-GAT AGA TTC GGG AGC CAT CC-3′ (forward) and 5′-CCA ACC CCA ACC CCG AGG TGA-3′ (reverse); vegf, 5′-GGG CTG AGT TGG GTC TTT TA-3′ (forward) and 5′-GGA CAC CAA CAT TTG GCC ATC TT-3′ (reverse); β-actin, 5′-CTG CTC CTT TGG TTG-3′ (forward) and 5′-AGC ACC CCG ATT ATG TGA GAA A-3′ (reverse); vegfr2, 5′-GGA CAC CAA CAT TTG GCC ATC TT-3′ (reverse); vegf, 5′-GGG CTG AGT TGG GTC TTT TA-3′ (forward) and 5′-GGA CAC CAA CAT TTG GCC ATC TT-3′ (reverse); β-actin, 5′-CTG CTC CTT TGG TTG-3′ (forward) and 5′-AGC ACC CCG ATT ATG TGA GAA A-3′ (reverse); vegfr2, 5′-GGA CAC CAA CAT TTG GCC ATC TT-3′ (reverse); vegf, 5′-GGG CTG AGT TGG GTC TTT TA-3′ (forward) and 5′-GGA CAC CAA CAT TTG GCC ATC TT-3′ (reverse); β-actin, 5′-CTG CTC CTT TGG TTG-3′ (forward) and 5′-AGC ACC CCG ATT ATG TGA GAA A-3′ (reverse); vegfr2, 5′-GGA CAC CAA CAT TTG GCC ATC TT-3′ (reverse); vegf.

The data were analyzed using the two-tailed Student’s t-test or ANOVA and the Scheffe’s test. A p-value of less than 0.05 was considered significant.

Results

Effect of vegf mRNA on Apoptosis Caused by Anti-Cancer Drugs

First, we investigated whether vegf mRNA possessed a tumor-promoting activity in HCT116 cells that expressed VEGFR-2 and Neuropilin-1, ~2 (Figure S6) and secreted spontaneously VEGF165 at 759 ± 32 pg/ml (mean ± SD, n = 4). Two different vegf-targeting siRNAs successfully knocked down endogenous vegf mRNA in HCT116 cells (Figure 1A). This silencing made the cells susceptible to apoptosis caused with a low concentration of 5-FU (20 μM). We then examined the effect of supplementation of rhVEGF165 in the knock-down cells. The amount of secreted VEGF165 increased to 1,018 ± 48 (n = 4) from the cells that were untransfected and treated with 5-FU (20 μM) for 48 h. An external supplementation of 1,000 pg/ml rhVEGF165 failed to block the 5-FU-induced apoptosis in vegf siRNA-transfected cells (Figure 1B). Treatment with rhVEGF165 at 2,000 pg/ml or higher significantly, but not completely, prevented the apoptosis.

In contrast, cells were transiently transfected with a plasmid expressing full-length vegf165 mRNA and exposed to a higher concentration of 5-FU (80 μM). These cells secreted VEGF165 (7,092 ± 23 pg/ml, n = 4) during the experimental period and exhibited resistance to 5-FU-induced apoptosis (Figure 1C). It should be noted that anti-VEGF165-neutralizing monoclonal Ab could not completely cancel the apoptotic activity even at concentrations of up to 5 μg/ml (about 250 molar excess amount of secreted VEGF165) (Figure 1C). Both knockdown and overexpression experiments suggest that both VEGF protein and vegf mRNA may possess an anti-apoptotic function. Similar or even clearer results were observed in the cells treated with other anti-cancer drugs, etoposide and doxorubicin (Figure S1A and S1B).

Effect of vegf mRNA 5′UTR on Apoptosis Caused by 5-FU

Next, we used the mutated full-length vegf165 mRNA-expressing plasmid that has mutations of two amino acid residues within the signal peptide, shown as “mSP” in construct II (Figure 2A). This plasmid-derived mRNA is translated into a nonsecreted VEGF165 protein [26]. We confirmed that cells transfected with this construct expressed full-length vegf mRNA and VEGF165 protein (Figure S2A), but did not secrete detectable amounts of the plasmid-derived VEGF165 (unpublished data). As was described above, HCT116 cells overexpressing full-length vegf165 mRNA without mutation were completely resistant to 80 μM 5-FU (Figure 1C). In contrast, cells transfected with construct II significantly, but not completely, blocked the 5-FU-induced apoptosis (Figure 2B and Figure S3A). This partial loss of anti-apoptotic activity was probably due to the lack of VEGF165-secreting activity. Next, we prepared the constructs lacking the 3′UTR (construct III), 5′UTR (construct IV), or both (construct I), and found that the 5′UTR was associated with the chemoresistant phenotype (Figure 2B). Similar results were observed in the cells treated with other anti-cancer drugs, etoposide and doxorubicin (Figure S1C and S1D).

To confirm the functional role of the 5′UTR, we used a plasmid expressing the vegf 5′UTR fused to CAT coding gene (Figure 2A, construct V), which does not express VEGF protein. This chimeric mRNA retained the resistance to 5-FU (Figure 2C). We then designed several deletion constructs of the chimeric mRNA (constructs VI–VIII in Figure 2A), and found that the 270-nt-long element delimited by positions nt 475 and nt 745 in the 5′UTR sequence was crucial for the drug-resistance phenotype (Figure 2C). This sequence is just upstream of IRES-A, but does not require the IRES activity [22].

It is known that the 5′UTR of human vegf mRNA encodes long N-terminal VEGF proteins (termed L-VEGF) that are translated from CUG codons upstream of and in-frame with the classical AUG start codon [24]. To eliminate the possibility that the L-VEGF proteins might induce the chemoresistance, we designed a mutated 5′UTR construct that produced L-VEGF protein, but modified the RNA structure by substituting the nucleic acid sequence between nt 591 and nt 746 (construct X in Figure 2A). This mutant did not produce L-VEGF protein (unpublished data). As was described above, HCT116 cells overexpressing full-length vegf165 mRNA without mutation were completely resistant to 80 μM 5-FU (Figure 1C). In contrast, cells transfected with construct II significantly, but not completely, blocked the 5-FU-induced apoptosis (Figure 2B and Figure S3A). This partial loss of anti-apoptotic activity was probably due to the lack of VEGF165-secreting activity. Next, we prepared the constructs lacking the 3′UTR (construct III), 5′UTR (construct IV), or both (construct I), and found that the 5′UTR was associated with the chemoresistant phenotype (Figure 2B). Similar results were observed in the cells treated with other anti-cancer drugs, etoposide and doxorubicin (Figure S1C and S1D).

To confirm the functional role of the 5′UTR, we used a plasmid expressing the vegf 5′UTR fused to CAT coding gene (Figure 2A, construct V), which does not express VEGF protein. This chimeric mRNA retained the resistance to 5-FU (Figure 2C). We then designed several deletion constructs of the chimeric mRNA (constructs VI–VIII in Figure 2A), and found that the 270-nt-long element delimited by positions nt 475 and nt 745 in the 5′UTR sequence was crucial for the drug-resistance phenotype (Figure 2C). This sequence is just upstream of IRES-A, but does not require the IRES activity [22].

It is known that the 5′UTR of human vegf mRNA encodes long N-terminal VEGF proteins (termed L-VEGF) that are translated from CUG codons upstream of and in-frame with the classical AUG start codon [24]. To eliminate the possibility that the L-VEGF proteins might induce the chemoresistance, we designed a mutated 5′UTR construct that produced L-VEGF protein, but modified the RNA structure by substituting the nucleic acid sequence between nt 591 and nt 746 (construct X in Figure 2A). Secondary structures of the vegf 5′UTR and its mutated 5′UTR were predicted by mFold program (Figure S5). The mutation changed the secondary structure of several parts and ablated a stem-loop structure that is located in the functional region of the vegf 5′UTR (nt 699 to nt 738) as described above. The mutated construct X produced L-VEGF protein (Figure S2B), but did not induce the resistance to 5-FU (Figure 2C). We also used a simply mutated 5′UTR, in which the first CUG codon was substituted into a noninitiating UUU codon (construct XI in Figure 2A) [24]. This mutant did not produce L-VEGF protein (Figure S2B), but was able to induce the resistance as efficiently as did the wild-type 5′UTR (Figure 2C). We further
examined the effect of a protein synthesis inhibitor, cycloheximide (CHX). To determine the inhibitory effect of CHX, HCT116 cells were transfected with plasmid encoding luciferase in the absence or presence of CHX, and luciferase activity was measured. Protein synthesis was blocked by 86 ± 5% (mean ± SD, n = 3) and 94 ± 3% (mean ± SD, n = 3) with 1 and 10 μg/ml CHX, respectively. As shown in Figure 2D, both full-length vegf mRNA (construct II) and vegf 5’UTR-CAT mRNA (construct V) efficiently inhibited 5-FU-induced apoptosis even in the presence with 10 μg/ml CHX. These results suggest that the 5’UTR-mediated chemoresistance may not require de novo protein synthesis.

In addition to the TUNEL-staining method, we evaluated the vegf 5’UTR-mediated anti-apoptotic effect by the Apop- \( \text{percentage-staining method (Figure S3C) and by measuring activities of caspase 3 and caspase 7 (Figure S3D), which are crucial effector enzymes for apoptosis induced by genotoxic agents. We confirmed that the results obtained by the Apop-\) \( \text{percentage-staining method were similar to those by the TUNEL assay (Figure S3B and S3C). Cells transfected with construct II, V, and XI significantly, but not completely, blocked the activation of caspase 3/7 (Figure S3D).}

To examine whether this anti-apoptotic function is peculiar to vegf mRNA 5’UTR, we used three 5’UTRs containing IRES (\(c\)-myc, bip, and \(fgf-2\) mRNA 5’UTRs) [30] and the \(fgf-2\) mRNA 5’UTR that is unusually long, but does not contain IRES activity [31]. After transfection with plasmid expressing one of the indicated 5’UTR-CAT mRNAs, apoptosis was assessed by the TUNEL-staining method. Only the vegf 5’UTR induced the resistance to 5-FU among 5’UTRs tested (Figure S4).

We also examined whether the vegf 5’UTR similarly induce the chemoresistance in other human cancer cell lines (RKO, HEK293, and AGS cells), and confirmed that both full-length vegf mRNA and vegf 5’UTR RNA induced 5-FU resistance in these cells. (Figure S6B–S6D). These observations suggest that the vegf 5’UTR function is not peculiar to HCT116 cells.

The vegf 5’UTR contains IRES sequences that interact with IRES-binding factors and regulate translation initiation in a cap-independent manner [22,23]. Many IRES-containing mRNAs encode proteins that regulate cell growth, differentiation, and apoptosis [30,32]. Thus, overexpression of the vegf 5’UTR raises the possibility that increased amounts of the vegf 5’UTR may trap IRES-binding factors, thereby non-specifically modifying IRES-independent expression of genes. To address this issue, we tested the effect of vegf 5’UTR on other IRES activities (Figure S7B). HCT116 cells were cotransfected with plasmids expressing vegf 5’UTR-CAT mRNA (construct V or mutated construct X in Figure 2A) and the bicistronic construct containing 5’UTR of the indicated genes. We confirmed that overexpression of the vegf 5’UTR RNA did not repress the IRES activity of bip, c-myec, or \(fgf-2\) 5’UTR (Figure S7B).

Characterization of HCT116 Cells Stably Expressing vegf 5’UTR

To investigate chronic effects of the vegf mRNA 5’UTR on malignant transformation of HCT116 cells both in vitro and in vivo, we established stable transfectants that expressed chimeric mRNAs of either the vegf 5’UTR or the mutant one (as described above) fused to the YFP coding gene. Use of these chimeric mRNAs had the following advantages: (i) they did not produce VEGF protein, (ii) expression of these elements could be monitored by YFP fluorescence, and (iii) the chimeric mRNAs were translated similarly as vegf mRNA in our cell system. Ectopic mRNAs were equally expressed in corresponding cell lines and did not modify the amounts of endogenous vegf mRNA and VEGF among the clones (Figure S8A and S8B).

The vegf 5’UTR-expressing cells (HCT116\(\text{vegf}\) 5’, clones G3 and G5) were again resistant to 5-FU-induced apoptosis (Figure 3A). In contrast, the mutant vegf 5’UTR-expressing cells (HCT116\(\text{vegf}\) mut, clones D5 and E4) or the transfection control cells (HCT116/YFP, clone G11) underwent apoptosis by 5-FU (Figure 3A). We also examined the growth rate of each clone and observed that the HCT116\(\text{vegf}\) 5’ exhibited a small, albeit significant increase in growth rate compared to the HCT116\(\text{vegf}\) mut and the control HCT116/YFP (Figure 3B). Furthermore, HCT116\(\text{vegf}\) 5’ displayed a distinctive, anchorage-independent growth, while HCT116/ vegf 5’mut and the control HCT116/YFP did not (Figure 3C and D).

Tumor-Forming Activity of the HCT116/vegf 5’ Clones

To demonstrate the malignancy of HCT116/vegf 5’ in vivo, athymic nude mice were injected subcutaneously with each clone, and growing tumor masses were measured. Both HCT116\(\text{vegf}\) mut and control HCT116/YFP formed small tumor masses that did not expand further (vegf 5’mut-tumor and YFP-tumor, respectively). In contrast, HCT116\(\text{vegf}\) 5’ rapidly and progressively formed tumors (vegf 5’-tumor). The average volumes of the vegf 5’-tumors were 4-fold and 41-fold larger than those of the control YFP-tumors on days 5 and 28, respectively (Figure 4B). There was no significant difference between the sizes of tumors derived from vegf 5’-G3 clone and -G5 clone at either time point.

Histological examination showed that the growing vegf 5’-tumors contained central necrotic areas, while the vegf 5’mut-tumors formed a small nest of tumors encapsulated by connective tissue (Figure 4C). We next tested whether the accelerated growth of the vegf 5’-tumors was a consequence of enhanced proliferation, decreased apoptosis, or both. Apop-\(\text{tosis and proliferation were assessed by quantifying TUNEL-}\) positive and BrdU-incorporating cells, respectively (Figure 4C). The vegf 5’-tumors showed a small, but significant increase in the numbers of BrdU-positive cells (Figure 4D) compared with the vegf 5’mut-tumors. The number of apoptotic cells in the vegf 5’-tumor was significantly lower than in the vegf 5’mut-tumor (Figure 4D). These results suggest that the vegf 5’UTR RNA may accelerate tumor growth by suppression of apoptosis and promotion of cell growth. We also examined the development of tumor vessels by staining with an Ab against an endothelial cell marker CD31. The vegf 5’-tumors significantly increased CD31-negative microvessels (Figure 4C) in association with enlargement of the tumors, compared with the vegf 5’mut-tumors.

Changes in Gene Expression in the vegf 5’-Tumors

Total RNA was prepared on day 14 from three different vegf 5’-tumors, and an equal amount of RNA from each tumor was mixed. Total RNA was also prepared from three different vegf 5’mut-tumors in the same manner. Using a microarray technique, gene expression profiles were compared between the two RNA samples. The vegf 5’-tumors up-regulated mRNA
expression of 377 genes and down-regulated 295 genes >2-fold, compared with the vegf 5’-mut-tumors (see gene lists in Tables S1 and S2). One of the distinctive identifying features of the listed genes was that the vegf 5’-tumors selectively down-regulated pro-apoptotic genes (eight genes) and up-regulated anti-apoptotic genes (five genes). Quantitative RT-PCR validated the significant up-regulation of an anti-apoptotic gene (*mia*) and down-regulation of pro-apoptotic genes (*fas*, *pde4c1*, *nrg1*, and *bax*) (Table 1).

It should also be noted that a major set of IFN-inducible genes (43 genes) were specifically down-regulated in the vegf 5’-tumors (Table 2), whereas none of IFN-inducible genes were included in the up-regulated genes (Table S1). Using quantitative RT-PCR, we compared mRNA levels of nine IFN-inducible genes between the vegf 5’-tumors and the vegf 5’-mut-tumors, and validated significant down-regulation of eight out of nine genes in the vegf 5’-tumors (Table 1). An IFN-inducible gene *stat1* encoding a critical transcription

---

**Figure 3. Characterization of HCT116 Cells Stably Expressing the vegf 5’UTR RNA**

(A) 5-FU resistance in stable transfectant expressing the vegf 5’UTR RNA. Stably transfected HCT116 clones (HCT116/YFP-G11, HCT116/vegf 5’-G3, HCT116/vegf 5’-G5, HCT116/vegf 5’-mut-D5, and HCT116/vegf 5’-mut-E4) were treated with 80 μM 5-FU for the indicated time, and then apoptotic cells were identified and quantitated by the TUNEL-staining method. Values are means ± SEM from three independent experiments.

(B) Growth rate of the stable transfectants. Cell growth was assessed by MTT assay at the indicated time points. Results are expressed as relative increases in the absorbance, compared with that at time 0. Values are means ± SEM, n = 4.

(C and D) Increase in anchorage-independent growth ability in the vegf 5’UTR-expressing clones. The stably transfected clones were cultured in semi-solid medium for 28 d as described in the Methods. Pictures of growing colonies on day 28 are shown in (C), and the number of colonies were counted (D). Values are means ± SEM from three independent experiments. Means with different superscripts are significantly different by ANOVA followed by Scheffe’s test (p < 0.05).

doi:10.1371/journal.pmed.0050094.g003
factor for both type I (α/β) and type II (γ) IFNs [33] was also significantly down-regulated, suggesting specific deactivation of the IFN/STAT1 pathway in the rapidly growing \textit{vegf} 5'-tumors.

STAT1 Expression and Responsiveness to IFNs

To confirm that the \textit{vegf} mRNA 5'UTR selectively deactivates the IFN-dependent pathway in vitro, we measured the levels of \textit{stat1} mRNA and its protein in each stable clone by
quantitative RT-PCR and immunoblotting, respectively. Similar amounts of stat1 mRNA were observed among all clones (Figure 5A). Both HCT116/YFP and HCT116/vegf 5’mut cells responded to rhIFNα and up-regulated stat1 mRNA expression, but this up-regulation was not observed in the HCT116/vegf 5’ clones (Figure 5A). Consistent with these, induction of STAT1 protein by IFNα was defect in the HCT116/vegf 5’cells (Figure 5B).

STAT1 plays a crucial role in the expression of the majority of IFN inducible genes [34]. We therefore tested whether the HCT116/vegf 5’ cells actually showed decreased STAT1-dependent transcriptional activity. For this purpose, we used two different STAT1-dependent reporter plasmids encoding a promoter that either contained ISRE-binding sites (pISRE-Luc) that mainly respond to IFNα or GAS-binding sites (pGAS-Luc) that preferentially respond to IFNγ. Each stable clone was transiently transfected with pISRE-Luc or pGAS-Luc reporter plasmid, and then they were treated with rhIFNα or rIFNγ for 24 h. In the HCT116/vegf 5’ clones, there was significant impairment of the IFNα-stimulated response of ISRE (Figure 5C) and the IFNγ-dependent response of GAS (Figure 5D) compared to the HCT116/vegf 5’mut clones or to the HCT116/YFP cells (Figure 5C and 5D).

We confirmed that the IFN receptors were equally expressed in all clones tested (Figure S9A). These results suggest that the IFNα pathway might be more severely impaired than the IFNγ pathway. We also measured the levels of endogenous IFNα, β, and γ mRNAs in each clone by quantitative RT-PCR. There was no difference in the level of IFNα mRNA in each clone (Figure S9B). Neither clone expressed detectable amounts of IFNβ or IFNγ mRNAs.

We further examined the effects of stable knockdown of endogenous vegf mRNA on STAT1 expression in HCT116 cells (HCT116/siVEGF). Endogenous vegf mRNA was successfully silenced in the HCT116/siVEGF clones (Figure 6A). As shown in Figure 6B, the basal and the IFNα-stimulated expression levels of STAT1 were significantly increased in the HCT116/siVEGF clones compared with the control clone (HCT116/siControl) and parental cells (HCT116). Consistent with these results, both the basal and the IFNα-stimulated ISRE-dependent transcriptional activities were increased 2- to 3-fold in the HCT116/siVEGF clones (Figure 6C).

**Impairment of the IFNα-Dependent Anti-apoptotic Pathway in Growing vegf 5’-Tumors**

To assess whether the reduced IFN signals were responsible for the malignant transformation of the HCT116/vegf 5’ cells, we examined the effect of IFNs on 5-FU-induced apoptosis, as IFNs are known to potentiate the pharmacological action of 5-FU [35]. Treatment with rhIFNα or rhIFNγ alone did not induce apoptosis in any of the clones tested, while both IFNα and IFNγ significantly enhanced 5-FU-induced apoptosis in the HCT116/vegf 5’mut cells or the HCT116/YFP cells (Figure 5E and 5F). In contrast, neither IFNα nor IFNγ had an effect in the HCT116/vegf 5’ cells.

Finally, we examined whether growing vegf 5’-tumors had impaired responses to IFNα in vivo. We therefore focused on the effects of rhIFNα on tumor growth. For these experiments, we implanted HCT116/vegf 5’ (5 × 10^6 cells) in the left flank and either HCT116/vegf 5’ (1 × 10^7 cells) or HCT116/vegf 5’mut (1 × 10^7 cells) in the right flank of the same animal. When both tumors reached approximately 40 mm³ on day 7, we started to treat the mouse once a day with 5-FU (30 mg/kg body weight) or with a combination of 5-FU (30 mg/kg body weight) and rhIFNα (50,000 U). At day 4 in the YFP-tumors (Figure 7A) or day3 in the vegf 5’mut-tumors (Figure 7C) after the 5-FU-treatment, the YFP-tumors and the vegf 5’mut-tumors had reduced their body weight (Figure 7B).
In the present study, we show a novel function of the \textit{vegf} 5’UTR in tumor cell survival and growth. Both treatment of \textit{vegf}-knockdown HCT116 cells with rhVEGF\textsubscript{165} and treatment of VEGF\textsubscript{165}-overexpressing HCT 116 cells with a neutralizing anti-VEGF \textit{Ab} suggest the presence of \textit{vegf} mRNA-mediated anti-apoptotic action against anti-cancer drugs (5-FU, etoposide, and doxorubicin). We determined that the anti-apoptotic action resided in a 270-nt-long element between positions nt 475 and nt 745 of the 5’UTR RNA, we showed that the 5’UTR RNA may function as a regulatory RNA.

To further clarify the potential role of \textit{vegf} 5’UTR RNA, we established HCT116\textit{vegf}\textsuperscript{−} and HCT116\textit{vegf}\textsuperscript{+} cell clones. HCT116\textit{vegf}\textsuperscript{−} cells, but not HCT116\textit{vegf}\textsuperscript{+} mut cells, showed anchorage-independent growth in vitro and rapidly grew when implanted in athymic nude mice, indicating that \textit{vegf} mRNA 5’UTR facilitates tumor progression. The rapid growth observed in the \textit{vegf} 5’-tumors might be due to the acquisition of resistance to apoptosis (the process that eliminates defective cells), which contributes to tumor development and resistance to drug therapy. Microarray and quantitative RT-PCR analyses demonstrated that expression of pro-apoptotic genes (\textit{fas}, \textit{pdcld1}, \textit{vegf}, and \textit{bax}) and an anti-apoptotic gene (\textit{min}) was up-regulated and markedly down-regulated, respectively, in the rapidly growing \textit{vegf} 5’-tumors. In addition, microarray analysis also showed that the expression of cell growth-promoting genes (\textit{cxcl1}, \textit{cxcl2}, \textit{cxcr4}, \textit{mia1}, and \textit{fgf-19}) was up-regulated in the rapidly growing \textit{vegf} 5’-tumors.

The combination of both dysregulated cell proliferation and suppressed cell death are required for neoplastic progression [36]. In addition, survival and growth under conditions that promote anchorage independence in vitro and rapid growth upon transplantation into athymic mice, strongly suggests that \textit{vegf} mRNA 5’UTR facilitates tumor progression.
anchorage-independent conditions are required for the progression [37]. This anchorage-independent property of tumor cells correlates with their in vivo oncogenic potential. Thus, we consider that the \textit{vegf} mRNA 5'UTR may increase the ability not only to suppress apoptosis but also to survive and grow in an anchorage-independent manner, resulting in the acceleration of tumor formation.

The precise sequence and structure of the 5'UTR of \textit{vegf} mRNA as well as the molecular target(s) that interact with the RNA remain to be elucidated. However, it seems likely that such a profound change in cell function induced by RNA would require a global change of gene expression in cells. Our results support this notion by showing that the \textit{vegf} mRNA 5'UTR modulated expression of numerous genes. One of the striking findings is that many of the down-regulated genes belong to a set of IFN-inducible genes, including \textit{stat1}.

Since it is reported that IFN down-regulates IRES-dependent translation of distinct mRNAs [38], overexpression of the IRES-containing 5'UTR might attenuate the IFN signal, leading to the down-regulation of IFN-inducible genes. We therefore tested whether IFNα affected IRES activity of \textit{c-myc}, \textit{fgf-2}, or \textit{bip} using a bicistronic luciferase assay system (Figure 5).

Figure 5. Impaired Responses to IFNs in the \textit{vegf} 5'UTR-Expressing Clones

(A and B) Repression of \textit{stat1} mRNA and its protein expression in the \textit{vegf} 5'UTR-expressing cells. Total RNA or whole cell extracts were prepared from the indicated clones before and 24 h after treatment with 500 U/ml rhIFNα. Levels of \textit{stat1} mRNA were measured using quantitative RT-PCR (A). Values were normalized for the amount of \textit{gapdh} mRNA (means ± SEM, n = 4). Means with different superscripts are significantly different by ANOVA and Scheffe’s test (p < 0.05). Amounts of STAT1α (91 kDa) and STAT1β (84 kDa) isoforms were measured by Western blot analysis using an anti-STAT1 Ab (B, upper panel). The levels of β-actin are shown as a loading control (B, lower panel).

(C and D) Suppression of IFN-mediated transactivation activities in the \textit{vegf} 5'UTR-expressing cells. Each stable transfectant was transiently cotransfected with pTK-Renilla (as a monitor for transfection efficiency) and with a luciferase reporter plasmid containing ISRE sequence (C) or GAS sequence (D). Twenty-four hours after transfection, the cells were treated with 500 U/ml rhIFNα (C) or 500 U/ml rhIFNγ (D) for 24 h. The luciferase activity of each construct was calculated as LucF/LucR activity. Results are expressed as fold changes compared to untreated cells. Means with different superscripts are significantly different by ANOVA and Scheffe’s test (p < 0.05).

(E) Resistance to 5-FU and IFNα in the \textit{vegf} 5'UTR-expressing cells. Stable transfectants were untreated or treated with 500 U/ml rhIFNα, 80 μM 5-FU, or 80 μM 3-FU plus 500 U/ml rhIFNα for 48 h. Percentages of apoptotic cells were calculated by the TUNEL-staining method. Values are means ± SEM, n = 4. Means with different superscripts are significantly different by ANOVA and Scheffe’s test (p < 0.05).

(F) Resistance to 5-FU and IFNγ in the \textit{vegf} 5'UTR-expressing cells. As in (E), but 500 U/ml rhIFNγ was used instead of 500 U/ml rhIFNα. Values are means ± SEM, n = 4. Means with different superscripts are significantly different by ANOVA and Scheffe’s test (p < 0.05).

doi:10.1371/journal.pmed.0050094.g005
in our experimental conditions, and found that IFNα did not modify at least these IRES activities (Figure S7C). At the same time, we also confirmed that IFNα signaling, estimated using the ISRE-dependent transcription reporter assay, was not inhibited in the cells overexpressing bip, c-myc, or fgf-2 5’UTR, nor was it inhibited in the cells overexpressing the pdgf-b 5’UTR that have no IRES activity (Figure S7D). Although these results suggest that the suppression of IFN signaling may be specific for the vegf 5’UTR, further studies are needed to clarify the molecular mechanism for the suppression.

The IFNs/STAT1 signal is one of the key pathways for tumor suppression. Mice with a targeted deletion of the STAT1 or IFNc receptor develop chemically induced or spontaneous tumors more rapidly than wild-type mice [39]. Similarly, mouse embryonic fibroblasts deficient in the IFNα receptor undergo spontaneous malignant transformation, and the IFNα receptor-deficient mice develop papillomas of the skin at a high rate when treated with a chemical carcinogen [40]. In contrast, the reconstitution of STAT1 suppressed the tumorigenicity of STAT1-deficient tumor cells in vivo, demonstrating that STAT1 acts as an important tumor suppressor [41]. Indeed, the majority of cancer cell lines and primary tumors are resistant to IFNs, often through inhibition of STAT1 expression [42,43]. We propose here that the vegf mRNA 5’UTR negatively regulates expression of STAT1, leading to suppression of STAT1-dependent transcriptional activities. It has been suggested that STAT1 directly regulates DNA damage-induced apoptosis by transcriptional activation of apoptosis modulating genes, such as TRAIL, Fas, and XAF-1 [33], all of which were down-regulated in the vegf 5’-tumors (Table 1). Thus, the suppression of STAT1 may be crucial for malignant transformation of...
HCT116/vegf cells. In fact, the vegf 5'-tumors did not respond to IFNα therapy (Figure 7).

Tumor cell growth and survival may require both expression of VEGF and down-regulation of the IFN pathway. Consistent with this notion, it is known that IFNα transcriptionally suppresses VEGF expression [44,45]. In contrast, we suggest here that vegf mRNA may negatively regulate the IFN signaling pathway by repressing STAT1. This reciprocal regulation of VEGF and STAT1 is entirely feasible as these two molecules exert opposing biological functions: VEGF promotes proliferation, metastasis, and angiogenesis [1–4], and inhibits apoptosis [1]; in contrast, STAT1 negatively regulates proliferation, metastasis, and angiogenesis [41,46], and promotes apoptosis [33,47]. Tumor cells abundantly expressing vegf mRNA [1,48,49] may use this negative regulatory mechanism to gain advantages of growth and survival to escape from the IFN-mediated anti-tumor machinery. Indeed, we found here that stable knockdown of endogenous vegf mRNA by siRNA increased STAT1 expression.

At present, the precise mechanisms for the vegf 5’-UTR-mediated anti-apoptotic action are still unknown. However, our preliminary experiments showed that the vegf 5’UTR RNA might interact with double-stranded RNA-activated protein kinase (PKR) protein in the cells expressing the full-length vegf mRNA or the vegf 5’UTR RNA, which were examined by the pull-down assay using an antisense oligonucleotide probe specific for the vegf 5’UTR sequence (unpublished data). It is reported that PKR binds several structured UTRs of mRNA, such as the 5’UTR of ifn-γ mRNA [50], the 3’UTR of α-tropomyosin mRNA [51], and the 3’UTR of tnf-α mRNA [52]. PKR associates with tumor suppressor, such as IRF-1 and p53 [53] and induces apoptosis. Our preliminary data suggest that the vegf 5’UTR might interact with PKR and lead to suppression of PKR-mediated apoptotic pathway. However, this is still an unproven hypothesis, and further experiments are being carried out in our laboratory.

Recent studies have revealed that a large number of non-coding RNAs play a critical role in tumorigenesis [17]. Our findings support the concept originally put forward by Blau and colleagues [18,54] that non-coding regions of mRNAs can act as RNA regulators for tumor malignancy. There is increasing evidence that the UTR of certain mRNAs significantly suppresses the tumorigenic properties of cancer cells both in vitro and in vivo. The 3’UTR of α-tropomyosin mRNA suppresses the proliferation, invasion, and destruction of muscle tissues characteristic of rhabdomyosarcoma cells [18]. The 3’UTR of ribonucleotide reductase, a key rate-limiting enzyme in DNA synthesis, and the 3’UTR of prohibitin mRNA suppresses the proliferation, invasion, and destruction of muscle tissues characteristic of rhabdomyosarcoma cells [18]. In addition, the 5’UTR of the human c-myc P0 transcript suppresses the malignant phenotype of human breast cancer cells with decreased anchorage-independent proliferation, enhanced susceptibility to programmed cell death, and complete loss of the ability to form tumors in the intact animal [21]. To the

![Figure 7. Effects of IFNα Treatment on Growth of Xenograft Tumors](image-url)
best of our knowledge, the 5’UTR vegf mRNA is the first example of tumor-promoting UTR RNA.

VEGF protein is considered to be an important therapeutic target for cancer treatment, and anti-VEGF strategies are undergoing clinical evaluation [14]. A number of preclinical studies have demonstrated that anti-VEGF therapy alone can suppress the growth of established tumors [55,56]. Unlike these preclinical studies, anti-VEGF-specific Ab (bevacizumab) alone has not been shown to increase survival in lung and colorectal cancer patients [14]. The combined use of bevacizumab with standard chemotherapy increased overall survival in metastatic colorectal cancer [57], but did not improve the clinical outcome in metastatic breast cancers in previously treated patients [58]. Furthermore, the combination of the VEGF receptor tyrosine kinase inhibitor, vatalanib, with chemotherapy did not show an increased survival rate in metastatic colorectal cancer patients [59]. Our finding that the novel intrinsic tumor-promoting activity presents in the vegf mRNA might explain, at least in part, the inconsistencies of outcome associated with VEGF-VEGFR strategies. The present study suggests that both vegf mRNA and VEGF protein may synergistically promote the malignancy of tumor cells. Thus anti-vegf transcript therapy, such as siRNA-based gene silencing, in combination with anti-VEGF therapy might provide optimal anti-tumor effects, including inhibition of angiogenesis, blockade of tumor cell survival, and enhanced sensitivity to radiation and drug therapies.

Supporting Information

Figure S1. Effect of vegf Transcript on Susceptibility to Anti-cancer Drugs

(A and B) Knockdown of vegf transcript increases apoptosis induced by etoposide and doxorubicin. Cells untransfected (−) or transfected with the indicated plasmids for 24 h were treated with 3 μM etoposide (A), 20 nM doxorubicin (B), or rhVEGF protein (1 or 2 ng/ml) combined with each drug for 40 h. Apoptosis was evaluated by calculating the percentages of TUNEL-positive cells. Values are means ± standard error of the mean (SEM) from four independent experiments. Means with different superscripts are significantly different by ANOVA followed by Scheffe’s test (p < 0.05).

Supporting Information

Figure S2. Expression of Transfected Construct Transcripts and Their Proteins

(A) Levels of transfected vegf mRNAs and their proteins were measured by Northern and Western analyses, respectively. Northern hybridization of vegf mRNA was performed using a cDNA probe specific for vegf ORF. Levels of HA-tagged VEGF and β-actin proteins were measured using a rabbit polyclonal anti-αHA Ab and a mouse monoclonal Ab against β-actin, respectively. (B) Levels of transfected vegf 5’UTR-CAT constructs were analyzed by Northern blot analysis using a cDNA probe specific for cat. Levels of L-VEGF protein were measured by Western blotting using a rabbit polyclonal anti-L-VEGF Ab.

Supporting Information

Figure S3. Effects of vegf 5’UTR on 5-FU-Induced Apoptosis

(A–C) HCT116 cells transfected with the indicated plasmids for 24 h were treated with 80 μM 5-FU for 40 h. Cells undergoing apoptosis were detected by the TUNEL method (A and B) or the APOPercentage assay (C). Values are means ± SEM, n = 4. Means with different superscripts are significantly different by ANOVA and Scheffe’s test (p < 0.05).

Supporting Information

Figure S4. Effects of Several 5’UTR RNAs on Resistance to 5-FU in HCT116 Cells

Cells transfected with the indicated plasmid for 24 h were treated with 80 μM 5-FU for 40 h. Percentages of apoptotic cells were calculated. Values are means ± SEM, n = 4. Means with different superscripts are significantly different by ANOVA and Scheffe’s test (p < 0.05).

Supporting Information

Figure S5. Prediction of RNA Secondary Structures

The sequences of vegf 5’UTR (A) and mutated vegf 5’UTR (B) were analyzed using the mfold algorithm 3.2 of Zuker. A circle shown in (A) indicates the stem-loop located in the region that is required for the vegf 5’UTR function.

Supporting Information

Figure S6. Effects of vegf 5’UTR RNA on Resistance to 5-FU in RKO, HEK293, and AGS Cells

(A) The levels of mRNAs for VEGF receptors (vegf1, vegf2, vegfr1, and vegfr2) in the indicated cell lines were analyzed by RT-PCR. (B–D) RKO cells (B), HEK293 cells (C), and AGS cells (D) were transfected with the indicated constructs for 24 h. The cells were untreated or treated with 150 μM 5-FU for 48 h. In the case of cells transfected with the plasmid expressing full-length vegf mRNA, the cells were treated with 150 μM 5-FU plus 5 μg/ml of a monoclonal mouse anti-human VEGF165-neutralizing Ab (+ Ab) for 48 h. Then apoptosis was evaluated by the TUNEL method. Values are means ± SEM from three independent experiments. Means with different superscripts are significantly different by ANOVA followed by Scheffe’s test (p < 0.05).

Supporting Information

Figure S7. Effect of the vegf 5’UTR RNA or IFNα on Cellular IRES Activities

(A) Schematic diagram of bicistronic plasmid. The bicistronic cassette expresses the LucR in a cap-dependent manner and LucF in an IRES-dependent manner. Each IRES or hairpin control is located between the two cistrons. (B) HCT116 cells were cotransfected with 0.5 μg of the indicated vegf 5’UTR-expressing plasmid and 0.25 μg of bicistronic plasmid containing the indicated 5’UTR. The each IRES activity was calculated as LucF/LucR activity. (C) IFNα has no effect on cellular IRES activity. HCT116 cells were transfected with the indicated bicistronic plasmid for 24 h, then they were treated with 500 U/ml IFNα for 24 h. The luciferase activity of each construct was calculated as described in (B). (D) Overexpression of IRES-containing 5’UTR has no effect on IFNα signaling. HCT116 cells were cotransfected with the indicated 5’UTR-CAT-expressing plasmid and luciferase reporter plasmid containing ISRE sequence. Twenty-four hours after transfection, the cells were treated with 500 U/ml rhIFNα for 24 h. The luciferase activity of each construct was measured and calculated as LucF/LucR activity.

Supporting Information

Figure S8. Expression Levels of Transfected mRNA and Endogenous vegf mRNA, and Secretion Levels of VEGF by Stable Transfectants

(A) Levels of exogenous vegf 5’UTR-yfp mRNA (upper panel), endogenous vegf mRNA (middle panel), and gapdh mRNA (loading control, lower panel) were measured by Northern hybridization using probes specific for yfp, vegf ORF, and gapdh, respectively. The transfectants expressed similar levels of chimeric RNA. The stable transfectants of different chimeric RNAs did not significantly change the expression of endogenous vegf mRNA. (B) Amounts of VEGF165 secreted into the culture medium for 24 h were measured by ELISA assay. Values are means ± SEM, n = 4.
Figure S9. Expression of IFN Receptors and IFN-α/β mRNAs in Stable Clones
(A) The mRNA levels of IFN receptors (ifnar1, ifnar2, ifngre1, and ifngre2) and β-actin in the transfectants were measured by RT-PCR. Each stable clone showed similar levels of expression of ifnar mRNAs. (B) The levels of ifna2 mRNA in each clone were measured by quantitative RT-PCR.

Figure S10. Confirmation of the Specificity of Antibody Reactivity Cell extracts were prepared from HCT116 cells treated with 500 U/ml IFN-α (A), or transfected with plasmid expressing L-VEGF (B), or HA-tagged VEGF (C). After each extract was either untreated (−) or treated (+) with antibody peptide, as described in the Methods section, they were subjected to Western blotting.

Table S1. List of All Genes Selectively Up-regulated in vegf 5’UTR-Expressing Tumors

| Accession Numbers
| The GenBank (http://www.ncbi.nlm.nih.gov/)| accession numbers of the genes discussed in this paper are vegfa (NM_001029368), mia (NM_006533), bcl6 (NM_138931), vegf (NM005888), vegf1 (NM_013902), pahb1 (NM_050181), fax (NM_000643), and hox (NM_138764). |

The Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) raw data files discussed in this paper are accession number GSE88888.

Acknowledgments
We thank Dr. Hervé Prats and Dr. Anne-Catherine Prats (INSERM U858, France) for providing polyclonal anti-L-VEGF Ab and plasmids (p165SmPHAS3, p165SmPHAS, pVC, pSCT-CAT, pFGF2-CAT, pMyc2-CAT, pBip-CAT, pPDGFB-CAT, pRFL, pRMM2L, and pRBL).

Author contributions. KM, ST-K, and KR designed the research. KM, ST-K, MM, NY, NN, KN, and TK performed the research. KM and ST-K analyzed the data. KM, ST-K, and KR wrote the paper. KM, ST-K, MM, NY, KN, TK, and KR discussed the results and commented on the manuscript.

References
1. Ferrara N, Davis-Smyth T (1997) The biology of vascular endothelial growth factor. Endocr Rev 18: 4–25.
2. Dvorak HF (2002) Vascular permeability factor/vascular endothelial growth factor. Endocr Rev 18: 4–25.
3. bergers G, benjamin LE (2003) Tumorigenesis and the angiogenic switch. Cancer Res 63: 5251–5256.
4. Han V, Villegas C, Huang A, Wright JA (1996) Suppression of malignancy by the 5’ untranslated regions of ribonucleotide reductase R1 and R2 messenger RNAs. Cancer Res 56: 4366–4369.
5. Blume SW, Miller DM, Guercello V, Shrestha K, Meng Z, et al. (2003) Inhibition of tumorigenicity by the 5’-untranslated RNA of the human c-myc p50 transcript. Exp Cell Res 298: 131–142.
6. Hsu I, Crenacier L, Audigier S, Genesis MC, Prats AC, et al. (1998) Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. Mol Cell Biol 18: 6178–6190.
7. Akiri G, Nahari D, Finkelstein Y, Le SY, Elroy-Stein O, et al. (1998) Regulation of vascular endothelial growth factor (VEGF) expression is mediated by internal initiation of translation and alternative initiation of transcription. Oncogene 17: 227–236.
8. Hsu I, Borens S, Bresson D, Crenacier L, Prats H (2001) New vascular endothelial growth factor isoform generated by internal ribosome entry site-driven CUG translation initiation. Mol Endocrinol 15: 2197–2210.
9. Teshima-Kondo S, Kondo K, Prado-Lourence L, Gonzalez-Herrera IG, Rokutan K, et al. (2004) Hyperglycemia upregulates translation of the fibroblast growth factor 2 mRNA in mouse aorta via internal ribosome entry site. FASEB J: 03–11168fje.
10. Borens S, Bouard M, Hichet C, Zanibellato I, Lacovoni JS, et al. (2004) Control of the vascular endothelial growth factor internal ribosome entry site (IRES) activity and translation initiation by alternatively spliced coding sequences. J Biol Chem 279: 18717–18726.
11. Martinet Y, Le Bec C, Monbrun L, Allo V, Chiu IM, et al. (2004) Internal ribosome entry site structural motifs conserved among mammalian fibroblast growth factor 1 alternatively spliced mRNAs. Mol Cell Biol 24: 7622–7635.
12. Zucker M (2003) Fmold web server for nucleic acid folding and hybridization prediction. Nucl Acids Res 31: 3406–3415.
13. Kawahara T, Kuwano Y, Teshima-Kondo S, Takeya R, Sumimoto H, et al. (2004) Role of nicotinamide adenine dinucleotide phosphate oxidase 1 in oxidative burst response to Toll-like receptor 5 signaling in large intestinal epithelial cells. J Immunol 172: 3051–3058.
14. Baird SD, Turkotte M, Korneluk RG, Holick M (2006) Searching for IRES RNA. IRE 12: 1755–1785.
15. Han B, Dong Z, Zhang JT (2003) Tight control of platelet-derived growth factor B-cis expression by interplay between the 5’-untranslated region sequence and the major upstream promoter. J Biol Chem 278: 40983–40993.
16. Prats AC, Prats H (2002) Translational control of gene expression: role of IRE5s and consequences for cell transformation and angiogenesis. Prog Nucleic Acid Res Mol Biol 72: 367–413.
17. Battle TE, Frank DA (2002) The role of STAT in apoptosis. Curr Med Mol Med 2: 381–392.
18. Stark GR, Kerr IM, Williams BR, Silverman KH, Schreiber RD (1998) How cells respond to interferons. Annu Rev Biochem 67: 227–245.
19. Wadler S, Schwartz EL (1990) Antineoplastic activity of the combination of Adriamycin and interferon and cytotoxic agents against experimental and human malignancies. Mt Sinai J Med 71: 361–367.
38. Dhar D, Roy S, Das S (2007) Translational control of the interferon regulatory factor 2 mRNA by IRES element. Nucleic Acids Res 35: 5409–5421.

39. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, et al. (1998) Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A 95: 7556–7561.

40. Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, et al. (2003) Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature 424: 516–525.

41. Huang S, Bucana CD, Van Arsdall M, Fidler IJ (2002) Stat1 negatively regulates angiogenesis, tumorigenicity and metastasis of tumor cells. Oncogene 21: 2504–2512.

42. Wong LH, Krauer KG, Hatzinisiriou I, Estcourt MJ, Hersey P, et al. (1997) Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. J Biol Chem 272: 28779–28783.

43. Abril E, Real LM, Serrano A, Jimenez P, Garcia A, et al. (1998) Unresponsiveness to interferon associated with STAT1 protein deficiency in a gastric adenocarcinoma cell line. Cancer Immunol Immunother 47: 113–120.

44. von Marschall Z, Scholz A, Cramer T, Schafer G, Schirner M, et al. (2003) Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis. J Natl Cancer Inst 95: 457–448.

45. Rosewicz S, Detjen K, Scholz A, von Marschall Z (2004) Interferon-alpha: regulatory effects on cell cycle and angiogenesis. Neuroendocrinology 80 Suppl 1: 85–93.

46. Battle TE, Lynch RA, Frank DA (2006) Signal transducer and activator of transcription 1 activation in endothelial cells is a negative regulator of angiogenesis. Cancer Res 66: 3649–3657.

47. Sironi JJ, Ouchi T (2004) STAT1-induced apoptosis is mediated by Caspases 2, 3, and 7. J Biol Chem 279: 4066–4074.

48. Chan AS, Leung SY, Wong MP, Yuen ST, Cheung N, et al. (1998) Expression of vascular endothelial growth factor and its receptors in the anaplastic progression of astrocytoma, oligodendrogloma, and ependymoma. Am J Surg Pathol 22: 816–826.

49. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, et al. (1995) Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res 55: 4727–4735.

50. Ben-Assouli Y, Banai Y, Pel-Or Y, Shir A, Kaempfer R (2002) Human interferon-gamma mRNA autoregulates its translation through a pseudo-knot that activates the interferon-inducible protein kinase PKR. Cell 108: 221–232.

51. Davis S, Watson JC (1996) In vitro activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3' untranslated regions of human alpha-tropomyosin. Proc Natl Acad Sci U S A 93: 508–513.

52. Osman F, Jarrous N, Ben-Assouli Y, Kaempfer R (1999) A cis-acting element in the 3'-untranslated region of human TNF-alpha mRNA renders splicing dependent on the activation of protein kinase PKR. Genes Dev 13: 3280–3293.

53. Garcia MA, Gil J, Ventoso I, Guerra S, Domingo E, et al. (2006) Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol Mol Biol Rev 70: 1032–1060.

54. Rastinejad F, Blau HM (1993) Genetic complementation reveals a novel regulatory role for 3' untranslated regions in growth and differentiation. Cell 72: 903–917.

55. Gerber HP, Ferrara N (2005) Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. Cancer Res 65: 671–680.

56. Presta LG, Chen H, O’Connor SJ, Chisholm V, Meng YG, et al. (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res 57: 4593–4599.

57. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, et al. (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350: 2335–2342.

58. Miller KD, Chap Ll, Holmes FA, Cobl. Leigh MA, Marcom PK, et al. (2005) Randomized phase III trial of capecitabine compared with bevacizumab plus capcitabine in patients with previously treated metastatic breast cancer. J Clin Oncol 23: 792–799.

59. Los M, Roodhart JML, Voest EE (2007) Target practice: lessons from phase III trials with bevacizumab and vatalanib in the treatment of advanced colorectal cancer. Oncologist 12: 443–450.
Background Normally, throughout life, cell division (which produces new cells) and cell death are carefully balanced to keep the body in good working order. But sometimes cells acquire changes (mutations) in their genetic material that allow them to divide uncontrollably to form cancers—disorganized masses of cells. When a cancer is small, it uses the body’s existing blood supply to get the oxygen and nutrients it needs for its growth and survival. But, when it gets bigger, it has to develop its own blood supply. This process is called angiogenesis. It involves the release by the cancer cells of proteins called growth factors that bind to other proteins (receptors) on the surface of endothelial cells (the cells lining blood vessels). The receptors then send signals into the endothelial cells that tell them to make new blood vessels. One important angiogenic growth factor is “vascular endothelial growth factor” (VEGF). Tumors that make large amounts of VEGF tend to be more abnormal and more aggressive than those that make less VEGF. In addition, high levels of VEGF in the blood are often associated with poor responses to chemotherapy, drug regimens designed to kill cancer cells.

Why Was This Study Done? Because VEGF is a key regulator of tumor development, several anti-VEGF therapies—drugs that target VEGF and its receptors—have been developed. These therapies strongly suppress the growth of tumor cells in the laboratory and in animals but, when used alone, are no better at increasing the survival times of patients with cancer than standard chemotherapy. Scientists are now looking for an addition, high levels of VEGF in the blood are often associated with poor responses to chemotherapy, drug regimens designed to kill cancer cells.

What Did the Researchers Do and Find? The researchers first used a technique called small interfering (si) RNA knockdown to stop VEGF expression in human colon cancer cells growing in dishes. siRNAs are short RNAs that bind to and destroy specific mRNAs in cells, thereby preventing the translation of those mRNAs into proteins. The treatment of human colon cancer cells with vegf-targeting siRNAs made the cells more sensitive to chemotherapy-induced apoptosis (a type of cell death). This sensitivity was only partly reversed by adding VEGF to the cells. By contrast, cancer cells engineered to make more vegf mRNA had increased resistance to chemotherapy-induced apoptosis. Treatment of these cells with an antibody that inhibited VEGF function did not completely block this resistance. Together, these results suggest that both vegf mRNA and VEGF protein have anti-apoptotic effects. The researchers show that the anti-apoptotic activity of vegf mRNA requires a noncoding part of the mRNA called the 5' UTR, and that whereas human colon cancer cells expressing this 5' UTR form tumors in mice, cells expressing a mutated 5' UTR do not. Finally, they report that the expression of several pro-apoptotic genes and of an anti-tumor pathway known as the interferon/STAT1 tumor suppression pathway is down-regulated in tumors that express the vegf 5' UTR.

What Do These Findings Mean? These findings suggest that some cancer cells have a survival system that is regulated by vegf mRNA and are the first to show that a 5' UTR of mRNA can promote tumor growth. They indicate that VEGF and its mRNA work together to promote their development and to increase their resistance to chemotherapy drugs. They suggest that combining therapies that prevent the production of vegf mRNA (for example, siRNA-based gene silencing) with therapies that block the function of VEGF might improve survival times for patients whose tumors overexpress VEGF.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0050094.

- This study is discussed further in a PLoS Medicine Perspective by Hughes and Jones
- The US National Cancer Institute provides information about all aspects of cancer, including information on angiogenesis, and on bevacizumab, an anti-VEGF therapeutic (in English and Spanish)
- CancerQuest, from Emory University, provides information on all aspects of cancer, including angiogenesis (in several languages)
- Cancer Research UK also provides basic information about what causes cancers and how they develop, grow, and spread, including information about angiogenesis
- Wikipedia has pages on VEGF and on siRNA (note that Wikipedia is a free online encyclopedia that anyone can edit; available in several languages)