Down-regulation of Vascular Endothelial Growth Factor in a Human Colon Carcinoma Cell Line Transfected with an Antisense Expression Vector Specific for c-src*

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Vascular endothelial growth factor (VEGF) is implicated in the angiogenesis of human colon cancer. Recent evidence suggests that factors that regulate VEGF expression may partially depend on c-src-mediated signal transduction pathways. The tyrosine kinase activity of Src is activated in most colon tumors and cell lines. We established stable subclones of the human colon adenocarcinoma cell line HT29 in which Src expression and activity are decreased specifically as a result of a transfected antisense expression vector. This study determined whether VEGF expression is decreased in these cell lines and whether the smaller size and reduced growth rate of antisense vector-transfected cell lines in vivo might result, in part, from reduced vascularization of tumors. Northern blot analysis of these cell lines revealed that VEGF mRNA expression was decreased in proportion to the decrease in Src kinase activity. Under hypoxic conditions, cells with decreased Src activity had a <2-fold increase in VEGF expression, whereas parental cells had a >50-fold increase. VEGF protein in the supernatants of cells was also reduced in antisense transfectedants compared with that from parental cells. In nude mice, subcutaneous tumors from antisense transfectants showed a significant reduction in vascularity. These results suggest that Src activity regulates the expression of VEGF in colon tumor cells.

Neovascularization is a critical requirement for tumor growth and metastasis formation. Numerous angiogenic factors that regulate this process have been identified (1). Among them is vascular endothelial growth factor (VEGF), which has been implicated in the neovascularization of a wide variety of tumors (2–8). VEGF, also known as vascular permeability factor, is a 36–45-kDa dimeric glycoprotein that has been identified in the conditioned media from numerous cell lines and that is expressed in many tumors (2–12). The gene for this angiogenic factor has ~20% homology to platelet-derived growth factor and ~50% homology to placenta growth factor (13, 14).

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The abbreviations used are: VEGF, vascular endothelial growth factor; PCR, polymerase chain reaction; HT29-P cells, HT29 parental cells; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT, reverse transcription.

Experimental Procedures

Construction of c-src Expression Vectors—A construct spanning the translation start site of c-src was generated by annealing two primers,
In a buffer consisting of 0.1% Triton X-100, 150 mM NaCl, and 10 mM Cowan strain; Calbiochem) for 30 min. Pellets were washed three times purified by agarose gel electrophoresis, recovered using a QIAEX gel CA). Following prehybridization, the membranes were probed for VEGF gan, UT). Stable HT29 subclones expressing c- were also maintained under these conditions, except that the medium serum-free medium for 6 h with 100 μM sodium orthovanadate in 20 mM HEPES. After 10 min at 25 °C, reactions were terminated by adding SDS sample buffer. The mixture was heated to 100 °C for 5 min, and the samples were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and radioactive proteins were detected by autoradiography

Transfection—HT29 parental (HT29-P) cells were grown to 70% confluence under the conditions described below. Cells were transfected in serum-free medium for 6 h with 100 μg of LipofectAMINE (Life Technologies, Inc.) and 16 μg of plasmid DNA. The medium was then replenished with medium supplemented with 250 μg/ml G418 (Life Technologies, Inc.). Colonies resistant to G418 were expanded, and the resulting clones were screened for expression and activity of Src, as described below.

Cell Culture—HT29 cells, derived from a colon adenocarcinoma (27), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with Earle’s salts and 2 mM glutamine (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). HT29 subclones expressing either “sense” (HT29-S8 and HT29-S33) or “antisense” (HT29-AS15 and HT29-AS33) constructs were also maintained under these conditions, except that the medium was supplemented with 250 μg/ml G418. For experiments examining the effect of tumor cell-conditioned medium on endothelial cell proliferation, HT29-P cells and subclones were grown to 100% confluence in DMEM supplemented with 1% FBS for 24 h.

Immunoprecipitation and Immune Complex Kinase Assays—Prior to lysis, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS). Detergent lysates were made in a standard radioimmuno precipitate assay buffer. Cells were homogenized and clarified by centrifugation at 10,000 × g. Cell lysates (250 μg of protein) were reacted for 2 h with either monoclonal antibody 327 (Oncogene Science Inc., Cambridge, MA) for immunoprecipitation of Src or monoclonal antibody 1H7 (Wako Bioproducts, Richmond, VA) for immunoprecipitation of Yes. Immune complexes were formed by incubation with 6 μg of rabbit anti-mouse IgG (Organon Teknika, Durham, NC) for 1 h and then with 50 μl of 10% (v/v) Formalin-fixed Pansorbin (Staphylococcus aureus, Cowan strain; Calbiochem) for 30 min. Pellets were washed three times in a buffer consisting of 0.1% Triton X-100, 150 mM NaCl, and 10 mM sodium orthovanadate in 20 mM HEPS. After 10 min at 25 °C, reactions were terminated by adding SDS sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 8% polyacrylamide gels, and radioactive proteins were detected by autoradiography.

Immunoblotting—Clariﬁed cell lysates (250 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and electrobotted onto nitrocellulose membranes (Schleicher & Schuell) using standard procedures (22). Membranes were blocked with 5% skimmed milk in PBS and then incubated with anti-Src or anti-Yes antibodies at a 1:1000 dilution followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. Specific binding of antibody was determined using the ECL detection system (Amersham Corp.).

mRNA Extraction and Northern Blot Analysis—Polyadenylated mRNA was extracted from tumor cells grown under confluent conditions in culture using the TRI reagent kit (Molecular Research Center, Inc. Cincinnati, OH). Twenty μg of total RNA was fractionated on 1% denaturing formaldehyde-agarose gels, transferred to a Hybond nylon membrane (Amersham Corp.) by capillary elution, and UV-cross-linked at 120,000 μCi/cm² using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Following prehybridization, the membranes were probed for VEGF and glyceraldehyde-3-phosphate dehydrogenase. Each cDNA probe was purified by agarose gel electrophoresis, recovered using a QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, CA), and radiolabeled by the random priming method using a kit (Amersham Corp.) that utilizes [α-32P]dCTP (Amersham Corp.). Nylon filters were washed at 65 °C with 30 mM NaCl, 3 mM sodium citrate (pH 7.2), and 0.1% (w/v) SDS. Autoradiography was then performed.

A 1.28-kilobase glyceraldehyde-3-phosphate dehydrogenase probe (American Type Culture Collection, Rockville, MD) was used as an internal control. The VEGF probe, a 204-bp pair fragment of human VEGF cDNA, was a gift from Dr. Brygida Berse (Harvard Medical School, Boston, MA) (17).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction—cDNA was synthesized from total RNA extracted from HT29-P cells and antisense cell lines (HT29-AS33 and HT29-AS15) by reverse transcription (RT) in 20 μl of reaction mixture containing 0.5 μg of random primers (Life Technologies, Inc.), 200 units of SuperScript™ RNase H− reverse transcriptase (Life Technologies, Inc.), 0.1 μg of mRNA, 4 μl of 5 × RT buffer (375 mM KCl, 250 mM Tris-HCl (pH 8.3 at room temperature), and 15 mM MgCl2), 5 μM dithiothreitol, 0.1 mM each dNTP, 20 units of Taq polymerase (Promega, Madison, WI), 50 pmol/μl VEGF primers, and 50 pmol/μl β-actin primers. The mixture was overlaid with mineral oil and then amplified with a TemTronic DNA amplification system using the following amplification profile: an initial denaturation at 94 °C for 5 min, denaturation at 93 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for varying cycle numbers, and a final elongation step at 72 °C for 10 min. The PCR products were then electrophoresed on a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide, visualized under UV light and photographed with Polaroid Type 55 positive/negative film. Specific amplification was determined by the size of the product on the gel relative to that of known DNA molecular weight marker V (Boehringer Mannheim). The amount of PCR product was determined by measuring the density of the bands on the negative film with a densitometer.

RT products were amplified by PCR using specific primers for β-actin (design of primers based on published sequence (GenBank™/EMBL Data Bank accession number M10277)), sense, 5′-ACACTTGCCGC-ATCTACGAGG-3′; and antisense, 5′-AGGGGCCGAGCTCGTACTACT-3′) and for VEGF (28), sense, 5′-CACATAGGAGATGACGTC-3′; and antisense, 5′-CCGCCTCGGCTGTTCAT-3′) in separate reactions for 18–28 cycles under the above conditions, and the products were quantitated by densitometry. Curves depicting product quantity relative to the number of PCR cycles were generated, and the PCR cycle number that generated product quantities representing the up-slope of the curve was selected for quantitative PCR of each specific set of primers. Semiquantitative PCR was performed for β-actin (22 cycles) and VEGF (22 cycles).

Densitometric Quantitation—Protein kinase activities and VEGF mRNA expression were quantitated by densitometry of autoradiograms using the ImageQuant™ software program (Molecular Dynamics, Inc., Sunnyvale, CA) in the linear range of the film. Semiquantitative PCR products were separated by agarose gel electrophoresis, and after ethidium bromide staining, they were exposed to UV light and photographed with Polaroid Type 55 positive/negative film. The positive bands on the film were quantitated by densitometry using β-actin as an internal control.

Determination of VEGF Protein Levels in Cell Supernatants—For these determinations, equal densities as opposed to equal numbers of cells were chosen because of previous experiments in colon tumor cells demonstrating that density affects VEGF expression (29, 30). Thus, HT29-P cells were seeded at 20 × 10⁶, HT29-AS33 cells at 25 × 10⁶, and HT29-AS15 cells at 40 × 10⁶ cells/100-mm tissue culture plate to compensate for size differences in these cells. Cells were grown for 24 h in DMEM/F-12 (1:1 mixture) supplemented with 10% FBS, washed three times with PBS, and changed to 7.5 ml of DMEM/F-12 (1:1 mixture) supplemented with 1% FBS. Cell supernatants were collected, filtered, and stored at −12 °C, and concomitantly, cell pellets were harvested by trypsinization, and cell number was determined. The amount of VEGF protein in the supernatant was determined with an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. VEGF was expressed as pg of VEGF protein/10⁶ cells/24 h.

Effects of Hypoxia on VEGF Expression in HT29-P Cells and Transfectants—HT29-P and HT29-AS15 cells were grown to confluence in standard medium. The medium was changed, and cells were then transfected, according to a hypoxia protocol available from The New York Instru
mants Co., Redfield, NY). Control cultures were harvested just prior to hypoxic exposure (t = 0), and protein and RNA were harvested. Cultures were then incubated for 4, 6, and 24 h, and cells were harvested at these time points for protein and RNA analysis.

Quantiﬁcation of Marine Tumor Microvasculature—Cells from clones to be tested were grown in tissue culture to log phase (~70% confluent),
trypsinized, and counted with the aid of a hemocytometer. 1E10541 tumors were compared by two-tailed, unpaired Student’s t-test.

dried on a 60 °C hot plate. Because heterogeneity in vascularization

sections of tissues previously frozen in O.C.T. compound were fixed

peroxidase

aceton. The sections were washed three times, and endogenous peroxi-

sections of tissues previously frozen in O.C.T. compound were fixed

in Src and Yes as described under “Experimental Procedures.”

A. Autophosphorylation

B. Enolase Phosphorylation

C. Protein Level

FIG. 1. Expression and activity of Src and Yes in HT29-P cells, HT29-S8 and HT29-S20 sense transfected subclones, and HT29-AS33 and HT29-AS15 antisense transfected subclones. Stable HT29 subclones were isolated, and cell lysates were subjected to immunoblotting and immune complex kinase assays for Src and Yes as described under “Experimental Procedures.” Row A, autophosphorylation of Src and Yes; row B, phosphorylation of the exogenous substrate enolase; row C, relative levels of Src and Yes.

decrease in VEGF mRNA expression correlated with the alteration in Src kinase activity: the transfected cell line that exhibited the greatest decrease in Src kinase activity (HT29-AS15) also exhibited the greatest decrease in VEGF mRNA expression.

Effect of Src on Expression of Various Isoforms of VEGF—Semi-quantitative PCR of the various isoforms of VEGF demonstrated a decrease in overall VEGF expression in both HT29-AS33 and HT29-AS15 cells (Fig. 3). The most abundant isoform expressed was VEGF-165. There was a relatively equal decrease in the expression of all VEGF isoforms in the antisense clones. The overall decrease in VEGF expression in the antisense clones by semi-quantitative RT-PCR confirmed our findings by Northern blotting.

Determination of VEGF Protein Levels in Cell Supernatants—To examine directly the amount of VEGF protein produced in the various clones, supernatants from cultures grown to identical confluences were harvested, and VEGF expression was determined by enzyme-linked immunosorbent assay. A decrease in VEGF protein was observed in the supernatant of cells with decreased Src activity (HT29-AS15 and HT29-AS33) compared with that in control cell lines (HT29-P, HT29-S8, and HT29-S20).

Effects of Hypoxia on VEGF Expression in HT29-P Cells and Transfectants—The above results demonstrate that the expression of Src in colon tumor cells leads to the constitutive expression of VEGF, a critical factor in tumor cell growth. However, neovascularization of tumors also results from local hypoxia as the tumor volume exceeds its blood supply. As hypoxic stimulation of VEGF in fibroblasts has been associated with activation of Src protein-tyrosine kinase, we examined the response to hypoxia in HT29-P and transfected subclones. Cells were grown to equal densities, transferred to hypoxic chambers,
harvested at specific times, and processed for RNA and protein as described under “Experimental Procedures.” For these studies, HT29 parental cells were compared with HT29-AS15, the antisense transfectant expressing the least Src. The ability of hypoxia to induce VEGF mRNA in these clones is compared in Fig. 5. VEGF mRNA was markedly induced in a time-dependent manner in HT29-P cells, with a >50-fold increase observed after 24 h. In contrast, a <2-fold increase in VEGF mRNA expression was observed in HT29-AS15 cells. These results demonstrate that not only is the constitutive production of VEGF reduced by lowering Src expression, the ability of hypoxia to induce further expression of VEGF is severely impaired. To confirm that induction of VEGF mRNA by hypoxia was Src-dependent, the expression and activity of Src were compared in these clones under identical conditions. In HT29-P cells, Src kinase activity was stimulated in a time-dependent manner, with a maximum stimulation of 4.5-fold occurring 4 h after the onset of hypoxic conditions, whereas only a 1.1-fold increase in activity was observed in HT29-AS15 cells under identical conditions (data not shown).

**Effect of Src on in Vivo Tumor Vascularity**—For this determination, HT29-P and HT29-AS15 cells were implanted in the subcutis of nude mice as described under “Experimental Procedures.” Tumors were harvested when they were ~1 cm in diameter. Mean vessel counts were determined by counting the most vascularized areas of the respective tumors, after immunostaining with rat CD31 antibody, as described. In tumors grown from HT29-AS15 cells, vessel counts were significantly reduced relative to those tumors grown from HT29-P cells (31.8 ± 2.8 versus 53.0 ± 1.5 (mean ± S.E.), respectively; p < 0.0001) (Fig. 6). These results demonstrate that reduction in constitutive and inducible VEGF production in c-src antisense transfected clones corresponds with decreased vascularization of the tumor. However, we cannot rule out that other factors, such as different growth rates and metabolic requirements, contribute to changes in tumor vascularization.

**DISCUSSION**

Angiogenesis is an essential step in tumor growth and metastasis, and this process is driven by the balance of positive and negative effector molecules (31). In human colon cancers and established cell lines, VEGF appears to be the angiogenic factor most closely associated with neovascularization. Several lines of evidence implicate VEGF production as important to colon tumorigenicity and/or metastatic potential. Increases in VEGF are observed in primary tumors relative to normal tissue (7, 8) and in metastatic tumors relative to nonmetastatic tumors (2, 3). Using colon tumor cell lines in mouse models, Warren et al. (16) found that a VEGF antibody greatly inhibits the growth of subcutaneous xenografts and the number and size of experimental metastases. These results suggest that the production of VEGF is important to colon tumor cell growth and progression. However, other factors have also been implicated in the process of colon cancer angiogenesis. Subcutaneous injection into nude mice of several HT29 subclones with varying degrees of differentiation has demonstrated a positive correlation between vessel counts and the ability of the cells to express platelet-derived growth factor-B in vitro (32). Thus, in different subclones from even the same cell line, different angiogenic factors might be important to induction of neovascularization.

The signal transduction pathways by which VEGF is induced remain to be elucidated fully. However, recent experiments have implicated specific activation of the protein-tyrosine kinase activities of the src family of proto-oncogenes as important in the induction of VEGF. Mukhopadhyay et al. (18) examined the role of activated c-src in hypoxic induction of VEGF. Hypoxia was found to increase VEGF expression in U87 glioma cells and 293 kidney cells, and this induction was inhibited by genistein, an inhibitor of tyrosine kinases. When the effects of hypoxia on src family protein-tyrosine kinases were analyzed, Src activity, but not Yes or Fyn activity, was increased. Transfection of v-src into U87 glioma cells and 293 kidney cells also increased the hypoxic induction of VEGF, whereas transfection of cells with a dominant-negative form of c-src partially inhibited VEGF induction. To examine the potential role of Src in hypoxic induction of VEGF, fibroblasts derived from mice with a c-src disruption were employed. These cells exhibited a 50–70% decrease in hypoxic induction of VEGF mRNA, with a compensatory activation of Fyn. These results strongly suggest a specific role for Src in promoting angiogenesis. In addition, Rak et al. (33) demonstrated that transfection with v-src increased VEGF expression and induced tumorigenicity in an immortalized rat intestinal epithelial cell line. Conditioned media from cells transfected with v-src were able to increase endothelial cell proliferation, and this increase was blocked by the addition of antibodies to VEGF. These results suggest that activation of Src is important to VEGF induction in several cell systems. In contrast, in Hep3b hepatoma cells, hypoxic induction of VEGF did not appear to be Src-dependent (25).

Several laboratories have demonstrated that the specific activity of Src is greatly increased in the majority of colon tumors.
and cell lines (22, 23, 34). The importance of this activation to colon cell tumorigenicity has been uncertain. Recently, we developed cell lines from HT29-P cells with reduced c-src expression and activity by transfection with an antisense expression vector specific for c-src (26). These cells proliferate more slowly in vitro, and tumorigenicity in nude mice is reduced (26). Furthermore, tumors from antisense transfec-
tants were limited in size, even after >1 year of growth in mice. Therefore, the present study was undertaken to determine whether limited growth, in part, resulted from reduced expres-
and/or induction of VEGF in the antisense transfected cell lines. The results in this paper demonstrate that reduction of Src expression and activity, but not those of the related Yes, directly corresponds to decreased levels of VEGF mRNA and decreased biologic activity of VEGF. Furthermore, fewer blood vessels are observed in tumors that form after injection of the cell lines transfected with the Src antisense constructs. These results suggest that in addition to the role of Src in regulating cell proliferation, activation of Src in colon tumorigenesis may promote tumor growth via induction of VEGF, which in turn induces neovascularization. Further evidence for this possibility was derived from studies on the effect of hypoxia on induc-
tion of VEGF mRNA expression in HT29 parental cells and the antisense transfected clone HT29-AS15. In the HT29 parental cells, a 50-fold induction of VEGF mRNA was observed. This induction far exceeded that observed by Mukhopadhyay et al. (18) in fibroblasts, where hypoxia resulted in a maximum 4.5-fold induction of VEGF mRNA. Our results therefore suggest that the higher expression and specific activity of Src kinase in colon tumor cells can augment the ability of hypoxia to induce VEGF. However, in HT29-AS15 cells, in which c-src expression has been reduced 4-fold, the ability of hypoxia to induce VEGF mRNA is severely impaired. These results suggest that in this colon tumor cell system, Src kinase regulates both inducible and constitutive pathways leading to VEGF production. Fur-
ther confirmation of the ability of Src kinase to regulate induc-
bile VEGF expression was derived from a study of Fleming et al. (30), in which the ability of cell density to up-regulate VEGF expression was diminished in the antisense transfected clones relative to HT29 parental cells.

While our results suggest that constitutive Src activation may be a primary pathway leading to production of angiogenic factors in colon cancer, other pathways resulting from genetic changes in colon cancer may also be responsible for the induc-
tion of angiogenic factors. For example, 40–50% of colon tu-
mors are known to have activating ras mutations (35, 36), and previous studies have demonstrated that Ras activation is suf-
cient to induce VEGF (33, 37). Additionally, in 293 kidney cells, induction of the promoter of VEGF by transfection of v-src is inhibited by overexpression of wild-type p53 (21). Approxi-
mately 50–60% of colon cancers exhibit p53 mutations (38), whereas >80% contain activated Src kinase. The relationship between p53 status, Src activation, and VEGF production in colon tumor cells thus requires further study. Nevertheless, the data presented in this paper suggest an important role for Src
activation and VEGF expression in the tumorigenicity, progression, and vascularization of human colon tumors.

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