Animal Model

Generation of a Syngeneic Mouse Model to Study the Effects of Vascular Endothelial Growth Factor in Ovarian Carcinoma

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Vascular endothelial growth factor (VEGF) performs multifaceted functions in the tumor microenvironment promoting angiogenesis, suppressing anti-tumor immune response, and possibly exerting autocrine functions on tumor cells. However, appropriate syngeneic animal models for *in vivo* studies are lacking. Using retroviral transfection and fluorescence-activated cell sorting, we generated a C57BL6 murine ovarian carcinoma cell line that stably overexpresses the murine VEGF164 isoform and the enhanced green fluorescent protein. VEGF164 overexpression dramatically accelerated tumor growth and ascites formation, significantly enhanced tumor angiogenesis, and substantially promoted the survival of tumor cells *in vivo*. *In vitro*, VEGF164 overexpression significantly enhanced cell survival after growth factor withdrawal and conferred resistance to apoptosis induced by cis-platin through an autocrine mechanism. VEGF/green fluorescent protein-expressing tumors were not recognized by the adaptive immune system. After vaccination, a specific anti-tumor T-cell response was detected, but tumor growth was not inhibited. This engineered murine carcinoma model should prove useful in the investigation of the role of VEGF in modulating the tumor microenvironment and affecting the complex interactions among angiogenesis mechanisms, anti-tumor immune mechanisms, and tumor cell behavior at the natural state or during therapy in ovarian carcinoma. (*Am J Pathol* 2002, 161:2295–2309)

Vascular endothelial growth factor (VEGF) plays a critical role in angiogenesis during embryonic development, growth, wound healing, and the cyclic regeneration of female reproductive tissues.^{1,2} Solid tumor growth also depends on the development of new blood supply, which is supported by VEGF.^{1,3} Human VEGF exists as five isoforms, VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206, produced by alternative splicing of a primary transcript from a single VEGF gene.⁴ Similarly, murine VEGF exists as at least four isoforms, VEGF120, VEGF144, VEGF164, and VEGF188, respectively.^{5,6} The larger molecular weight isoforms, VEGF206 and VEGF189 are cell-associated, whereas the smaller isoforms VEGF165 and VEGF121 are secreted.⁷ The different isoforms may be differentially regulated and may exert different functions during development.^{8,9} VEGF164 has been shown to be primarily responsible for tumor angiogenesis in mouse models.^{10,11} Two shared tyrosine-kinase receptors, VEGF receptor-1 (flt-1) and VEGF receptor-2 (KDR/flk-1)^{12,13} and two co-receptors, neuropilin-1 and neuropilin-2^{14,15} mediate the effects of VEGF.

Accumulating evidence indicates that VEGF exerts multifaceted functions in tumors and its overexpression of VEGF by tumors has been correlated with poor outcome.^{16–21} VEGF receptors have been detected in a variety of tumor cells^{22–29} and VEGF promotes the growth, proliferation, survival and/or migration of tumor cells.^{24,26,27,30–32} In addition, VEGF exerts a local intra-tumoral as well as systemic immune suppression by inhibiting the differentiation and maturation of dendritic

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cells (DCs),^{33,34} a process that is necessary for tumor antigen presentation and stimulation of anti-tumor T cells. Although the angiogenic effects of VEGF have been thoroughly documented in animal models, the role of VEGF in modulating the tumor microenvironment and affecting the complex interactions among angiogenesis mechanisms, anti-tumor immune mechanisms, and tumor cell behavior at the natural state or during tumor therapy remains elusive. Such studies necessitate dependable animal models fulfilling specific requirements. First, the growth of experimental tumors needs to be angiogenesis-dependent. Second, a syngeneic model is necessary to study molecular and cellular interactions in the immunocompetent host. Furthermore, experimental tumors need to mimic human tumors in their immunological behavior, namely they should harbor potential antigens but be able to evade immune recognition and/or attack. Finally, to study the direct effects of VEGF, tumor cells should be susceptible to the autocrine effects of VEGF. Ideally, an animal model should recapitulate a human tumor in which VEGF may exert important biological effects.

Epithelial ovarian cancer is the most frequent cause of gynecological cancer-related mortality and accounts for ~15,000 deaths in the United States yearly.³⁵ Increased levels of tumor VEGF have been reported in invasive ovarian carcinoma compared to benign tumors or tumors of low malignant potential.^{36–38} Besides tumor growth, VEGF has been implicated in the pathogenesis of ovarian cysts and ascites,^{39,40} where markedly elevated levels of VEGF are seen.³⁸ Serum levels of VEGF are 10-fold higher in patients with advanced ovarian cancer compared to healthy controls.³⁸ Importantly, increased serum and/or tumor levels of VEGF have been associated with poor clinical outcome.^{16,41,42} Finally, ovarian carcinoma cells express the VEGF receptor-2 KDR/flk-1.²² Ovarian carcinoma therefore offers important opportunities to investigate the multifaceted functions of VEGF.

In the present study, we report the generation of a syngeneic model of ovarian carcinoma in the C57BL6 mouse overexpressing murine VEGF164. This engineered murine model offers a valuable tool to investigate the effects of VEGF in the biology of ovarian carcinoma and its response to therapy in the immunocompetent host. This model is also valuable for the investigation of tumor-host interactions and anti-tumor immune mechanisms as well as cellular and molecular mechanisms underlying tumor spread and metastasis in ovarian cancer.

Materials and Methods

Cell Culture and Reagents

ID8, a cell line derived from spontaneous *in vitro* malignant transformation of C57BL6 mouse ovarian surface epithelial cells, was a generous gift from Dr. Paul F. Terranova, University of Kansas.⁴³ ID8 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 4% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml sodium

selenite (Roche, Indianapolis, IN) in a 5% CO₂ atmosphere at 37°C. In some experiments, ID8 cells were cultured in serum-free and insulin-free media overnight, or in serum-free conditions in the presence or absence of *cis*-platin for 24 hours. Dose-response experiments were performed to define the sensitivity of ID8 cells to the drug, and final experiments were performed at a 50 μ mol/L concentration. Select cells treated with *cis*-platin were exposed to recombinant murine VEGF (100 ng/ml; R&D Systems, Minneapolis, MN). All experiments were repeated three times. All reagents were analytical grade and purchased from Sigma (St. Louis, MO) unless otherwise specified.

Construction of VEGF/Green Fluorescent Protein (GFP) Retroviral Vector and Cell Transfection

The cDNA of murine VEGF164 was a generous gift from Dr. Patricia D'Amore, Harvard University.⁵ A murine stem cell retroviral vector MigR1 containing a coding sequence for enhanced GFP and an internal ribosome entry site, as well as BOSC23, a murine kidney 293T-derived packaging cell line, were generously provided by Dr. Warren Pear, University of Pennsylvania.⁴⁴ The VEGF/GFP retroviral vector was constructed as follows: plasmid containing VEGF164 cDNA was digested with *Xba*I and *Xho*I to release VEGF164 cDNA; MigR1 vector was digested with *Eco*RI and *Xho*I. VEGF164 cDNA fragment was inserted into MigR1 upstream of internal ribosome entry site and GFP using T4 DNA ligase. The sequence of the above constructs was confirmed by multiple restriction digestion analysis and direct sequencing (not shown). Retroviral vector containing GFP alone or VEGF164 plus GFP was transfected into BOSC23 cells using the calcium phosphate method. ID8 cells were infected with retrovirus in the presence of 8 μ g/ml of polybrene. After 72 hours, infected cells were examined under a fluorescent microscope.

Flow Cytometry and Cell Sorting

Cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA) using CellQuest flow cytometry analysis software (Becton Dickinson). GFP was detected using a 530/30-nm bandpass filter. Ascites leukocytes were detected with allophycocyanin (APC)-labeled rat anti-mouse CD45 monoclonal antibody (BD Pharmingen, San Diego, CA). Monoclonal antibodies against major histocompatibility complex class I molecules (MHC-I) (H-2kb/H-2Db, biotinylated), MHC-II (KH74, biotinylated), isotype control (IgG2ak, biotinylated), CD11c (HL3, APC-conjugated), CD86 (GL-1, fluorescein isothiocyanate-conjugated), and CD3 (17A2, fluorescein isothiocyanate-conjugated) were purchased from BD Pharmingen. Data were recorded and analyzed with CellQuest software (Becton Dickinson). Sorting was performed on a MoFlo cell sorter (Cytomation, Fort Collins, CO) equipped with an argon laser beam. All flow cytometry data were analyzed by upload-

ing data files into FlowJo (TreeStar, Inc.). Cells were sorted at a flow rate of 1000 to 3000 cells/second as GFP-negative, GFP-low-positive, and GFP-high-positive polyclonal cell populations.

Animals

Six- to 8-week-old female C57BL6 mice (Charles River Laboratories, Wilmington, MA) were used in protocols approved by the Institutional Review Board of the Wistar Institute and the University of Pennsylvania.

In Vivo Tumor Generation

Subconfluent ID8 cells were trypsinized, washed twice, and harvested by centrifugation at $1000 \times g$ for 5 minutes. A single-cell suspension was prepared in phosphate-buffered saline (PBS), or PBS mixed with an equal volume of cold Matrigel (BD Biosciences, Bedford, MA) at 10 mg/ml. For flank injections, a total volume of 0.5 ml containing 5×10^6 VEGF/GFP-transfected cells was injected subcutaneously into the flank of 8-week-old C57BL6 mice, whereas in some experiments the other flank was injected with the same number of control GFP-transfected cells ($n = 7$) or wild-type cells ($n = 7$). Tumors were detectable 2 weeks later and tumor size was measured weekly thereafter using a Vernier caliper. Tumor volumes were calculated by the formula $V = \frac{1}{2}(L \times W)^2$, where L is length (longest dimension) and W is width (shortest dimension).⁴⁵ Mice were sacrificed 5 weeks after flank injection. For intraperitoneal injections, a total volume of 0.7 ml of PBS containing 7×10^6 VEGF/GFP-transfected cells ($n = 10$), GFP-transfected cells ($n = 10$), or wild-type cells ($n = 10$) cells was inoculated into the mouse peritoneal cavity. Animals were followed for survival or were sacrificed 8 weeks after inoculation to evaluate tumor growth. Moribund animals were euthanized according to the protocols of the Wistar Institute and the University of Pennsylvania. To measure serum VEGF levels, whole blood samples were obtained by retro-orbital bleed and allowed to clot for 1 hour at room temperature. Intraperitoneal VEGF levels were determined in ascites supernatants collected 6 weeks after inoculation of intraperitoneal tumor or from healthy control animals.

Animal Vaccinations

For the preparation of whole tumor cell vaccine, ID8 cells transfected with VEGF/GFP-positive retrovirus were rinsed with PBS twice, cultured in serum-free media for 48 hours to minimize fetal bovine serum xenoantigens, and subsequently subjected to UVB rays at increasing energy to identify the dose of UVB that induces apoptosis in 100% of cells. Apoptosis was quantified through annexin-V and propidium iodide staining by flow cytometry, whereas killing efficacy was confirmed by cell proliferation assay using CellTiter96 kits (Promega, Madison, WI) according to the manufacturer. Immediately after exposure to lethal UVB, cells were rinsed twice in PBS and $1 \times$

10^5 apoptotic tumor cells suspended in 0.3 ml of PBS were injected subcutaneously to 6-week-old healthy female mice. Control mice received PBS injection. Mice were immunized twice, 1 week apart, and challenged with subcutaneous inoculations of 5×10^6 live VEGF/GFP-transfected ID8 cells 1 week after the second vaccination. Animals were sacrificed 8 weeks later and tumors were resected and measured as above.

Live Fluorescent Stereo Microscopy

The gross morphology of tumors was observed using a fluorescence stereo microscope (SMZ800; Nikon, Tokyo, Japan) equipped with a 100-W mercury lamp. Emitted fluorescence was acquired through a long pass filter Ex 480/20 on a CoolSNAP Pro color digital camera (Media Cybernetics, Silver Spring, MD).

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 1×10^6 cultured cells or 100 to 500 mg of fresh tissue with TRIzol reagent (Invitrogen). After treatment with RNase-free DNase (Invitrogen) for 15 minutes at room temperature, RNA was further purified with the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Total RNA ($2 \mu\text{g}$) was reverse-transcribed in a 20- μl reaction system using the Superscript first-strand synthesis kit for RT-PCR (Invitrogen) under conditions described by the supplier. Reverse-transcribed cDNA ($2 \mu\text{l}$) was amplified in 25 μl of PCR reaction system containing 200 $\mu\text{mol/L}$ each dNTP, 20 pmol of each primer, the standard buffer supplemented with 1.5 U *Taq* polymerase (Roche), and 1.5 mmol/L MgCl_2 . After initial denaturation at 94°C for 4 minutes, 30 cycles of PCR were performed with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds. The last extension was at 72°C for 7 minutes. Specific oligonucleotide primers (Table 1) were synthesized based on published sequences. To avoid false-positive results because of amplification of contaminated genomic DNA in the cDNA preparation, all primers were designed to span two exons separated by an intron.

DNA Isolation

Total DNA was isolated from 1×10^6 cultured cells or 100 to 500 mg of fresh tissue lysed in 10 mmol/L of Tris/HCl (pH 7.4) containing 0.2% Triton X-100 and 15 mmol/L of ethylenediaminetetraacetic acid. Lysates were treated with 50 μg of proteinase K overnight at 50°C and 10 μg of RNase for 1 hour at 37°C . DNA was gently extracted with phenol/chloroform.

Real-Time TaqMan PCR

The VEGF isoform and GFP was quantified by real-time PCR on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR was per-

Table 1. Primer Sequence

Primer name	Sequence
VEGF outer F	GAA GTC CCA TGA AGT GAT CAA G
VEGF outer R	TCA CCG CCT TGG CTT GTC A
VEGF common F	GCC AGC ACA TAG AGA GAA TGA GC
VEGF120 R	CGG CTT GTC ACA TTTT TCT GG
VEGF164 R	CAA GGC TCA CAG TGA TTT TCT GG
VEGF188 R	AAC AAG GCT CAC AGT GAA CGC T
VEGF probe	ACA GCA GAT GTG AAT GCA GAC CAA AGA AAG
GAPDH F	CCT GCA CCA CCA ACT GCT TA
GAPDH R	TCA TGA GCC CTT CCA CAA
GAPDH probe	CCT GGC CAA GGT CAT CCA C
GFP F	AAG AAC GGC ATC AAG GTG AAC T
GFP R	ACT GGG TGC TCA GGT AGT GGT T
GFP probe	CGT GCA GCT GGC CGA CCA CTA C
flt-1 F	ACC TGT CCA ACT ACC TCA AGA GC
flt-1 R	CTG GTT CCA GGC TCT CTT TCT T
KDR/flk-1 F	CGA CAT AGC CTC CAC TGT TTA TG
KDR/flk-1 R	TTT GTT CTT GTT CTC GGT GAT GT
Neuropilin-1 F	ATT TGA AGT TTA TGG CTG CAA GA
Neuropilin-1 R	ATT GGA TGC TGT AAT CTG GGA GT

formed using TaqMan PCR Core Reagents (Applied Biosystems) according to the manufacturer's instructions. PCR cycles consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of at 95°C for 15 seconds and at 60°C for 60 seconds. PCR amplification of the housekeeping gene, mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed for each sample as control for sample loading and to allow normalization among samples. A standard curve was constructed with PCR-II TOPO cloning vector (Invitrogen) containing the same inserted fragment and amplified by the TaqMan system. The relative expression units in each sample were calculated with respect to the standard calibration curve. Each sample was run twice and each PCR experiment included two nontemplate control wells. PCR products were confirmed as single bands using gel electrophoresis.

Western Blotting

Cultured cells (8×10^6) were lysed in 1 ml of lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 8 μ mol/L aprotinin, 0.22 mmol/L leupeptin, 0.4 mmol/L bestatin, 0.15 mmol/L pepstatin A, and 0.14 mmol/L E-64. Protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions and transferred to nitrocellulose membrane. Nonspecific binding was blocked by overnight incubation at 4°C in 0.1 mol/L of PBS (pH 7.4) containing 3% bovine serum albumin and 0.1% Tween-20. Membranes were then incubated with an anti-VEGF goat polyclonal antibody (C-20; 2 hours, 37°C, 1:200 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA), followed by incubation in horse anti-goat secondary antibody conjugated with horseradish peroxidase (1 hour, room temperature, 1:5000). Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Enzyme-Linked Immunosorbent Assay

Capture enzyme-linked immunosorbent assay was performed using anti-mouse VEGF antibody (BAF493, R&D Systems) as capture antibody and anti-VEGF164 biotinylated antibody (AF-493-NA, R&D Systems) as detection antibody in the concentrations described by the manufacturer. The reaction plate was revealed by the 2,2'-aziridine-bis-(3-ethylbenzothiazoline-g-sulfonic acid) diammonium salt (ABTS) detection system (Roche) after streptavidin-horseradish peroxidase (Pharmingen) incubation. Optical densities were read at 405 nm and VEGF concentrations were determined by comparison with standard curves generated with recombinant mouse VEGF164 (R&D Systems). Media from exponentially growing cultures were collected every 24 hours. The rate of VEGF secretion was calculated as previously described.⁴⁶

Immunostaining

Immunohistochemical staining was performed using the avidin-biotin-peroxidase method. Sections were in cold acetone for 10 minutes, pretreated with 3% H₂O₂ for 20 minutes to block endogenous peroxidase activity and incubated in matched normal sera (Vector Laboratories, Burlingame, CA). Rat anti-mouse CD31 (Pharmingen) was diluted at 1:200. The Vectastain ABC kit was applied as described by the manufacturer (Vector Laboratories). Sections were counterstained with Gill's hematoxylin (Vector Laboratories). Images were acquired through Cool SNAP Pro color digital camera (Media Cybernetics). CD31-staining density was analyzed using Image-Pro Plus 4.1 software (Media Cybernetics). For immunofluorescent staining, sections were sequentially incubated in 5% normal serum, anti-mouse CD31 antibody (1:100), biotin-labeled anti-rat immunoglobulin (Ig)G, and avidin-Texas Red (Vector Laboratories). Sections were counterstained with propidium iodide before being inspected under the fluorescent microscope.

Apoptosis Assays

DNA Ladder Assay

DNA was extracted as above, separated by 1.2% agarose gel electrophoresis, and visualized with ethidium bromide staining.

Annexin-V Assay

Annexin-V staining was detected by flow cytometry using an apoptosis detection kit (R&D Systems). Both floating and adherent cells were collected and processed as recommended by the manufacturer. After 15 minutes of incubation with annexin-V-biotin at room temperature, cells were resuspended and incubated in binding buffer containing 4 $\mu\text{g/ml}$ of streptavidin Red 670 (Invitrogen) for 15 minutes. Cells were analyzed using a FACScan flow cytometer (Becton Dickinson). For annexin-V cytochemistry, cells cultured on glass coverslips were incubated in annexin-V-biotin for 15 minutes at room temperature, incubated in binding buffer containing streptavidin-Texas Red (Vector Laboratories) for 15 minutes, washed with PBS, and immediately analyzed under the fluorescent microscope.

In Situ Terminal dUTP Nick-End Labeling (TUNEL) Assay

The ApopTag peroxidase *in situ* detection kit (Intergen, Purchase, NY) was used to visualize apoptotic cells *in vivo* and *in vitro*. The procedure was performed according to the manufacturer's instructions. Briefly, cells cultured on glass coverslips or tumor tissue sections were fixed with 1% paraformaldehyde in PBS, followed by cold ethanol and acetic acid after fixation. After incubation with residues of digoxigenin nucleotide and terminal deoxynucleotide transferase for 1 hour at 37°C, cells were incubated with peroxidase-labeled anti-digoxigenin antibody. DNA fragments were visualized with diaminobenzidine and H_2O_2 .

ELISPOT Assay

Generation of Tumor-Pulsed DCs

DC precursor cells were procured through bone marrow flushing of hind legs from 6-week-old healthy female C57BL/6 mice, rinsed once, and plated in RPMI media under standard conditions in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml; Peprotech, Rocky Hill, NJ) for 8 days.⁴⁷ Differentiation into immature DCs was assessed by flow cytometry detection of specific DC marker expression including Cd11c, MHC-II, and CD86.⁴⁷ VEGF/GFP-positive ID8 cells were rinsed twice in PBS to eliminate fetal bovine serum xenoantigens, cultured in serum-free media overnight, and then exposed to UVB rays (1500 $\mu\text{W/cm}^2$) to induce apoptosis as described earlier and 12 hours later were co-incubated with immature DCs

at a 1:1 ratio (tumor cells:DCs) for 48 hours. Tumor necrosis factor- α (50 U/ml, Preprotech) was added for 3 days. DCs were harvested, rinsed, and counted by trypan blue exclusion. Control DCs included unpulsed DCs matured with tumor necrosis factor- α as above; DCs pulsed as above with autologous murine splenocytes cultured on serum-free media overnight and exposed to UVB to induce apoptosis.

Isolation of Splenic T Cells

To determine the frequency of peripheral tumor-reactive T cells, T cells were isolated from splenocytes procured from tumor-naïve nonvaccinated mice as well as tumor-vaccinated or mock-vaccinated mice bearing flank tumors. Animals were vaccinated with apoptotic tumor cells or mock-vaccinated with PBS (control) as described above and subsequently challenged with flank injections of live tumor cells. Eight weeks after injection of live tumor, mice were euthanized and spleens were resected and minced in a sterile manner to yield a single cell suspension. Splenocytes were also obtained from control age-matched healthy female mice. Erythrocytes were eliminated by hypotonic shock. Splenocytes were plated in culture dishes in RPMI media under standard conditions for 30 minutes and a 95% pure population of T cells (as assessed by flow cytometry) was isolated by collecting the nonadherent fraction.

Interferon (IFN)- γ ELISPOT Assays

For ELISPOT, 10^7 autologous nonadherent T cells were cultured with tumor-pulsed DCs prepared as above at a 10:1 ratio in RPMI medium supplemented with 3% mouse serum. Control DCs and live tumor cells were also used as controls. Plates (MultiScreen-IP, Millipore, Bedford, MA) were coated overnight at 4°C with 50 μl /well of monoclonal anti-mouse IFN- γ (Pharmingen) at 1 $\mu\text{g/ml}$ in sodium carbonate buffer (2.93 mg/ml sodium bicarbonate, 1.59 mg/ml sodium carbonate, 0.2 mg/ml sodium azide in distilled water). Plates were washed three times in sterile PBS and blocked with RPMI 3% mouse serum for 1 hour at room temperature. T cells generated as above were washed three times in RPMI, resuspended in RPMI 3% mouse serum at 4×10^5 T cells/ml with DCs at a ratio of 10:1 (T cell:DC) and plated in triplicate at 100 μl /well. After 20 hours of co-incubation in standard culture conditions, cells were removed by washing with PBST (PBS, 0.1% Tween-20). Anti-mouse IFN- γ biotinylated monoclonal antibody (2 $\mu\text{g/ml}$, Pharmingen) was added to each well for 2 hours in PBS containing 0.5% mouse serum and 0.1% Tween-20. After additional washing, streptavidin-alkaline phosphatase at 1:10,000 dilution in PBS was added and incubated for 1 hour at room temperature. After washing, diaminobenzidine was added for 20 to 30 minutes at room temperature. The reaction was stopped by immersion in distilled water. Spots were scanned and counted by computer-assisted ELISPOT image analysis (Hitech Instruments, Edgemont, PA). Digitized images were analyzed for the presence of

areas in which color density, spot size, and circularity exceeded background by a factor set on the basis of the comparison of control wells.

Statistical Analysis

Data statistical analysis was performed using SPSS statistics software package (SPSS, Chicago, IL). All of the results are expressed as mean $\pm$ SD, and $P < 0.05$ was used for significance.

Results

Stable VEGF164 Overexpression in ID8 cells

The murine VEGF164 cDNA was successfully inserted in the murine stem cell retrovirus backbone upstream of enhanced GFP, from which it was separated by an internal ribosome entry site, ensuring the transcription of two separate products. After 24 hours of incubation with MigR1 vector carrying VEGF plus GFP or GFP alone, BOSC23 supernatants containing retrovirus were harvested and immediately used to infect ID8 cell monolayers. More than 15% GFP-positive cells were detected by flow cytometry analysis after two passages (Figure 1, A and B). Cell populations with high GFP expression were sorted by fluorescence-activated cell sorting from cultures transfected with VEGF/GFP-positive or control GFP-positive retrovirus. The purity of each population was examined immediately by flow cytometry and was revealed to be more than 99.7% (Figure 1A).

Total intracellular VEGF protein was assessed by Western blotting. A specific band was detected in all cell populations examined. When antibody was preincubated with recombinant murine VEGF, no band was detected (not shown). Total intracellular VEGF protein level was threefold higher in VEGF/GFP-transfected cells compared to wild-type or GFP-transfected ID8 cells by Western blot (Figure 1C), whereas secretion of VEGF protein in culture media was 12-fold higher by enzyme-linked immunosorbent assay (Table 2). Flow cytometry analysis proved that GFP was stably expressed in more than 90% of cells transfected with GFP or VEGF/GFP retrovirus after 20 passages (Figure 1D). VEGF164 and total VEGF mRNA levels were more than 11-fold and 4.5-fold higher, respectively, by real-time quantitative RT-PCR in VEGF/GFP-transfected cells compared to wild-type cells or control cells transfected with GFP only (Figure 1E).

VEGF164 Overexpression Dramatically Accelerates Ascites Formation and Tumor Growth *in Vivo*

Animals inoculated intraperitoneally with VEGF/GFP-positive ID8 cells, displayed diffuse peritoneal carcinomatosis consisting of multiple tumor nodules of 1 to 10 mm, which were dispersed on the parietal and visceral surfaces of the peritoneal cavity at 8 weeks. Resembling

human ovarian carcinoma, tumor nodules were particularly prevalent in the diaphragmatic peritoneum, the porta hepatis, and the pelvis (not shown). Control animals injected intraperitoneally with GFP-transfected or wild-type ID8 cells displayed occasional nodules <2 mm on the diaphragmatic peritoneum and porta hepatis at 8 weeks. Resembling human ovarian carcinoma, animals inoculated intraperitoneally with ID8 cells formed cellular ascites, which in late stages of disease became hemorrhagic. Ascites accumulation was markedly higher in mice bearing VEGF/GFP-transfected intraperitoneal tumors (10 to 12 ml) compared to mice bearing GFP-transfected tumors (1 to 3 ml) 8 weeks after intraperitoneal inoculation (Figure 2A). Furthermore, resembling human malignant ascites associated with ovarian carcinoma, $\sim 33\%$ cells isolated from ascites were CD45⁺ leukocytes (data not shown). Animals bearing VEGF/GFP intraperitoneal tumors exhibited 12.9-fold higher ascites levels and 2.6-fold higher serum levels of VEGF compared to animals bearing control GFP tumors 2 weeks after inoculation of cells (Table 2). After intraperitoneal inoculation of 1×10^7 cells, animals injected with VEGF/GFP-positive cells displayed a median survival of 8 weeks, whereas control animals injected intraperitoneally with GFP-transfected or wild-type cells displayed a median survival of 16 weeks ($P < 0.05$) (Figure 2B).

In the flank model, VEGF/GFP-transfected ID8 cells were injected subcutaneously into one flank, whereas the same number of control GFP-transfected ID8 cells ($n = 7$ /group) or wild-type ID8 cells ($n = 7$) were injected to the other flank in the presence of Matrigel. The tumor volume of VEGF/GFP-transfected cells was significantly larger (0.587 ± 0.083 cm³) compared to control contralateral GFP-transfected cells (0.033 ± 0.01 cm³, $P < 0.01$) 5 weeks after inoculation (Figure 2; C to E). Wild-type ID8 cells yielded similar tumors to GFP-transfected cells (not shown). Cell injection without Matrigel led to initially slower flank tumor growth, but similarly significant differences were noted between tumors formed by VEGF/GFP-transfected cells and contralateral control GFP-transfected cells (not shown). To confirm the stable *in vivo* expression of VEGF164, we examined the mRNA level of total VEGF in the tumor tissue by both RT-PCR and real-time RT-PCR (Figure 2, F and G). Tumors formed by VEGF/GFP-transfected cells displayed approximately fivefold higher mRNA levels (relative expression units 194.7 ± 34.0) compared to contralateral control tumors formed by GFP-transfected cells (37.2 ± 11.4 , $P < 0.05$).

To eliminate possible interactions between tumors with different VEGF expression growing in opposite flanks of the same animal, animals were inoculated with only one type of tumor cells in one flank ($n = 7$ /group). Identical results were obtained as above: VEGF/GFP tumors grew at a dramatically faster rate compared to control GFP tumors. The volume of VEGF/GFP-positive tumors was significantly larger (0.862 ± 0.252 cm³) compared to control GFP-positive tumors (0.046 ± 0.016 cm³, $P < 0.01$) 5 weeks after inoculation.

Figure 1. Stable overexpression of VEGF164 in ID8 cells after retroviral transfection and sorting. ID8 cells were transfected with retrovirus containing GFP or GFP plus VEGF164. **A: Top:** flow cytometry analysis of GFP expression of cells transfected with VEGF164/GFP-positive retrovirus during sorting. Approximately 7.5% of the cells express low-medium levels of GFP (gate R2, red), whereas ~7% express high levels of GFP (gate R3, blue). **Bottom:** Flow cytometry assessment of the purity of cells sorted based on high GFP expression. The purity of the population is >99.7%. **B: Top:** GFP-positive ID8 cells are observed 72 hours after transfection under a fluorescent microscope. **Bottom:** After sorting, most cells express high levels of GFP. **C:** Total intracellular VEGF protein level analyzed by Western blotting after 20 passages *in vitro*. Intracellular VEGF protein level in cells transfected with VEGF/GFP-positive retrovirus is threefold higher compared to control cells. **D:** Assessment of the stability of GFP expression by flow cytometry. More than 90% of transfected cells stably express GFP after 20 passages (red, control nontransfected cells; blue, VEGF/GFP transfected cells). **E:** Quantification of VEGF164 expression by real-time quantitative RT-PCR. Cells transfected with GFP/VEGF-positive retrovirus and sorted based on high expression of GFP (GFP/VEGF) express 18-fold higher levels of VEGF164 mRNA (**left**) and eightfold higher levels of total VEGF mRNA (**right**) compared to wild-type control ID8 cells (WT) or ID8 cells transfected with GFP-positive retrovirus (GFP).

GFP Expression Is Stable in Vivo and Provides a Sensitive Tool to Monitor Tumor Growth and Metastasis

In both the flank and intraperitoneal model, GFP or VEGF/GFP-transfected tumors were clearly visible under a fluorescent stereo microscope (Figure 3). The borders between the tumor and normal tissue could be easily observed owing to the distribution of GFP fluorescence. Furthermore, the nonluminescent tumor-associated blood vessels were clearly observed against the fluorescent background of GFP-expressing tumors under the fluorescent

Figure 2. VEGF164 overexpression in tumor dramatically accelerates ascites formation and tumor growth *in vivo*. **A:** Ascites accumulation is markedly higher in C57BL/6 mice inoculated intraperitoneally with 1×10^7 ID8 cells transfected with VEGF/GFP-positive retrovirus (**right**) compared to mice inoculated with 1×10^7 ID8 cells transfected with GFP-positive retrovirus (**left**) 8 weeks after tumor inoculation. **B:** Animals inoculated with ID8 cells transfected with VEGF/GFP-positive cells display a median survival of 8 weeks, whereas control animals injected intraperitoneally with GFP-transfected cells display a median survival of 14.8 weeks ($P < 0.05$, $n = 10/\text{group}$). **C:** VEGF/GFP-transfected ID8 cells give rise to markedly larger flank tumors (left flank) compared to control GFP-transfected ID8 cells injected to the contralateral (right) flank. **D:** Comparison of contralateral tumors resected from three mice. **Left:** Tumors from GFP-transfected cells growing in the right flanks. **Right:** Tumors from VEGF/GFP-transfected cells growing in the left flanks. **E:** Growth of flank tumors after injection of VEGF/GFP-transfected cells (VEGF) and contralateral control GFP-transfected cells (control). Cells were suspended in Matrigel ($n = 7/\text{group}$; *, $P < 0.05$; **, $P < 0.01$). **F:** Stable overexpression of VEGF164 *in vivo* is demonstrated by RT-PCR. Results from three mice bearing flank tumors are shown. In each mouse, the **left** RNA sample is from the tumor generated with GFP-transfected, whereas the **right** sample is from the contralateral tumor generated with VEGF/GFP-transfected tumor. The **rightmost** lane represents RNA from cultured VEGF/GFP-transfected ID8 cells (positive control). VEGF isoforms 188, 164, and 120 were amplified with isoform-specific primers. Only VEGF164 is overexpressed *in vivo*. β -actin RNA documents equal amount of RNA used for all samples. **G:** Real-time quantitative RT-PCR confirms stable overexpression of total VEGF mRNA *in vivo*. Tumors formed by VEGF/GFP-transfected cells (VEGF) display fourfold higher total VEGF mRNA levels compared to contralateral control tumors formed by GFP-transfected cells (control). Data were normalized with the housekeeping gene GAPDH.

stereomicroscope. Prominent vessels were readily seen in VEGF-overexpressing tumors (Figure 3, B and D). Notably, in the intraperitoneal model, early metastatic tumors, which were very difficult to identify grossly or under normal light stereomicroscope because of their considerably small size and random distribution, could be readily detected under epifluorescence owing to GFP

Table 2. Summary of VEGF Protein Expression Levels by Enzyme-Linked Immunosorbent Assay

	GFP	VEGF/GFP
Conditioned medium	$34 \pm 16 \text{ pg}/(10^6 \text{ cells} \times \text{hour})$	$408 \pm 45 \text{ pg}/(10^6 \text{ cells} \times \text{hour})$
Serum	$52 \pm 6 \text{ (pg/ml)}$	$135 \pm 26 \text{ (pg/ml)}$
Ascites	$245 \pm 58 \text{ (pg/ml)}$	$3172 \pm 230 \text{ (pg/ml)}$
Solid tumor	$4 \pm 3 \text{ (pg/mg)}$	$112 \pm 36 \text{ (pg/mg)}$

Figure 3. GFP expression is stable *in vivo* and provides a sensitive tool to monitor tumor growth and metastasis. Stable expression of enhanced GFP *in vivo* allows for rapid identification of tumors in both flank and intraperitoneal models. **A–F:** Flank tumors resected from both sides of the same mouse. The borders between the tumor and normal tissue can be easily observed owing to the distribution of GFP fluorescence. Furthermore, the nonluminescent tumor-associated blood vessels are clearly observed against the fluorescence of the GFP-expressing tumors under the fluorescent stereomicroscope. In control tumors from GFP-transfected ID8 cells (**A** and **B**), few blood vessels grow into the tumor; whereas in tumors from VEGF/GFP-transfected ID8 cells (**C** and **D**), prominent blood vessels growing into the tumor from nearby normal tissue are observed. Tumor from GFP-transfected ID8 cells under light microscope (**E**) and fluorescence stereomicroscope (**F**). A large central necrosis area is observed. Note the absence of prominent vessels in comparison with (**D**). Areas of necrosis were absent in tumors from VEGF/GFP-transfected ID8 cells. **G** and **H:** In the intraperitoneal tumor model, microscopic tumor nodules are detected on the spleen by stereomicroscopy. GFP expression allows for accurate detection of tumors. **I:** Real-time PCR revealed the presence of GFP gene, the genomic tumor marker, in multiple normal tissues of mice bearing VEGF164/GFP flank tumors, whereas in control mice, GFP was only detected in the lung.

expression (Figure 3, G and H). To test the use of GFP in detecting extraperitoneal metastasis, mice were sacrificed 10 weeks after inoculation of flank tumors and the presence of metastatic tumor in the lungs was examined by fluorescent stereo microscopy. Tumor metastasis to lungs was observed in one of seven mice in the VEGF164/GFP group, but in no mice in the GFP group. To detect microscopically invisible metastasis, Genomic GFP was quantified by real-time PCR in DNA extracted from four normal tissues (ie, lung, liver, kidney, and heart). GFP gene was detected in lung (five of seven), liver (two of seven), and kidney (one of seven) in animals inoculated with VEGF164/GFP ID8 cells, whereas it was only detected in lung (three of seven) of animals inoculated with control GFP tumors (Figure 3I).

Overexpression of VEGF164 Is Associated with Enhanced Angiogenesis and Increased Survival of Tumor Cells *In Vivo*

We analyzed angiogenesis *in vivo* by microvascular density (MVD). Tumors grown from VEGF/GFP-transfected cells were associated with more prominent microvasculature (Figure 3, A to D, and Figure 4) compared to GFP-transfected tumors. Quantitative analysis of CD31-

Figure 4. Overexpression of VEGF164 is associated with enhanced angiogenesis *in vivo*. **A** and **B:** Immunohistochemical detection of CD31 (brown) in flank tumors, followed by hematoxylin counterstaining. MVD calculated by the expression of CD31 was dramatically lower in control tumors generated with GFP-transfected cells (**A**, control) compared to tumors generated with VEGF/GFP-transfected cells (**B**, VEGF). **C:** Immunofluorescent staining of CD31 (red) shows prominent vascularization in association with VEGF/GFP-positive tumor cells (green). Nuclei are counterstained by 4,6-diamidino-2-phenylindole (DAPI) (blue). Most of CD31⁺ microvasculature is confined within the tumor (GFP⁺/DAPI⁺) and does not extend into the GFP⁻/DAPI⁺ surrounding stroma. **D:** Quantitative analysis of CD31 staining density by image analysis. Tumors generated by VEGF/GFP-transfected cells (VEGF) show significantly higher density of CD31⁺ cells than control tumors (control, $P < 0.05$).

Figure 5. VEGF164 overexpression promotes the survival of tumor cells *in vivo*. A pair of flank tumors from the same mouse is analyzed for detection of apoptosis *in vivo* with the TUNEL assay. MVD is detected by CD31 immunofluorescent staining. **A** and **B**: Tumor generated with GFP-transfected cells analyzed by TUNEL and hematoxylin counterstaining (**A**) and CD31 (**B**). A large necrotic area can be seen **A**, in which decreased MVD is appreciated with CD31 staining (**B**). **Insets F** and **G** are magnified below. **C–E**: Tumor generated with VEGF/GFP-transfected cells analyzed by CD31 (**C**) shows dramatically more pronounced MVD in the area occupied by tumor cells, as assessed by detection of GFP (**D**) and DAPI (**E**). **F** and **G**: Magnification of **insets F** and **G** from **A**. TUNEL assay reveals marked apoptosis in tumor cells in proximity to the necrotic area (**F**) as well as distal to the necrotic site (**G**) in control tumor generated with GFP-transfected cells. Tissue is counterstained with hematoxylin. **H**: Fewer TUNEL-positive apoptotic cells are detected in contralateral tumor generated with GFP/VEGF-transfected cells.

staining density revealed that in tumors generated by VEGF/GFP-transfected cells, MVD was significantly higher (5.63 ± 0.76) compared to control tumors (1.35 ± 0.45) ($P < 0.05$) (Figure 4; A, B, and D). Large necrotic areas were observed in GFP-positive tumors ($n = 5/7$, 71.5%) by stereomicroscopy (Figure 3, E and F) and histology (Figure 5, A and B), whereas no necrosis was observed in VEGF/GFP-transfected tumors ($n = 0/7$, 0%). The prevalence of tumor cell apoptosis was compared in tumors generated with GFP-transfected cells and contralateral tumors generated with VEGF/GFP-transfected ID8 cells by *in situ* TUNEL assay (Figure 5). An inverse association was seen between MVD and areas of necrosis in control tumors (Figure 5, A and B). Only scattered apoptotic cells were detected in tumors overexpressing VEGF164 (Figure 5H), whereas in control GFP-transfected tumors, a significantly higher number of apoptotic cells was detected both proximal (Figure 5F) as well as distant to necrotic areas (Figure 5G).

VEGF164 Promotes the Survival of Murine Ovarian Cancer Cells Through an Autocrine/Paracrine Mechanism

We examined the expression of VEGF receptors flt-1, KDR/flk-1, and co-receptor neuropilin-1 by RT-PCR. We

readily detected neuropilin-1 but not flt-1 or KDR/flk-1 in ID8 cells by this method. To enhance the sensitivity of RT-PCR, we used nested PCR to detect the expression of VEGF receptors. We found that flt-1 was expressed at low levels in ID8 cells, but KDR/flk-1 was still undetectable (Figure 6). KDR/flk-1 and flt-1 were detected in whole tumor RNA, which was used as positive control.

The abundance of different VEGF isoform transcripts was examined by RT-PCR. After starvation of serum and insulin for 18 hours, mRNA levels of VEGF188, VEGF164, and VEGF120 were significantly reduced in control wild-type and GFP-transfected cells, whereas cells transfected with VEGF/GFP retrovirus exhibited suppressed

Figure 6. VEGF receptor flt-1 but not flk-1/KDR is expressed in ID8 cells. Expression of VEGF receptors is assessed by nested RT-PCR in wild-type ID8 cells (WT) and ID8 cells transfected with VEGF/GFP-positive retrovirus (GFP/VEGF). In ID8 cells, VEGFR1/flt-1 is detected at low levels, whereas VEGFR2/flk-1/KDR is not detectable. The VEGF164-specific co-receptor neuropilin-1 is detected at high levels in ID8 cells. Transfection of VEGF164 did not change the expression levels of the above receptors. As positive control, flt-1 and KDR/flk-1 are detected in whole tumor RNA.

Figure 7. VEGF overexpression promotes survival of ID8 cells *in vitro* and confers resistance to *cis*-platin-induced apoptosis in ID8 cells. **A:** Detection of apoptosis by flow cytometry analysis of annexin-V staining. **Top:** Control. Markedly fewer ID8 cells transfected with VEGF/GFP-positive retrovirus (GFP/VEGF) exhibit annexin-V staining (apoptosis) after growth factor withdrawal compared to control wild-type ID8 cells (WT) or ID8 cells transfected with GFP-positive retrovirus (GFP). **Bottom:** *cis*-platin. Markedly fewer ID8 cells transfected with VEGF/GFP-positive retrovirus exhibit apoptosis after exposure to 50 μ mol/L of *cis*-platin for 24 hours compared to control wild-type ID8 cells or ID8 cells transfected with GFP-positive retrovirus. **B:** Summary of flow cytometry data from three different experiments. ID8 cells transfected with VEGF/GFP-positive retrovirus exhibit significantly less apoptosis after growth factor withdrawal (**left**) or exposure to *cis*-platin (**right**) compared to control wild-type ID8 cells (WT) or ID8 cells transfected with GFP-positive retrovirus (GFP; *, $P < 0.05$). **C:** *In situ* TUNEL and annexin-V apoptosis assay. **Top:** Control. *In situ* TUNEL assay detects apoptosis in control WT or GFP ID8 cells cultured under growth factor withdrawal conditions. Apoptotic TUNEL-positive cells are also detected in VEGF/GFP-positive cells. **Middle:** *cis*-platin. *In situ* TUNEL assay detects apoptosis in control WT or GFP ID8 cells exposed to 50 μ mol/L of *cis*-platin for 24 hours. Note markedly increased prevalence of apoptosis and decreased number of cells after exposure to *cis*-platin compared to cells grown under growth factor deprivation (above). Apoptosis and cell number reduction is markedly less prominent in VEGF/GFP-positive cells. **Bottom:** *cis*-platin. *In situ* fluorescent annexin-V assay using biotinylated annexin-V followed by streptavidin-Red 670 staining. Apoptotic annexin-V-positive cells (red) are noted among control WT ID8 cells and GFP⁺ ID8 cells (green) transfected with GFP-positive or VEGF/GFP-positive retrovirus.

VEGF188 and VEGF120 and constitutively elevated levels of VEGF164. The addition of recombinant murine VEGF did not alter the expression of endogenous VEGF (not shown). Growth factor withdrawal induced marked increase in apoptosis in control ID8 cells as well as ID8 cells transfected with GFP-positive retrovirus compared to growth factor-supplemented standard culture conditions (<3%, not shown). However, cells overexpressing VEGF164 displayed twofold to threefold lower amount of apoptosis under conditions of growth factor deprivation

(10 $\pm$ 2%) compared to ID8 cells transfected with GFP-positive retrovirus (29 $\pm$ 3%) or control ID8 cells (22 $\pm$ 7%, $P < 0.05$), as assessed by annexin-V staining (Figure 7, A and B). To assess whether the observed effect on apoptosis was because of an autocrine/paracrine effect of VEGF or to genetic alterations induced in ID8 cells by retroviral insertional mutagenesis,⁴⁸ several VEGF/GFP-transfected subclones were tested under these conditions and were found to display significantly increased resistance to growth factor deprivation-induced apopto-

Figure 8. VEGF overexpression reduces *cis*-platin-induced apoptosis of ID8 cells *in vitro*. DNA laddering analysis demonstrates that ID8 cells transfected with VEGF/GFP-positive retrovirus exhibit markedly less DNA fragmentation after exposure to *cis*-platin compared to control wild-type ID8 cells (WT) or ID8 cells transfected with GFP-positive retrovirus (GFP). M1 and M2 are two molecular markers.

sis compared to control cells (not shown). Furthermore, control GFP-transfected cells or wild-type ID8 cells were exposed to serum and insulin deprivation in the presence or absence of recombinant murine VEGF. A threefold reduction in apoptosis was observed in the presence of exogenous VEGF ($P < 0.05$, not shown). These results indicate that VEGF inhibits apoptosis in ID8 ovarian cancer cells directly through an autocrine/paracrine mechanism. Interestingly, no apoptotic cells were found expressing GFP, in agreement with a recent report that GFP expression is lost in cells undergoing apoptosis.⁴⁹

Overexpression of VEGF164 Protects Murine Ovarian Cancer Cells from Apoptosis Induced by *cis*-Platin

GFP-transfected and VEGF/GFP-transfected ID8 cells were exposed to increasing doses of *cis*-platin for increasing periods of time under serum and insulin-free conditions. *cis*-Platin was found to induce apoptosis in ID8 cells in a dose- and time-dependent manner (data not shown). Under insulin and serum starvation, up to $41 \pm 5\%$ of GFP-transfected cells (wild-type ID8, $36 \pm 4\%$) underwent apoptosis when exposed to $50 \mu\text{mol/L}$ of *cis*-platin for 24 hours, whereas only $14 \pm 2\%$ of VEGF/GFP-transfected cells underwent apoptosis with *cis*-platin treatment ($P < 0.05$) (Figure 7, A and B). Similar results were obtained with *in situ* TUNEL assay and *in situ* fluorescent annexin V staining (Figure 7C). Furthermore, a marked reduction in DNA laddering was observed in VEGF/GFP-positive cells after exposure to $50 \mu\text{mol/L}$ of *cis*-platin for 24 hours compared to wild-type or GFP-transfected cells (Figure 8). Several VEGF/GFP-transfected subclones were tested under these conditions and were found to display significantly increased resistance to platinum-induced apoptosis compared to control cells

Figure 9. Exogenous VEGF partially reduces *cis*-platin-induced apoptosis in ID8 cells *in vitro*. **A:** Detection of apoptosis by flow cytometry analysis of annexin-V staining. Addition of *cis*-platin markedly increases apoptosis in wild-type ID8 cells compared to control cells cultured under serum-free, insulin-free conditions. Addition of recombinant murine VEGF partially reduces *cis*-platin-induced apoptosis. **B:** Summary of flow cytometry data from three different experiments. Addition of recombinant murine VEGF induces a significant reduction in apoptosis after exposure of cells to *cis*-platin.

(not shown). Furthermore, control GFP-transfected cells or parental ID8 cells were exposed to *cis*-platin in the presence or absence of recombinant murine VEGF (Figure 9). Exogenous VEGF conferred partial resistance to apoptosis induced by *cis*-platin in ID8 cells, with an approximate 25% reduction in the prevalence of apoptotic cells compared to cells exposed to *cis*-platin in the absence of VEGF ($P < 0.05$) (Figure 9, A and B).

The Syngeneic Mouse Model of Ovarian Carcinoma Overexpressing VEGF Is Suitable for Immunological Studies

We examined the expression of major complex histocompatibility molecules by ID8 cells or cells transfected by GFP/VEGF or GFP-positive retrovirus. Approximately 50% of cells expressed surface MHC class I molecules (Figure 10A), whereas no cells expressed MHC class II molecules (not shown). Similar expression of MHC class I and II was observed in cells transfected by GFP/VEGF or GFP-positive retrovirus (not shown). ELISPOT analysis was performed to quantify the frequency of peripheral tumor-reactive T cells. In the absence of tumor vaccination, control animals exhibited no evidence of tumor-reactive T cells compared to healthy tumor-naïve non-vaccinated C57BL6 female mice of matched age. To generate tumor antigen for *in vivo* vaccination, ID8 cells transfected by GFP/VEGF-positive retrovirus were irradiated with UVB rays at increasing energy. Approximately 99% of cells were apoptotic by annexin-V and propidium iodide staining when exposed to UVB (not shown). Mice were immunized with two subcutaneous injections of apoptotic tumor cells or saline and subsequently challenged with subcutaneous inoculations of live tumor cells. Both animal groups developed flank tumors. A minimal, non-significant reduction in tumor volume was noted in immu-

Figure 10. Genetically engineered tumors may be recognized by the adaptive immune system. **A:** Flow cytometry detection of major histocompatibility complex class-I (MHC-I) molecule expression in VEGF/GFP-positive ID8 cells. Approximately 50% of cells exhibit expression of surface MHC class-I molecules (red). White: isotype-negative control. **B** and **C:** ELISPOT analysis of tumor-reactive T cells in spleens of tumor-naïve nonvaccinated mice (ctrl) as well as mock-vaccinated (nonvaccinated, NV) or tumor-vaccinated (V) mice bearing flank tumors. **B:** Summary of three different experiments. A threefold increase in the frequency of tumor-reactive T cells secreting IFN- γ is noted in vaccinated animals (V) compared to control mice (*, $P < 0.05$). **C:** Depiction of one representative well per condition in one representative experiment. In the absence of tumor vaccination, control animals (NV) exhibit no evidence of tumor-reactive T cells compared to healthy tumor-naïve nonvaccinated C57BL/6 female mice of matched age (ctrl). Marked increase in the number of spots staining for IFN- γ is noted, representing clones of antigen-specific (tumor-reactive) T cells recognizing tumor antigen presented by autologous DCs.

nized compared to control animals 8 weeks after inoculation of flank tumors (not shown). Remarkably, a significant increase in the frequency of tumor-reactive T cells secreting IFN- γ was noted after tumor vaccination in these animals compared to control mice ($P < 0.05$; Figure 10, B and C).

Discussion

VEGF may exert multifaceted functions on tumor cells, angiogenesis, and host immune mechanisms that may not only affect the natural course of ovarian carcinoma but also modify its response to therapy. Although such interactions may be partly studied in xenograft models, syngeneic models are best suited to investigate these events. In this study, we developed a syngeneic model of ovarian carcinoma with stable overexpression of murine VEGF164 in the C57BL/6 mouse. The rationale for choosing isoform VEGF164 was based on the secretory nature of this isoform⁷ and the evidence that VEGF164 is primarily responsible for the angiogenic effects of VEGF in tumors.^{10,11} The model that was generated exhibits marked similarities with human ovarian carcinoma. ID8 cells were originally developed from murine ovarian surface epithelium⁴³ and therefore represent the epithelial ovarian lineage, a true murine surrogate of human epithelial ovarian carcinoma. Intraperitoneal inoculation of genetically modified ID8 cells yielded peritoneal carcinomatosis that closely resembled stage III human ovarian carcinoma (the most frequent form of disease) with widespread nodules on the parietal and visceral peritoneum.

In addition, genetically modified tumors were associated with malignant ascites that contained leukocytes and tumor cells.

VEGF expression in tumor cells may be up-regulated by hypoxic conditions or glucose deprivation via hypoxia-inducible factor.^{6,50} On the other hand, genetic alterations such as loss of p53, p73 alterations, or overexpression of src may induce constitutive overexpression of VEGF in tumors.^{51–53} Expression of VEGF may vary among ovarian carcinomas, and in fact, several human ovarian carcinoma cell lines constitutively exhibit elevated VEGF expression even under standard oxygen and glucose conditions *in vitro* (unpublished observations from our laboratory). Our model used genetically modified tumor cells with constitutively elevated expression of VEGF and control tumor cells. In the former, overexpression of VEGF was stable *in vivo* and resulted in markedly elevated levels of VEGF protein in ascites and moderately elevated serum levels compared to animals bearing control tumors. In the latter, VEGF mRNA levels were similar to those detected in normal tissues with pronounced vascularity such as kidney, liver, and the heart.⁶ The serum or ascites content of VEGF detected with the two tumor types falls within the range of VEGF protein levels reported in serum (or ascites from patients with ovarian carcinoma).^{38,41,54}

Increased serum and/or tumor levels of VEGF have been associated with poor clinical outcome.^{16,41,42} The animal model presented in this study provides a suitable tool to dissect the molecular mechanisms underlying the effects of VEGF. As expected, tumors overexpressing VEGF164 exhibited significantly higher MVD compared to controls and significantly decreased tumor cell apoptosis *in vivo*, in agreement with others.^{55,56} Overexpression of VEGF164 significantly accelerated tumor growth and ascites formation, resulting in significantly shorter median survival. These findings prove the validity of the present model as one of angiogenesis-dependent ovarian carcinoma in which to investigate the biological effects of VEGF. Our findings are in contrast with the results recently reported with a xenograft model using human ovarian cancer cells overexpressing human VEGF165 in immunodeficient mice⁵⁷ and underscore the significance of experimentation in a syngeneic setting.

Tumor cells are faced with significant proapoptotic insults *in vivo* including hypoxia, acidosis, substrate starvation, growth factor deprivation, and immune-mediated attack, which may be partly mediated through apoptosis.⁵⁸ In addition, a variety of chemotherapy agents rely on apoptosis for their pharmacological cytotoxic effect.⁵⁹ Overexpression of VEGF164 not only increased angiogenesis, but also directly supported tumor cell survival through an autocrine/paracrine mechanism and conferred resistance to apoptosis induced by growth factor deprivation or chemotherapy. The present data taken together suggest that VEGF represents an important adaptation of tumor cells to adverse conditions within the tumor microenvironment, not only promoting nutrient supply through angiogenesis but also protecting tumor cells from the proapoptotic tumor environment. Our results are in agreement with observations that VEGF protects cells

against apoptosis including that induced by ionizing radiation or chemotherapeutic drugs.^{55,60} In leukemia cells, VEGF acts via KDR/flk-1 and downstream PI3-K, Akt kinase, and nitric oxide to prevent apoptosis.²⁹ Select human ovarian cancer cells may express KDR/flk-1,²² but we were unable to detect KDR/flk-1 in murine ID8 cells. In endothelial cells, KDR/flk-1 seems to be primarily responsible for the mitogenic effects of VEGF, whereas flt-1 may function as a decoy receptor.⁶¹ However, flt-1 is able to interact with a variety of signal transduction proteins, including the p85 subunit of PI3-K and mitogen-activated protein kinase, generating mitogenic signals. In fact, flt-1 was recently shown to participate in intracellular autocrine regulatory loops mediating the survival of hematopoietic stem cells.⁶² Furthermore, Flt-1 exhibits a significantly higher affinity for VEGF165 compared to KDR/flk-1,⁶¹ suggesting that even if expressed at low levels, Flt-1 may still be functional. Although there is yet no evidence for direct signaling mediated by neuropilins, a recent report indicates that in breast carcinoma cells lacking KDR/flk-1 but expressing neuropilin-1, VEGF induced activation of the PI3-K pathway.⁶³ Based on the above information, we hypothesize that VEGF164 regulates, via flt-1 and/or possibly neuropilins, signaling pathways such as PI3-K to support ID8 cell survival in an autocrine/paracrine manner.

An increasing bulk of evidence suggests that VEGF exerts important immunological functions. VEGF has been shown to inhibit the differentiation and maturation of DCs *in vitro* and *in vivo*,^{33,34,64,65} inhibiting the development of anti-tumor T cell responses. Furthermore, VEGF may also suppress cytokine-induced leukocyte-endothelial interactions *in vivo*⁶⁶ or decrease leukocyte transendothelial migration.⁶⁷ The immune biology of ovarian carcinoma has not been adequately investigated partly because of the lack of suitable syngeneic animal models. The present model fills this gap, because it is suitable for immunological studies related to ovarian cancer biology and therapy, and lends itself to investigation of the immunological effects of VEGF in cancer. Similarly to human ovarian carcinoma, genetically engineered ID8 cells were found to exhibit heterogeneous expression of surface MHC-I molecules. Our findings indicate that insertion of the murine VEGF164 isoform and enhanced GFP via a retrovirus did not significantly alter the immunogenicity of ID8 cells. In fact, in the absence of vaccination, no tumor-specific T cells were detected in mice using the highly sensitive ELISPOT method. These findings are in agreement with a recent report showing that enhanced GFP is not immunogenic in the C57BL6 mouse.⁶⁸ After repeated vaccination with apoptotic tumor cells, a significant tumor-specific T cell response was documented that however did not result in significant inhibition of tumor growth. Taken together, these findings suggest that ID8 tumors express antigens that may be recognized by the adaptive immune system if presented at a distant site from the tumor, but in nonimmunized animals the tumors entirely evade immune recognition. Furthermore, tumors evade immune attack by tumor-specific T cells after vaccination. These findings closely resemble the immunological behavior of human ovarian carcinoma in which

tumor-reactive T cells are documented among peripheral lymphocytes in patients with advanced disease.⁶⁹

An additional advantage offered by the present model relates to the expression of GFP. This facilitates rapid detection of tumor cells by fluorescent microscopy in histological specimens or by flow cytometry in analysis of cell suspensions. Furthermore, it allows for the sensitive detection of tumor cells *in vivo* using live fluorescent stereo microscopy. The molecular mechanisms underlying ovarian cancer extraovarian spread and intraperitoneal or retroperitoneal lymph node metastasis have been poorly elucidated, partly because of the lack of a suitable animal model. Successful orthotopic injection of tumor cells has been reported in mouse ovary.^{70,71} Our model combined with orthotopic injection of tumor cells offers opportunities for the investigation of early mechanisms of ovarian cancer intraperitoneal spread in the immunocompetent host and evaluation of the role of VEGF in this process. Furthermore, besides VEGF, basic fibroblast growth factor, interleukin-8, and transforming growth factor- β have been implicated in tumor angiogenesis and have been detected at high levels in ovarian cancer.^{72,73} Genetic manipulation of ID8 cells inserting additional or alternate proangiogenic factors has the potential to shed light on their individual function and possible synergistic interactions in promoting angiogenesis and progression of ovarian carcinoma in the immunocompetent host.

In summary, we present the development of a syngeneic mouse model of ovarian carcinoma with stable overexpression of murine VEGF164. The growth of these tumors was proven to be angiogenesis-dependent. This model provides a useful tool for the study of the multifaceted functions of VEGF on tumor cells, angiogenesis, and anti-tumor immune mechanisms. Furthermore, it offers a suitable tool for the investigation of the impact of VEGF on the efficacy of therapeutic strategies in ovarian carcinoma. Because of the expression of GFP, this model also offers new opportunities for the investigation of the molecular mechanisms underlying ovarian cancer spread in the immunocompetent host.

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