Vascular Endothelial Growth Factor-165 Overexpression Stimulates Angiogenesis and Induces Cyst Formation and Macrophage Infiltration in Human Ovarian Cancer Xenografts

Monique C. A. Duyndam,* Marion C. G. W. Hilhorst,*
Hennie M. M. Schlüper,* Henk M. W. Verheul,*
Paul J. van Diest,[†] Georg Kraal,[‡]
Herbert M. Pinedo,* and Epie Boven*

From the Departments of Medical Oncology,* Pathology,[†] and
Cell Biology and Immunology,[‡] Vrije Universiteit Medical Centre,
Amsterdam, The Netherlands

Vascular endothelial growth factor (VEGF) is suggested to be an important regulator of angiogenesis in ovarian cancer. We have evaluated the effects of VEGF overexpression on the histology and growth rate of human ovarian cancer xenografts. OVCAR-3 human ovarian cancer cells were stably transfected with an expression vector encoding the 165-amino acid isoform of VEGF. As subcutaneous xenografts, moderately and highly VEGF₁₆₅-overexpressing OVCAR-3 cells formed tumors with large cysts. Immunohistochemistry demonstrated an increase in the number of CD31-positive microvessels, some of which were larger in diameter than those in the parental tumors, as well as extensive vascular rimming around the cysts. Weakly VEGF₁₆₅-overexpressing tumors also contained an increased number of CD31-positive microvessels and occasional vascular rimming, but cysts were not present. Immunohistochemistry further revealed the presence of monocytes and macrophages in both parental and VEGF₁₆₅-overexpressing xenografts. Interestingly, the number of monocytes/macrophages was greatly increased in moderately and highly VEGF₁₆₅-overexpressing xenografts and large areas populated with monocytes/macrophages were detected within the tumor stroma. Although the higher number of CD31-positive cells would suggest a better vascularization pattern in VEGF₁₆₅-overexpressing xenografts, tumor growth rates were not increased when compared with that of parental xenografts. These data provide functional evidence for a role of VEGF₁₆₅ in cyst formation and monocyte/macrophage infiltration. (*Am J Pathol* 2002, 160:537–548)

Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis and has originally been iden-

tified by its ability to enhance vascular permeability.¹ The VEGF gene encodes five different isoforms that are generated by alternative splicing from a single gene.^{2–4} VEGF isoforms are active as homodimers and consist of monomers of 206, 189, 165, 145, or 121 amino acids.^{2,4} VEGF₁₂₁ is the only isoform that does not bind heparin and is efficiently secreted from the cell.⁵ VEGF₁₆₅ is partially secreted and partially retained on the cell surface as a result of binding to heparan-sulfate proteoglycans.⁵ The other VEGF isoforms are primarily retained on the cell surface.^{4,5} Besides differences in heparin-binding affinity, the VEGF isoforms also differ in their receptor recognition pattern. All VEGF isoforms induce proliferation of endothelial cells,^{2,4,6} presumably through binding to the endothelial cell receptor KDR/flk-1.^{4,7} VEGF₁₆₅ also binds to the endothelial receptor flt-1 and the recently identified receptor VEGF165-R.^{7,8} The latter receptor has a sequence identical to that of neuropilin-1 and seems to enhance the binding of VEGF₁₆₅ to the KDR receptor.^{7,8} In addition to its specific activities on endothelial cells, VEGF is also known to induce cellular responses in non-endothelial cells. VEGF binding to the flt-1 receptor induces migration of monocytes and macrophages,⁹ whereas binding to KDR and/or flt-1 receptors may enhance proliferation of certain tumor cells.^{10,11}

VEGF seems to play an important role in the process of angiogenesis in many types of cancer, including ovarian cancer. Expression of VEGF₁₂₁ and VEGF₁₆₅ mRNAs has been detected in normal ovaries,^{12,13} whereas transcripts of VEGF₁₄₅ and VEGF₁₈₉ have also been observed in ovarian cancer cell lines.^{4,14} VEGF mRNA levels can be increased in ovarian cancer tissue of patients.^{15,16} In addition, high VEGF expression and microvessel density have been correlated with poor disease-free survival and overall survival in patients with early or advanced stage ovarian cancer.^{15–17} VEGF also seems to be involved in the pathogenesis of ascitic fluid accumulation associated with peritoneal metastases by

Supported by the "Spinoza grant," awarded to Prof. H. M. Pinedo in 1997.

Accepted for publication October 26, 2001.

Address reprint requests to Dr. Monique C. A. Duyndam, Dept. of Medical Oncology, Vrije Universiteit Medical Centre, De Boelelaan 1117, 1081 HV Amsterdam, NL. E-mail: mca.duyndam.oncol@med.vu.nl.

increasing the vascular permeability of the microvessels in the peritoneal wall.^{18,19}

In addition to its role in tumor growth, VEGF may also be of negative influence on the response of tumors to conventional anti-cancer therapy. Node-positive breast cancer patients seem to have the highest likelihood of favorable outcome after treatment with adjuvant chemotherapy or hormone therapy, when VEGF levels in the primary tumor are low.²⁰ Moreover, high pretreatment serum VEGF concentrations are associated with poor response to combination therapy and unfavorable survival in patients with small-cell lung cancer.²¹ So far, the exact mechanisms that explain the above-mentioned phenomena are primarily unknown and have hardly been explored in *in vivo* tumor systems.

We have developed a human ovarian cancer xenograft model in which OVCAR-3 ovarian cancer cells were stably transfected with VEGF₁₆₅. Here, we report on the histology, the immunohistochemistry, and on the *in vitro* and *in vivo* growth properties of parental and weakly, moderately, and highly VEGF₁₆₅-overexpressing OVCAR-3 xenografts.

Materials and Methods

Cell Culture

Isolation of human umbilical vein endothelial cells (HUVECs) was performed according to the protocol of Van Hinsbergh and colleagues.²² HUVECs were grown in M199 medium (Gibco/Life Technologies, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum, 10% human serum, 2 mmol/L glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml endothelial cell growth factor. OVCAR-3 human ovarian cancer cells²³ and stably transfected derivatives were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco/Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin.

Plasmid Constructs and Transfection

pCMVVEGF₁₆₅ was constructed by cloning the *KpnI*-*XbaI* fragment of pBSVEGF₁₆₅²⁴ into the *KpnI* and *XbaI* polylinker sites of pcDNA3 (Invitrogen, Groningen, The Netherlands).

OVCAR-3 cells were transfected with pCMVVEGF₁₆₅ or with the empty pcDNA3 vector by the calcium-phosphate precipitation method.²⁵ After a 6-hour incubation period, the cells were washed, refed with medium, and left for 16 hours. Subsequently, the medium was replaced with medium containing 400 µg/ml of geneticin (G418, Gibco/Life Technologies) to select for geneticin-resistant cells. After 10 days, the concentration of geneticin was increased to 600 µg/ml. Two weeks later, isolated colonies were expanded and characterized. Transfected cell lines were grown in medium containing 300 µg/ml of geneticin for maintenance.

Figure 1. Schematic representation of possible fragments protected by the VEGF₁₆₅ RNA probe by the RNase protection assay. The mRNAs of the different VEGF isoforms are indicated by **dotted arrows** and the numbers 1 to 8 indicate the different exons of the VEGF gene. The VEGF₁₆₅ RNA probe is indicated by a **solid arrow**. On hybridization, noncomplementary exon sequences in the probe or in the mRNAs of the VEGF isoforms may form single-stranded loop structures. At the position where the sequences loop out, a nonprotected site may also be present in the opposite RNA strand. Regions of the VEGF₁₆₅ RNA probe that are susceptible for digestion by RNase A are indicated by **small arrows**. Fragments that may be protected by the mRNAs of the different VEGF isoforms and their respective sizes are indicated.

RNase Protection

pBSVEGF₁₆₅ was linearized with Styl and 161-nucleotide sense and 301-nucleotide anti-sense probes were generated with T3 and T7 polymerase, respectively. The sense probe included 106 nucleotides from the VEGF₁₆₅ cDNA (nucleotide positions -23 to 83) and 55 nucleotides of vector sequence. The anti-sense probe included 252 nucleotides from the VEGF₁₆₅ cDNA (nucleotide positions +341 to +593) and 49 nucleotides of vector sequence. In both cases, +1 was the first nucleotide of the first codon. Because of alternative splicing of VEGF transcripts, hybridization with the anti-sense probe allowed the possible detection of fragments of different sizes. VEGF₁₆₅ mRNA is expected to protect a fragment of 252 nucleotides, whereas VEGF₂₀₆ and VEGF₁₈₉ mRNAs may protect fragments of 82 nucleotides and 170 nucleotides. Furthermore, VEGF₁₄₅ and VEGF₁₂₁ mRNAs may give rise to protected fragments of 82 nucleotides and 38 nucleotides as shown in Figure 1.^{3,4} An anti-sense γ -actin probe was used as an internal control and has been described elsewhere.²⁶ RNase protection assays were performed as described.²⁷

Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blotting

Conditioned media of OVCAR-3 cells and stably transfected derivatives for ELISA and Western blotting were obtained by seeding 2×10^6 cells in a 96-mm culture dish. After 16 hours, the medium was replaced by serum-free

medium. Seventy-two hours later, the conditioned media were collected and the cells were trypsinized and counted.

ELISA was performed using the reagents and protocol supplied with the Quantikine Human VEGF Immunoassay kit (R&D Systems/ITK Diagnostics, Uithoorn, The Netherlands). The ELISA data were normalized for the number of cells counted at the time the conditioned media were collected.

For Western blot analysis, 2 ml of the conditioned media were incubated overnight at 4°C with 20 μ l of heparin-agarose beads (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Protein-heparin complexes were washed, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride-membrane (Immobilon; Millipore, Etten-Leur, The Netherlands). The membrane was blocked for 1 hour in TBST (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.025% Tween-20)/5% milk and incubated overnight at 4°C with rabbit polyclonal human VEGF-directed antiserum (1:1000 dilution, catalog no. sc-152; Sanver Tech/Santa Cruz Biotechnology, Heerhugowaard, The Netherlands) in the same solution. After washing with TBST, the membrane was incubated for 1 hour at room temperature with horseradish peroxidase-conjugated pork anti-rabbit antiserum (DAKO/ITK Diagnostics) in TBST/5% milk. The membranes were washed again with TBST and VEGF proteins were visualized by electro-chemiluminescence.

HUVEC Proliferation Assay

Conditioned media of OVCAR-3 cells and transfected derivatives were obtained as described above. Three thousand HUVECs per well were plated in 100 μ l of medium in 96-well tissue culture plates. After 24 hours, 100 μ l of nondiluted and 2, 4, 8, 16, and 32 times in serum-free medium diluted samples of the different conditioned media were added in three replicate wells. Seventy-two hours later, the number of living cells was estimated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,6-dimethyl-morpholino]-2,5-diphenyl-tetrazolium bromide] viability assay.²⁸

For the proliferation-inhibition assays, 100 μ l of the conditioned media samples of the VEGF-overexpressing cell lines inducing maximal proliferation of HUVECs (clone 19, nondiluted; clone 26, 16 times diluted; clone 31, 4 times diluted) was added to HUVECs in the absence or presence of 1 μ g/ml of normal goat serum or 1 μ g/ml of a neutralizing goat polyclonal human VEGF₁₆₅-directed antiserum in duplicate wells. As a control, 100 μ l of serum-free Dulbecco's modified Eagle's medium with or without 40 ng/ml of recombinant VEGF or 20 ng/ml of recombinant basic fibroblast growth factor (bFGF) was also tested on HUVECs in the absence or presence of 1 μ g/ml of normal goat serum or 1 μ g/ml of a neutralizing goat polyclonal human VEGF₁₆₅-directed antiserum in duplicate wells. Recombinant VEGF, bFGF, normal goat serum, and the VEGF₁₆₅-directed antiserum were all purchased from R&D Systems/ITK Diagnostics.

Cell-Cycle Distribution

Exponentially growing OVCAR-3 cells and transfected derivatives were washed twice with phosphate-buffered saline (PBS) and were detached from the culture flasks by incubation with PBS/0.2% ethylenediaminetetraacetic acid. Cells were collected, centrifuged, and resuspended in 1 ml of 70% ethanol. After centrifugation and removal of the ethanol, the cells were resuspended in PBS and centrifuged again. Subsequently, the cells were resuspended in 30 μ l of PBS and 200 μ l of RNase A (250 μ g/ml in 0.1% Triton X-100) was added. After a 20-minute incubation period at room temperature, 200 μ l of propidium iodide (100 μ g/ml) was added and the samples were incubated for another 20 minutes at 4°C in the dark. Cells were analyzed by using a FACScan flow cytometer. Results were expressed as the percentage of cells of each sample in the G₀/G₁, S, and G₂/M phase.

In Vitro Cell Growth

For each cell line a standard curve was obtained by plating 100,000, 50,000, 25,000, 12,500, 6250, 3125, 1562, and 781 cells per well in 100 μ l of medium in three replicate wells in a 96-well microtiter plate. After 3 hours, the MTT assay was performed.²⁸ Mean absorbances measured were plotted against the number of cells using regression analysis. In addition, 5000 cells per well of each cell line were plated in 12 replicate wells of 96-well microtiter plates. These cells were grown for 24, 48, 72, and 96 hours, after which the MTT assay was performed. Mean absorbances measured at each time point were related to the cell number from the standard curves. Doubling times were calculated by the following formula: $N_t = N_{te} \cdot e^{\lambda \cdot t}$ where λ is the growth constant and N_t and N_{te} are the number of cells on the last day and the first day that the cells are in exponential growth phase, respectively. Except from the first day after plating, the cells were growing exponentially during the whole test period.

Xenografts

Female nude mice (Hsd, athymic nude-*nu*) were purchased at the age of 6 weeks (Harlan, Horst, The Netherlands). The animals were maintained in cages with paper filter covers under controlled atmospheric conditions. Cages, bedding, food, and water were changed and sterilized weekly. The OVCAR-3 human ovarian cancer xenograft is a poorly differentiated serous adenocarcinoma and has been described previously.²⁹ VEGF₁₆₅-overexpressing OVCAR-3 xenografts were established as follows: VEGF₁₆₅-overexpressing OVCAR-3 cells were grown for 2 days in medium without G418 and were in the exponential growth phase at the time of harvesting. Cells were harvested by brief trypsinization, washed twice in PBS, and resuspended in PBS. The presence of single cells was confirmed by light microscopy and cell viability was confirmed by trypan blue exclusion. Cells (0.5×10^7) were injected subcutaneously in both flanks of the mice. The solid tumors arising at the inoculation site (passage 1) were transferred as tissue fragments with a

diameter of 2 to 3 mm through a small skin incision into both flanks of 8- to 10-week-old mice.

Immunohistochemistry

Indirect immunoperoxidase staining was performed on 4- μ m frozen xenograft tissue sections stored at -70°C . On thawing, sections were fixed with acetone (ice-cold), incubated for 10 minutes at 4°C , and air-dried. Sections were preincubated for 15 minutes at room temperature with 10% normal serum of the animal species from which the primary antibody, as used in the next step, was obtained. The serum was decanted and the sections were incubated for 60 minutes at room temperature with PBS/0.2% bovine serum albumin (negative control), a rabbit polyclonal human VEGF-directed antiserum (1:40 dilution, catalog no. sc-152; Santa Cruz Biotechnology), a goat polyclonal antiserum directed against the endothelial cell marker CD31 (1:40 dilution, catalog no. SC-1506; Santa Cruz Biotechnology), or rat monoclonal antibodies directed against monocytes and macrophages (MOMA-2, 1:10 dilution and F4/80 1:1 dilution, catalogue nos. MCA519 and MCA497, respectively; Serotec, UK). In all staining series, appropriate negative control antisera were used, which were irrelevant same species polyclonal antisera for VEGF and CD31 and an irrelevant isotype (IgG2b) rat monoclonal antibody for MOMA-2 and F4/80. Subsequently, the sections were washed three times for 5 minutes at room temperature with PBS. These washing conditions were repeated after all incubation periods thereafter. To inactivate endogenous peroxidase activity, sections were incubated for 10 minutes at room temperature with methanol/0.006% H_2O_2 . Incubations with biotin rabbit anti-goat serum, biotin goat anti-rabbit serum, and biotin rabbit anti-rat streptavidin-horseradish peroxidase conjugate and substrate mixture were performed as described by the manufacturer's protocol (Zymed/Sanbio, Uden, The Netherlands). Counterstaining was performed by incubation of the sections with hematoxylin for 5 minutes at room temperature followed by extensive rinsing with tap water. The sections were enclosed by a coverslip and a drop of aquamount.

Description of histopathological features and scoring of immunohistochemistry was performed by an experienced pathologist in a blinded manner in two to three separate tumors of parental OVCAR-3 xenografts and xenografts from transfected derivatives (size $<500\text{ mm}^3$). Histopathology of xenograft tissue included description of tissue compactness, cyst formation, and the presence of dilated capillaries. Intensity of VEGF staining was semiquantitatively expressed as negative (–), weakly positive (+), positive (++), or strongly positive (+++). Vascular density of the CD31-stained slides was difficult to score because of vascular rim formation around cysts and tumor cell islands, and was therefore limited to noting the presence of vascular rimming (as previously described by Guidi et al³⁰) and dilated capillaries. The number of tumor-associated monocytes/macro-

Figure 2. Assessment of VEGF₁₆₅ mRNA expression by RNase protection. **A:** OVCAR-3 tumors (OT) and the corresponding OVCAR-3 cell line (OC) express VEGF₁₆₅ mRNA. **B:** VEGF₁₆₅-transfected OVCAR-3 clones 19, 26, and 31 overexpress VEGF₁₆₅ mRNA. For **A** and **B** the total RNA of OVCAR-3 tumor tissue (**A**, lanes 4 and 5), the parental OVCAR-3 cell line (P; **A**, lanes 6 and 7; **B**, lane 2), control-transfected clones (**B**, lanes 3 and 4), VEGF₁₆₅-transfected clones (**B**, lanes 6 to 8), and tRNA as a negative control (T; **A**, lanes 8 and 9; **B**, lane 9) was hybridized to the indicated sense (S) or anti-sense (AS) probes. Fragments protected by the mRNAs of γ -actin, endogenous VEGF₁₆₅, the other possibly expressed VEGF isoforms and exogenous VEGF₁₆₅ are indicated by arrows. Full-length probes are shown in lanes 1 and 2.

phages was semiquantitatively expressed as low (+), moderate (++), or high (+++).

Results

VEGF mRNA in OVCAR-3 Cells and Tumor Tissue

Expression of VEGF₁₆₅ and VEGF₁₂₁ mRNAs in the human ovarian cancer cell line OVCAR-3 has been demonstrated previously.¹² We further examined the expression of VEGF mRNAs in the OVCAR-3 cell line and in subcutaneous OVCAR-3 tumor tissue by the RNase protection

assay. Hybridization of total RNA to the VEGF₁₆₅ anti-sense probe resulted in the protection of a 252-nucleotide fragment and confirmed the expression of VEGF₁₆₅ mRNA in the OVCAR-3 cell line and in OVCAR-3 tumor tissue (Figure 1 and Figure 2A, lanes 5 and 7). Fragments of 170 nucleotides and 82 nucleotides were also detected, which may be because of protection by mRNAs of VEGF₂₀₆/VEGF₁₈₉ and VEGF₁₄₅/VEGF₁₂₁, respectively (Figure 1 and Figure 2A, lanes 5 and 7). Another protected fragment of ~78 nucleotides was also observed. This fragment was most likely also protected by mRNAs of VEGF₁₄₅/VEGF₁₂₁, but the size of this fragment could not be explained on a theoretical basis. All of the protected fragments were not detectable when the VEGF₁₆₅ sense control probe was used (Figure 2A, lanes 4 and 6) and were not observed after hybridization of the VEGF anti-sense probe with control tRNA (Figure 2A, lane 9). These data are indicative for mRNA expression of human VEGF₁₆₅ and of longer and shorter human VEGF isoforms in the OVCAR-3 cell line and in OVCAR-3 tumor tissue.

Identification of VEGF₁₆₅-Overexpressing OVCAR-3 Clones

OVCAR-3 cells were stably transfected with pCMV-VEGF₁₆₅ and with the empty control vector. G418-resistant clones from each transfection were analyzed for VEGF₁₆₅ expression by the RNase protection assay. It should be noted that the pCMVVEGF₁₆₅ was cloned by insertion of a VEGF₁₆₅ cDNA fragment isolated from pBSVEGF₁₆₅. The VEGF₁₆₅ anti-sense RNA probe was transcribed from linearized pBSVEGF₁₆₅. Because of the presence of complementary pBS vector sequences, the fragment protected by overexpressed VEGF₁₆₅ mRNAs is expected to be 11 nucleotides longer than that protected by endogenous VEGF₁₆₅ mRNA. Figure 2B shows the results of a subset of the transfected clones analyzed. The intensity of the 252-nucleotide fragment relative to the γ -actin-protected fragment was similar in all cell lines, indicating that the endogenous VEGF₁₆₅ mRNA levels in the parental cell line and the stably transfected sublines were comparable (lanes 3 to 8). A protected fragment of 263 nucleotides was indeed present in the case of VEGF₁₆₅-transfected clones 19, 26, and 31 (lanes 6 to 8), but not in the case of the parental cells (lane 3) or the control-transfected clones (lanes 4 and 5). As judged by the intensity of the 263-nucleotide fragments, overexpression of VEGF₁₆₅ mRNA was weak in clone 19, moderate in clone 31, and high in clone 26.

Secretion of VEGF₁₆₅ Protein

The parental OVCAR-3 cells and the subset of the control- and VEGF₁₆₅-transfected clones were analyzed for the secretion of VEGF₁₆₅ protein by Western blotting. As can be seen in Figure 3, only a short exposure time of the Western blot was required to detect the glycosylated (23 kd) and nonglycosylated (21 kd) monomeric forms of the VEGF₁₆₅ protein³¹ isolated from the conditioned media of the VEGF₁₆₅-transfected clones 26 and 31 (top panel,

Figure 3. VEGF₁₆₅-protein secretion by OVCAR-3 cells and transfected derivatives. VEGF₁₆₅ proteins were isolated from the conditioned media of the parental OVCAR-3 cell line (lane 1), control-transfected clones (lanes 2 and 3) and VEGF₁₆₅-transfected OVCAR-3 clones (lanes 4 to 6) by heparin-agarose beads and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using a VEGF₁₆₅-directed antiserum. **Top:** Short exposure of an experiment performed with nondiluted conditioned media. **Bottom:** A long exposure of an experiment with nondiluted media and with 20 times and 10 times diluted medium of clones 26 and 31, respectively. The numbers on the left and right refer to the positions of the molecular weight markers and the (non)glycosylated forms of the VEGF₁₆₅ protein in kd, respectively.

lanes 5 and 6). Endogenous VEGF₁₆₅ protein secreted by the parental cells and control-transfected cells and overexpressed VEGF₁₆₅ protein produced by VEGF₁₆₅-transfected clone 19 were only detectable after long exposure (bottom panel, lanes 1 to 4).

The concentration of VEGF₁₆₅ protein in the conditioned media of the parental cell line, control-transfected clones, and VEGF₁₆₅-overexpressing clones 19, 26, and 31 was determined more accurately by ELISA. Table 1

Table 1. VEGF₁₆₅ Concentrations in the Conditioned Media of the Parental OVCAR-3 Cells and Transfected Derivatives

Cell line	[VEGF] (ng/ml/10 ⁶ cells)*
Parent	0.314
Control-transfected clone 7	1.05
Control-transfected clone 15	0.595
VEGF ₁₆₅ -transfected clone 19	6.6
VEGF ₁₆₅ -transfected clone 26	1432.2
VEGF ₁₆₅ -transfected clone 31	83.6

*Values represent the mean of triplicate samples of one representative experiment as determined by ELISA.

shows that the VEGF₁₆₅-overexpressing clones 19, 26, and 31 secreted ~50, 225, and 5000 times more VEGF₁₆₅ than the parental cell line. The control-transfected clones 7 and 15 also secreted slightly more VEGF₁₆₅, but it is questionable if this small increase is significant. In summary, these data demonstrated that the VEGF₁₆₅-transfected clones 19, 26, and 31 overexpressed VEGF₁₆₅ protein of the correct size.

Biological Activity of Overexpressed VEGF₁₆₅ Protein

To assess whether the overexpressed VEGF₁₆₅ proteins were functional, we compared the potency of different diluted samples of conditioned media from the parental cell line, the control-transfected clones, and the VEGF₁₆₅-overexpressing clones in a HUVEC proliferation assay (Figure 4). Although the parental cells and the control-transfected cells secreted VEGF₁₆₅ protein (Figure 3, Table 1), the amount was not sufficient to induce proliferation of HUVECs (Figure 4A). In contrast, stimulation of proliferation was observed by the conditioned media of the VEGF₁₆₅-overexpressing clones 19, 26, and 31 (Figure 4A). Maximal stimulation was induced with 12, 4, and 2 times diluted samples of clones 26, 31, and 19, respectively. Clearly, the media of clone 26 was most potent, followed by that of clones 31 and 19, which was likely related to the amount of secreted VEGF₁₆₅ protein. Complete inhibition of proliferation occurred by addition of a VEGF-neutralizing antiserum, but not by addition of a control serum (Figure 4B). This was not because of a general toxic effect of the VEGF-neutralizing antiserum, because this antiserum did not inhibit proliferation induced by the potent angiogenic factor bFGF (Figure 4C). These data demonstrated that induction of HUVEC proliferation by the media of clones 19, 26, and 31 was dependent on the presence of overexpressed VEGF₁₆₅ protein and confirmed the biological activity of these proteins.

Proliferation in Vitro

To examine whether overexpression of VEGF₁₆₅ affected the growth of OVCAR-3 cells *in vitro*, we determined the doubling times and the cell-cycle distribution of the parental OVCAR-3 cells, the control-transfected cells, and the VEGF₁₆₅-overexpressing cells. As can be seen in Table 2, the doubling times of the different cell lines were comparable and no major differences were observed between the percentages of cells in G₀/G₁, S, and G₂/M phase. From these results we concluded that VEGF₁₆₅ overexpression had no influence on the growth rate of OVCAR-3 cells *in vitro*.

Immunohistochemistry for VEGF and CD31

To analyze whether VEGF₁₆₅ overexpression had an effect on the angiogenic phenotype of OVCAR-3 xenografts when grown subcutaneously in nude mice, we

visualized the vasculature of highly, moderately, and weakly VEGF₁₆₅-overexpressing tumors by immunohistochemical staining. Semiquantitative scoring of VEGF ex-

Figure 4. Biological activity of overexpressed VEGF₁₆₅ proteins in a HUVEC proliferation assay. **A:** Conditioned media of the VEGF₁₆₅-overexpressing OVCAR-3 clones induced proliferation of HUVECs. Nondiluted and diluted samples of the conditioned media of the parental OVCAR-3 cell line, control sublines, and VEGF₁₆₅-overexpressing sublines were added to HUVECs. After 72 hours, proliferation was estimated by the MTT assay. The y axis represents the measured absorbance and reflects the number of cells. The x axis represents the diluted samples that are expressed as the percent of conditioned medium in the sample. **B and C:** Induction of proliferation by the conditioned media of the VEGF-overexpressing OVCAR-3 sublines is dependent on VEGF₁₆₅. Conditioned media of VEGF₁₆₅-overexpressing OVCAR-3 sublines, VEGF (20 ng/ml), or recombinant bFGF (10 ng/ml) were tested in the absence or presence of a control serum (1 µg/ml) or a neutralizing VEGF₁₆₅-directed antiserum (1 µg/ml) for their effects on HUVEC proliferation as in **A**.

Table 2. *In Vitro* Characteristics of Parental OVCAR-3 Cells and Stably Transfected Derivatives

Cell line	Doubling time*	Cell-cycle distribution (%) [*]		
		G ₀ /G ₁	S	G ₂ M
Parent	25.9	38.7	43.4	17.9
Control 7	28.7	36.1	41.6	22.3
Control 15	26.2	36.8	37.8	25.4
VEGF ₁₆₅ 19	30.5	36.7	36.6	26.6
VEGF ₁₆₅ 26	29.2	36.9	39.7	23.6
VEGF ₁₆₅ 31	26.9	37.6	33.6	28.8

*Values are obtained from one representative experiment.

pression and the presence of CD31-positive microvessels is presented in Table 3. Figure 5A shows the CD31 staining of the vasculature of the parental OVCAR-3 xenografts. The constitutive expression of VEGF in tumor cells and a relatively intense staining of VEGF in the tumor stroma are demonstrated in Figure 5B. Appropriate control antisera confirmed specific staining for VEGF and CD31.

CD31 staining in weakly VEGF₁₆₅-overexpressing xenografts (derived from clone 19) was more intense than in the parental xenografts (Figure 5C). Some dilated capillaries were detected. The structure of the xenografts appeared to be less compact than that of the parental xenografts. VEGF staining of the tumor cells was also slightly more intense than that of the parental xenografts (Figure 5D).

The moderately and highly VEGF₁₆₅-overexpressing xenografts (derived from clones 31 and 26, respectively) had a less compact structure than the parental and the weakly VEGF₁₆₅-overexpressing xenografts (Figure 5, compare E and G with A and C) and contained round to oval cysts as often seen in human serous papillary ovarian adenocarcinomas. These cysts were lined with tumor cells, sometimes forming papillary structures into the lumen. The cysts in xenografts <500 mm³ varied in size of 1 to 10 mm and were separated by varying amounts of stroma. Around the cysts within the stroma there was extensive rimming of CD31-positive capillary structures (Figure 5, E and G). Some of these capillary structures were larger in diameter than those detected in the parental xenografts. As expected, VEGF was highly expressed in the tumor cells that lined the cysts and in areas of solid tumor tissue (Figure 5, F and H). On growth of the xeno-

grafts >500 mm³ the cysts gradually increased in size becoming macroscopically visible and containing hemorrhagic fluid.

These data demonstrated that VEGF₁₆₅ overexpression induces an increase of the angiogenic response in OVCAR-3 xenografts and that VEGF₁₆₅ expression levels may be rate-limiting in the normal process of angiogenesis in these xenografts. In addition, these results are indicative for a role of VEGF₁₆₅ in cyst formation.

Immunohistochemistry for Monocytes and Macrophages

On more detailed examination of the histology of the parental and VEGF₁₆₅-overexpressing OVCAR-3 tumors, it was observed that a significant number of small mononuclear cells was present at the periphery and in the stroma of the tumors. These cells were clearly smaller in size than the tumor cells and were nonendothelial because no staining was observed with the CD31-specific antiserum. Staining with an antiserum directed against the cell marker CD45 confirmed the hematopoietic origin of the cells (data not shown). To further identify the mononuclear cells, stainings were performed with antibodies directed against granulocytes and B lymphocytes. Although staining of a limited number of granulocytes and B lymphocytes was detected in the tumor stroma of both parental and VEGF₁₆₅-overexpressing xenografts, no staining was observed of the small mononuclear cells (data not shown).

As tumors are known to be often infiltrated by monocytes and macrophages, we performed stainings with two different antibodies recognizing both monocytes and macrophages (MOMA-2 and F4/80). A similar staining pattern was observed for both antibodies identifying the small mononuclear cells as cells of the monocyte/macrophage lineage. Results obtained with the MOMA-2 antibody are shown in Figure 6. Semiquantitative scoring of the number of monocytes/macrophages is presented in Table 3. The control rat monoclonal antisera of the IgG2b isotype indicated the specificity of MOMA-2 and F4/80 staining. Areas of monocytes/macrophages were detected at the periphery of parental and VEGF₁₆₅-overexpressing OVCAR-3 xenografts. In parental xenografts, a relatively small number of infiltrated monocytes/macrophages was also detected in the stroma between tumor islets (Figure 6A). A similar pattern of monocyte/macrophage staining was observed in weakly VEGF₁₆₅-overexpressing xenografts (Figure 6B).

Interestingly, moderately and highly VEGF₁₆₅-overexpressing xenografts were found to have a different monocyte/macrophage content than parental and weakly VEGF₁₆₅-overexpressing xenografts (Figure 6, C and D). The overall staining of monocytes and macrophages in the former xenografts was much more pronounced and large areas of monocyte/macrophage infiltrates were not only observed at the tumor periphery, but also within the tumor stroma. Infiltrating monocytes and macrophages were particularly observed in areas where CD31 staining was relatively intense. Note that in all xenografts, mono-

Table 3. Semiquantitative Scoring of Immunohistochemistry of OVCAR-3 Parental and VEGF₁₆₅-Overexpressing Xenografts

Tissue	VEGF [*]		CD31 [*]		MOMA-2 [†]
	Cells	Stroma	Rimming	Dilation	
Parent	+	++	—	—	+/++
VEGF ₁₆₅ 19	+/++	+	+	—/+	+
VEGF ₁₆₅ 31	++	+++	++	+	+++
VEGF ₁₆₅ 26	+++	++	+++	+	++/+++

*Staining intensity expressed as negative (—), weakly positive (+), positive (++), or strongly positive (+++).

†Number of monocytes/macrophages expressed as low (+), moderate (++), or high (+++).

Figure 6. Immunohistochemical analysis of monocytes and macrophages in parental and VEGF₁₆₅-overexpressing OVCAR-3 tumors. Monocytes and macrophages were visualized by the brown color. Note that in parental (**A**) and weakly VEGF₁₆₅-overexpressing xenografts (**B**) relatively few macrophages and monocytes are detected in the tumor stroma. In moderately and highly VEGF₁₆₅-overexpressing xenografts (**C** and **D**), abundant areas of infiltrating monocytes and macrophages are detected in the stroma between the tumor islets.

cytes and/or macrophages were also stained with the VEGF-directed antiserum (Figure 5; B, D, F, and H). These data suggest that VEGF₁₆₅ overexpression stimulates the infiltration of monocytes and macrophages in OVCAR-3 xenografts.

Growth Rates of VEGF₁₆₅-Overexpressing OVCAR-3 Xenografts

The higher number of CD31-positive cells would suggest increased vascularization in the VEGF₁₆₅-overexpressing xenografts. This did not result, however, in increased tumor growth when compared to the growth of parental xenografts. The mean volume doubling time ($\pm$ SEM) of parental tumors was 7.9 ± 2.1 days, whereas that of the moderately and highly VEGF₁₆₅-overexpressing tumors was 12.9 ± 1.4 days and 7.5 ± 1.5 days, respectively. Weakly VEGF₁₆₅-overexpressing tumors grew slightly

slower than the parental tumors with a mean volume doubling time of 8.8 ± 0.7 days. These data suggest that additional parameters, such as monocyte/macrophage content or cyst formation, determine the tumor growth rate of VEGF₁₆₅-overexpressing xenografts.

Discussion

It has become clear that the growth of solid tumors is dependent on the process of angiogenesis and that VEGF is a central positive regulator of this process. In this study, we describe the effects of weak, moderate, and high VEGF₁₆₅ overexpression on the histology, the immunohistochemistry, and the growth rate of OVCAR-3 human ovarian cancer xenografts.

The histological pattern of the VEGF₁₆₅-overexpressing tumors was different from that of the parental tumors.

Figure 5. Immunohistochemical analysis of CD31 and VEGF expression in parental and VEGF₁₆₅-overexpressing OVCAR-3 tumors. VEGF and CD31 expression was visualized by the brown color. **A, C, E, and G:** CD31 staining. Little CD31 staining of endothelial cells is detected in the parental OVCAR-3 tumors (**A**). The number of CD31-positive cells in the weakly VEGF₁₆₅-overexpressing tumors (clone 19, **C**) appears to be higher than that in the parental tumors. In addition, the weakly VEGF₁₆₅-overexpressing tumors contain some dilated capillaries (**C**). Moderately and highly VEGF₁₆₅-overexpressing tumors contain the highest number of CD31-positive cells (clones 31 and 26, respectively; **E** and **G**). Many small and dilated CD31-positive microvessels can be detected around cysts as vascular rimming (**E** and **G**). **B, D, F, and H:** VEGF staining. In parental OVCAR-3 tumors, weak VEGF staining is visible in the tumor cells and a relatively intense staining is observed in the tumor stroma (**B**). VEGF staining of tumor cells in weakly VEGF₁₆₅-overexpressing tumors is somewhat more pronounced than that in parental xenografts (**D**). As expected, VEGF staining of tumor cells in moderately and highly VEGF₁₆₅-overexpressing tumors is intense (**F** and **H**). Note that intensely stained tumor cells are lining the cysts. The weakly VEGF₁₆₅-overexpressing tumors do not contain cysts (**D**).

All VEGF₁₆₅-overexpressing tumors contained an increased number of capillaries, some of which were wider in diameter than those observed in the parental tumors. In contrast to the weakly VEGF₁₆₅-overexpressing tumors, the moderately and highly overexpressing VEGF₁₆₅-overexpressing tumors also contained large cysts that were lined with tumor cells and surrounded by CD31-positive cells. The formation of cysts in the moderately and highly VEGF₁₆₅-overexpressing OVCAR-3 xenografts is intriguing because cysts are often present in human serous papillary ovarian adenocarcinomas. In fact, high levels of VEGF protein have been measured in ovarian cyst fluid and were shown to be associated with ovarian malignancy.³² It has been suggested previously that VEGF may contribute to the accumulation of cyst fluid in ovarian cancer by increasing the permeability of the tumor vasculature.³² High concentrations of VEGF have also been measured in fluid of other types of tumors.³³ Our study provides functional evidence that VEGF₁₆₅ indeed plays a role in the formation of cysts.

Another interesting finding in this study is that the moderately and highly VEGF₁₆₅-overexpressing tumors had a much higher content of monocytes and macrophages than parental and weakly VEGF₁₆₅-overexpressing tumors. Although numerous monocytes and macrophages were detected at the periphery of all tumors, the intratumoral density of these cells was significantly higher in moderately and highly VEGF₁₆₅-overexpressing tumors. These data show an interesting parallel with clinical studies of Leek and colleagues^{34,35} demonstrating a positive correlation between macrophage infiltration and VEGF expression levels or the vascular grade in breast cancer. As VEGF is reported to be a chemotactic agent for monocytes,⁹ it is interesting to speculate that high VEGF overexpression by tumor cells directly leads to an increased recruitment of these cells from the peripheral circulation. VEGF₁₆₅ overexpression in OVCAR-3 tumor cells may also indirectly stimulate macrophage/monocyte infiltration through induction of the expression of other chemoattractants. An example is macrophage chemoattractant protein (MCP)-1, which can be induced in endothelial cells on VEGF treatment.³⁶ In this respect, it is interesting to mention that MCP-1 concentrations have been found to be positively correlated with VEGF expression levels and macrophage accumulation in primary breast cancer.³⁷

The growth rate of the highly VEGF₁₆₅-overexpressing tumors was comparable with that of the parental tumors, whereas weakly and moderately VEGF₁₆₅-overexpressing xenografts grew slower. This is in contrast to other cancer xenograft models in which an increase in vascularization because of VEGF₁₆₅ overexpression resulted in accelerated tumor growth.^{38–40} It should be noted that tumor growth was measured by volume measurements. Moderately and highly VEGF₁₆₅-overexpressing xenografts developed large cysts on growth and had a less compact structure. As mentioned above, the intratumoral density of monocytes/macrophages was also considerably increased. Hence, the absolute number of the tumor cells in these xenografts was lower, suggesting that the proliferation rate of these cells was reduced.

The *in vitro* growth rate and cell-cycle distribution of VEGF₁₆₅-overexpressing and parental OVCAR-3 cells was comparable. Interestingly, VEGF has been demonstrated to act as a growth factor and as a survival factor for some tumor cell types expressing KDR or flt-1 receptors *in vitro*.^{10,11} We have also detected KDR mRNA expression in OVCAR-3 parental and VEGF₁₆₅-overexpressing cell lines and the respective xenografts by reverse transcriptase-polymerase chain reaction (data not shown). The fact that VEGF₁₆₅ overexpression has no effect on OVCAR-3 cell growth *in vitro*, does not exclude a role for KDR receptor signaling in this process. Auto-crine stimulation of KDR receptor activity by VEGF₁₆₅ overexpression may influence the expression levels of other angiogenic regulators in OVCAR-3 cells. Changes in the levels of these regulators may not be manifested in altered growth properties of OVCAR-3 cells *in vitro*, but may contribute to the process of angiogenesis and thereby influence tumor growth *in vivo*. Alternatively, maximal activation of KDR receptors may already be induced by endogenous levels of VEGF₁₆₅ protein. To further explore the role of the KDR receptor in OVCAR-3 cells and OVCAR-3 xenografts, we are currently performing experiments that are aimed at inhibition of KDR receptor activity.

The role of monocytes and macrophages in the growth and angiogenesis in OVCAR-3 xenografts is unclear. Tumor-associated monocytes/macrophages can both negatively and positively regulate tumor growth.⁴¹ On the one hand, these cells can negatively influence tumor growth by eliciting a cytostatic effect against tumor cells. On the other hand, they are believed to contribute to angiogenesis and tumor growth by producing angiogenic growth factors on activation within the tumor environment.⁴² One such a factor is VEGF, which is expressed in activated, tumor-associated macrophages.⁴³ The latter hypothesis is supported by clinical studies in breast cancer and endometrial cancer in which macrophage infiltration has been reported to be positively correlated with angiogenesis and/or reduced survival.^{34,44} So far, the effect of monocytes and macrophages on tumor growth and angiogenesis has not been extensively studied in *in vivo* model systems. In a human melanoma xenograft model, however, it was described that overexpression of interleukin-10 led to a decrease in tumor growth rate and angiogenesis, presumably by inhibiting angiogenic cytokine production in macrophages.⁴⁵

In the VEGF₁₆₅-overexpressing OVCAR-3 xenograft models we present here, there is no clear relationship between monocyte/macrophage content and tumor growth. Although the density of infiltrated monocytes/macrophages was strongly increased within the tumor stroma of highly and moderately VEGF₁₆₅-overexpressing OVCAR-3 xenografts, this did not result in an increase in the tumor growth rate. In fact, the number of tumor cells in these xenografts was even reduced (see above). A possible explanation may be that the tumoricidal capacity of the infiltrating macrophages dominates their tumor growth-promoting effects in these xenografts. To further analyze the contribution of macrophages to tumor growth and angiogenesis in parental and weakly VEGF₁₆₅-over-

expressing tumors, it would be interesting to assess effects of agents that inhibit macrophage function.^{45,46}

The xenograft model we have presented is an excellent tool to further determine the function of VEGF in monocyte/macrophage infiltration and offers the possibility to study whether VEGF₁₆₅ expression levels or/and the degree of vascularization influences the efficacy of anti-cancer agents.

Acknowledgment

We thank Dr. W. P. J. Leenders (Nijmegen, The Netherlands) for providing us with the BSVEGF₁₆₅ plasmid.

References

1. Brown LF, Detmar M, Claffey K, Nagy JA, Feng D, Dvorak AM, Dvorak HF: Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. *EXS* 1997, 79:233–269
2. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991, 5:1806–1814
3. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA: The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991, 266:11947–11954
4. Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E, Neufeld G: VEGF₁₄₅, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. *J Biol Chem* 1997, 272:7151–7158
5. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N: Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 1992, 267:26031–26037
6. Park JE, Keller GA, Ferrara N: The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* 1993, 4:1317–1326
7. Gitay-Goren H, Cohen T, Tessler S, Cohen T, Tessler S, Soker S, Gengrinovitch S, Rockwell P, Klagsbrun M, Levi BZ, Neufeld G: Selective binding of VEGF₁₂₁ to one of the three vascular endothelial growth factor receptors of vascular endothelial cells. *J Biol Chem* 1996, 271:5519–5523
8. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M: Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998, 92:735–745
9. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D: Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 1996, 87:3336–3343
10. Katoh O, Tauchi H, Kawaiishi K, Kimura A, Satow Y: Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. *Cancer Res* 1995, 55:5687–5692
11. Masood R, Cai J, Zheng T, Smith DL, Naidu Y, Gill PS: Vascular endothelial growth factor/vascular permeability factor is an autocrine growth factor for AIDS-Kaposi sarcoma. *Proc Natl Acad Sci USA* 1997, 94:979–984
12. Olson TA, Mohanraj D, Carson LF, Ramakrishnan S: Vascular permeability factor gene expression in normal and neoplastic human ovaries. *Cancer Res* 1994, 54:276–280
13. Sowter HM, Corps AN, Evans AL, Clark DE, Charnock-Jones DS, Smith SK: Expression and localization of the vascular endothelial growth factor family in ovarian epithelial tumors. *Lab Invest* 1997, 77:607–614
14. Boock CA, Charnock-Jones DS, Sharkey AM, McLaren J, Barker PJ, Wright KA, Twentyman PR, Smith SK: Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst* 1995, 87:506–516
15. Paley PJ, Staskus KA, Gebhard K, Mohanraj D, Twigg LB, Carson LF, Ramakrishnan S: Vascular endothelial growth factor expression in early stage ovarian carcinoma. *Cancer* 1997, 80:98–106
16. Yamamoto S, Konishi I, Mandai M, Kuroda H, Komatsu T, Nanbu K, Sakahara H, Mori T: Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. *Br J Cancer* 1997, 76:1221–1227
17. Hollingsworth HC, Kohn EC, Steinberg SM, Rothenberg ML, Merino MJ: Tumor angiogenesis in advanced stage ovarian carcinoma. *Am J Pathol* 1995, 147:33–41
18. Nagy JA, Masse EM, Herzberg KT, Meyers MS, Yeo KT, Yeo TK, Sioussat TM, Dvorak HF: Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. *Cancer Res* 1995, 55:360–368
19. Mesiano S, Ferrara N, Jaffe RB: Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. *Am J Pathol* 1998, 153:1249–1256
20. Gasparini G, Toi M, Miceli R, Vermeulen PB, Dittadi R, Biganzoli E, Morabito A, Fanelli M: Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy or hormone therapy. *Cancer J Sci Am* 1999, 5:101–111
21. Salven P, Ruotsalainen T, Mattson K, Joensuu H: High pre-treatment serum level of vascular endothelial growth factor (VEGF) is associated with poor outcome in small-cell lung cancer. *Int J Cancer* 1998, 79:144–146
22. Van Hinsbergh VWM, Draijer R: In vitro models and functional studies of endothelia culture and characterization of human endothelial cells. Cell culture models of epithelial tissues. A practical approach. Oxford, Oxford University Press, 1996, pp 87–110
23. Hamilton TC, Young RC, McKoy WM, Grotzinger KR, Green JA, Chu EW, Whang-Peng J, Rogan AM, Green WR, Ozols RF: Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. *Cancer Res* 1983, 43:5379–5389
24. Potgens AJ, Lubsen NH, Van Altena MC, Vermeulen R, Bakker A, Schoenmakers JG, Ruiter DJ, De Waal RM: Covalent dimerization of vascular permeability factor/vascular endothelial growth factor is essential for its biological activity. Evidence from Cys to Ser mutations. *J Biol Chem* 1994, 269:32879–32885
25. Graham FL, Van der Eb AJ: A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973, 52:456–467
26. Enoch T, Zinn K, Maniatis T: Activation of the human beta-interferon gene requires an interferon-inducible factor. *Mol Cell Biol* 1986, 6:801–810
27. Giacccone G, Van Ark-Otte J, Scagliotti G, Capranico G, Van der Valk P, Rubio G, Dalesio O, Lopez R, Zunino F, Walboomers J, Pinedo HM: Differential expression of DNA topoisomerases in non-small cell lung cancer and normal lung. *Biochim Biophys Acta* 1995, 1264:337–346
28. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983, 65:55–63
29. Molthoff CF, Calame JJ, Pinedo HM, Boven E: Human ovarian cancer xenografts in nude mice: characterization and analysis of antigen expression. *Int J Cancer* 1991, 47:72–79
30. Guidi AJ, Fischer L, Harris JR, Schnitt SJ: Microvessel density and distribution in ductal carcinoma in situ of the breast. *J Natl Cancer Inst* 1994, 86:614–619
31. Ferrara N, Houck K, Jakeman L, Leung DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 1992, 13:18–32
32. Hazelton D, Nicosia RF, Nicosia SV: Vascular endothelial growth factor levels in ovarian cyst fluid correlate with malignancy. *Clin Cancer Res* 1999, 5:823–829
33. Verheul HM, Hoekman K, Lupu F, Broxterman HJ, Van der Valk P, Kakkar AK, Pinedo HM: Platelet and coagulation activation with vascular endothelial growth factor generation in soft tissue sarcomas. *Clin Cancer Res* 2000, 6:166–171
34. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL: Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res* 1996, 56:4625–4629

35. Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL: Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J Pathol* 2000, 190:430–436
36. Marumo T, Schini-Kerth VB, Busse R: Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes* 1999, 48:1131–1137
37. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, Matsushima K: Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res* 2000, 6:3282–3289
38. Zhang HT, Craft P, Scott PA, Ziche M, Weich HA, Harris AL, Bicknell R: Enhancement of tumor growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cells. *J Natl Cancer Inst* 1995, 87:213–219
39. Benjamin LE, Keshet E: Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc Natl Acad Sci USA* 1997, 94:8761–8766
40. Oku T, Tjuvajev JG, Miyagawa T, Sasajima T, Joshi A, Joshi R, Finn R, Claffey KP, Blasberg RG: Tumor growth modulation by sense and antisense vascular endothelial growth factor gene expression: effects on angiogenesis, vascular permeability, blood volume, blood flow, fluorodeoxyglucose uptake, and proliferation of human melanoma intracerebral xenografts. *Cancer Res* 1998, 58:4185–4192
41. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L: The origin and function of tumor-associated macrophages. *Immunol Today* 1992, 13:265–270
42. Joseph IB, Isaacs JT: Macrophage role in the anti-prostate cancer response to one class of antiangiogenic agents. *J Natl Cancer Inst* 1998, 90:1648–1653
43. Lewis CE, Leek R, Harris A, McGee JO: Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. *J Leukoc Biol* 1995, 57:747–751
44. Salvesen HB, Akslen LA: Significance of tumour-associated macrophages, vascular endothelial growth factor and thrombospondin-1 expression for tumour angiogenesis and prognosis in endometrial carcinomas. *Int J Cancer* 1999, 84:538–543
45. Huang S, Xie K, Bucana CD, Ullrich SE, Bar-Eli M: Interleukin 10 suppresses tumor growth and metastasis of human melanoma cells: potential inhibition of angiogenesis. *Clin Cancer Res* 1996, 2:1969–1979
46. Vukanovic J, Isaacs JT: Linomide inhibits angiogenesis, growth, metastasis, and macrophage infiltration within rat prostatic cancers. *Cancer Res* 1995, 55:1499–1504