Circulating plasma vascular endothelial growth factor in mice bearing human ovarian carcinoma xenograft correlates with tumor progression and response to therapy

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Abstract
Vascular endothelial growth factor (VEGF) performs as an angiogenic and permeability factor in ovarian cancer, and its overexpression has been associated with poor prognosis. However, models to study its role as a marker of tumor progression are lacking. We generated xenograft variants derived from the A2780 human ovarian carcinoma (1A9), stably transfected with VEGF_{121} in sense (1A9-VS-1) and antisense orientation (1A9-VAS-3). 1A9, 1A9-VS-1, and 1A9-VAS-3 disseminated in the peritoneal cavity and ascites in mice, but only 1A9-VS-1, the VEGF_{121}-overexpressing tumor variant, produced ascites. Tumor biopsies from 1A9-VS-1 showed alterations in the vascular pattern and caused an angiogenic response in the chorioallantoic membrane assay. A significant level of soluble VEGF was detectable in the plasma of mice bearing 1A9-VS-1 even at an early stage of tumor growth. Plasma VEGF correlated positively with tumor burden in the peritoneal cavity and ascites accumulation. Cisplatin reduced the tumor burden and ascites in mice bearing 1A9-VS-1; the response was associated with a significant decrease of VEGF in plasma. This 1A9-VS-1 xenograft model reproduces the behavior of human ovarian cancer by growing in the peritoneal cavity, being highly malignant, and producing ascites. Plasma VEGF as a marker of tumor progression offers a valuable means of detecting early tumor response and following up treatments in an animal model. [Mol Cancer Ther 2005;4(5):715–25]

Introduction
Epithelial ovarian cancer has been described as a silent killer because in most cases the disease has already spread outside the pelvis when it is first diagnosed. The most common form of dissemination is invasion of the organs and membranes in the peritoneal cavity. The circulation of peritoneal fluid facilitates the dissemination of malignant cells onto the peritoneal surfaces (1).

Vascular endothelial growth factor (VEGF), initially known as vascular permeability factor, was recognized as responsible for peritoneal fluid accumulation (2). Ascites associated with VEGF has been reported in experimental models (3) and in patients with ovarian cancer (4), and inhibition of malignant ascites by the blockade of VEGF activities has been achieved in several preclinical models (5, 6).

VEGF is considered the major factor involved in physiologic and pathologic angiogenesis, such as embryonic development, wound healing, tissue regeneration and reorganization, retinal disorders, solid tumors, and metastasis formation (7). VEGF exists in four main differently spliced variants (VEGF_{121}, VEGF_{165}, VEGF_{189}, VEGF_{206}) according to their amino acid residues (8). The isoforms differ in their ability to bind to heparan sulfate and extracellular matrix. VEGF_{121}, which lacks the region encoded by exons 6 and 7, does not bind heparin and is secreted from cells in a soluble and freely diffusible form, whereas the VEGF_{165} isoform, the predominant isoform in tissues, has intermediate properties, being in part secreted and in part retained on the cell surface to bind heparan sulfate proteoglycan. VEGF_{189} and VEGF_{206} are highly basic, have heparin-binding properties, and are strongly bound to the extracellular matrix (9, 10). In view of their
free diffusibility, the smaller weight isoforms play a major role in angiogenesis and tumorigenesis (8). They have also been associated with vasodilation and vascular permeability (2).

There is evidence that VEGF exerts multiple functions in tumors and its overexpression is associated with poor prognosis in some tumor types (11–13). In ovarian carcinoma the overexpression of VEGF has been correlated with clinicopathologic features and patient survival (14). Elevated serum/plasma VEGF levels have been described in these patients (4, 15, 16).

The importance of VEGF as a marker of tumor progression is debated. Studies have been conducted to profile VEGF and other growth factors with local and metastatic disease and ultimately with the response to therapy (11, 12, 17). Human tumor xenografts are useful for studying the role of tumor-produced cytokines and growth factors (18). However, VEGF is rarely detectable in plasma of mice bearing tumors and hardly ever in early stage tumors.

In this study we used a human ovarian carcinoma xenograft overexpressing the VEGF121 isoform to investigate whether tumor-produced VEGF can serve as a marker of tumor progression. This model is highly angiogenic in the chorioallantoic membrane and produces ascites in the peritoneal cavity of nude mice. The plasma level of VEGF correlates with tumor burden and ascites formation and dissemination in the organs of the peritoneal cavity; it is a reliable marker of tumor response to therapy. This model is valuable for investigating the role of VEGF in the biology of ovarian cancer and in the response to therapy.

Materials and Methods
Cell Culture and Transfection

The human ovarian carcinoma cell line 1A9, a variant derived from A2780 (19), was maintained in RPMI 1640 supplemented with 10% FCS (Life Technologies, Inc., Paisley, United Kingdom). 1A9 clones expressing different levels of VEGF were obtained by stable transfection of the parental cell line with the human VEGF121 cDNA in the sense and antisense orientations (20) by the calcium phosphate technique. Forty-eight hours after transfection, cells were seeded at concentration of 5,000 cells/mL and clones were selected by growing cells in complete medium supplemented with 500 μg/mL of G418 as selection antibiotic. Different clones were picked, the level of VEGF protein assessed, and two of them, 1A9-VS-1 and 1A9-VAS-3, expressing VEGF CDNA in the sense and the antisense orientation, respectively, were selected for additional studies. Clones were routinely cultured in RPMI 1640 supplemented with 1% glutamine and 500 μg/mL G418.

Real-time Reverse Transcription – PCR

VEGF transcript was analyzed by real-time reverse transcription–PCR (RT-PCR). Briefly, total RNA was purified from cell culture with the Trizol protocol (Invitrogen, Gaithersburg, MD). Total RNA from frozen tumor biopsies was purified after tumor homogenization with an Ultraturrax, using the SV total RNA isolation kit following the manufacturer’s instructions (Promega, Milan, Italy). For real-time RT-PCR analysis, 200 ng of total RNA were reverse-transcribed with the TaqMan Reverse Transcription Kit (Applied Biosystems, Cheshire, United Kingdom) and 2 μL further amplified by real-time RT-PCR.

Human or mouse VEGF, actin primers, and TaqMan probes were purchased as ready to use solutions (Assay on Demand, Applied Biosystems). Experiments were run in triplicate.

Growth Curve and Supernatant Collection

To monitor the growth, tumor cells were plated in 125 flasks (2 × 10^5 cells per flask) in RPMI 1640 supplemented with 10% FCS (Life Technologies, Inc.). Cells were maintained at 37°C and the medium replaced every 2 days. Cells were detached daily with trypsin-EDTA and counted with a Coulter counter ZM (Coulter Electronics Limited, Luton, United Kingdom). For supernatant collection, cells were plated in 24-well culture plates at a concentration of 6 × 10^4 cells per well in RPMI supplemented with 10% FCS. After 48 hours at 37°C, monolayers were washed with Ca- and Mg-free PBS and 500 μL per well of RPMI supplemented with 1% FCS added. For each time point, the supernatant was collected, centrifuged at 1200 rpm for 10 minutes at 4°C, aliquoted, and stored at −80°C.

Chorioallantoic Membrane Assay

Fertilized White Leghorn chicken eggs (20 per group) were incubated at 37°C at constant humidity. On day 3 of incubation, a square window was opened in the shell and 2 to 3 mL of albumen were removed to detach the developing chorioallantoic membrane from the shell. The window was sealed with glass of the same size and the eggs were returned to the incubator. The experiments were done between days 8 and 12 of incubation because it is generally accepted that implants made from days 8 to 10 are strongly angiogenic (21). On day 8, a 1-mm³ tumor fragment from xenografted 1A9 or 1A9-VS-1 and 1A9-VAS-3 was grafted onto the chorioallantoic membrane according to Ribatti et al. (22).

Sterilized gelatin sponges (Gelfoam, Upjohn Co., Kalamazoo, MI) loaded with 1 μL of PBS with or without 500 ng of recombinant human VEGF121 (R&D Systems, Abingdon, United Kingdom) implanted on top of the growing chorioallantoic membrane were used as negative or positive controls, respectively (22).

All experiments were done under sterile conditions in a laminar flow hood. Chorioallantoic membranes were examined daily until day 12 in ovo with a stereomicroscope equipped with an MC 63 camera system (Zeiss, Oberkochen, Germany). On day 12, blood vessels entering the implants within the focal plane of the chorioallantoic membrane were counted by two observers in a double-blind fashion at ×50 magnification. Results were expressed as mean (± SD) vessel counts.

Mice and Human Ovarian Carcinoma Xenografts

Female NCr-nu/nu mice were obtained from the animal production colony of the National Cancer Institute (NCI-BTB-DTP), Frederick Cancer Research and Development Center (Frederick, MD). Mice were used at 6 to 8 weeks of age, with a mean body weight of 21 ± 2 g. They were

Plasma VEGF in Mice and Tumor Progression

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housed in filtered-air laminar-flow cabinets and handled using aseptic procedures. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (Degreto Legge No. 116, Gazzetta Ufficiale, Suppl. 40, February 18, 1992; Circolare No. 8, Gazzetta Ufficiale, July 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

A suspension of $10 \times 10^6$ tumor cells from 1A9, 1A9-VS-1, and 1A9-VAS-3 in 0.2 mL HBSS was injected s.c. (subcutaneous tumor growth) or i.p. (orthotopic growth) into nude mice.

The tumors growing s.c. were measured with a Vernier caliper, and tumor weights were calculated from the formula: tumor weight = (length $\times$ width$^2$)/2 (23). When tumor weight reached $\sim$ 200, 500, and 1500 mg, mice were killed, the tumors were harvested (five per size), fixed in a solution of zinc acetate and zinc chloride in Tris-Ca acetate buffer (24, 25) for 24 hours, embedded in paraffin, sectioned at 5 $\mu$m and stained with H&E or processed for immunohistologic analysis as described below. Specimens were also harvested for RNA extraction as described above.

Nude mice bearing the different 1A9 tumor variants i.p. were euthanized by carbon dioxide inhalation on days 7, 14, and 21 (five per time point) after tumor transplantation and autopsied. Peripheral venous blood samples were collected from all mice on the day of autopsy in sterile plastic tubes containing 3.8% trisodium citrate dihydrate, collected from all mice on the day of autopsy in sterile plastic tubes containing 3.8% trisodium citrate dihydrate, final volume 1/10, and immediately centrifuged at 3,000 rpm for 20 minutes. Plasma was aliquoted and stored at $-80^\circ$C until further processing. Ascites was harvested, centrifuged at 1200 rpm for 10 minutes, and the volume of fluid recorded by subtracting the pellet volume. Ascitic fluid was stored at $-80^\circ$C until further processing. The ovaries, uterus, pancreas, omentum, liver, and diaphragm were collected, fixed in 10% phosphate-buffered formalin, and processed for histologic analysis as described below. Another group of eight mice was euthanized when they became moribund, the day of death being considered the limit of survival. At autopsy the peritoneal cavity was macroscopically examined to ascertain the presence of tumor. Results are plotted as the percentage survival per days after tumor transplant. The increment of life span (percent ILS) was calculated as $100\left( \frac{\text{median survival day of control group}}{\text{median survival day of treated group}} \right) - 100$ (6).

**ELISA**

Human VEGF was measured by an ELISA (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, MN) that recognizes human VEGF$_{121}$ and VEGF$_{165}$. The sensitivity of the assay was 9.0 pg/mL (samples below this limit were considered negative). Assays were conducted according to the manufacturer’s directions, as described previously (4). Standards and samples were tested in duplicate. Results were calculated from a standard curve, generated by a four-parameter logistic curve fit, and expressed in picograms per milliliter.

**Drug and Treatment Evaluation**

Drug response was evaluated in nude mice bearing 1A9 tumor variants i.p. cis-Diammine platinum(II) dichloride (cDDP, Sigma Aldrich, Milan, Italy) was dissolved in saline solution and injected i.v. at the dose of 4 mg/kg every 4 days for four times. The vehicle was given by the same schedule. Treatment started 7 days after tumor transplantation.

At the end of the treatment, five nude mice per group were euthanized by carbon dioxide inhalation and autopsied to establish the tumor burden. Serum, ascites, and the organs of the peritoneal cavity were collected and processed for further analysis, as described above.

**Histological and Immunohistochemical Analysis**

To establish microvessel density, zinc-fixed sections from each tumor were immunostained with the rat monoclonal anti-mouse CD31 antibody MEC 13.3 (PharMingen, San Diego, CA), a specific marker for endothelial cells (26). The reaction was revealed by incubating the sections with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Hematoxylin counterstaining was done.

Vascular hotspots (three to four fields in each sample) were selected at $40 \times$ to $100 \times$ and microvessels were counted at high magnification ($\times$400; refs. 27, 28). The median value for each sample was reported and the microvessel density of each group was calculated as the mean of these medians.

For computer-assisted analysis of tumor vessel calibers, CD31-immunostained sections were captured with a digital camera at $\times100$ magnification and morphometric analysis was done with Image-Pro Plus software. At least four different 0.1-mm$^2$ fields in each section were examined for vessel caliber. Tumor burden in the peritoneal cavity was classified, according to size of metastases, as small nodules (when composed of <10 tumor cells), nodules (10–100 tumor cells), nodes (>100 tumor cells), and masses (macroscopically detectable nodular lesions of at least 1 mm). Abdominal organs were considered metastasized if tumor cells were detected within the parenchyma or on the peritoneal surface. Results were presented according to the following pathologic score:

- $T_0$ = no metastasis or histologic detection of small omental nodules; no peritoneal organ invasion;
- $T_1$ = histologically detectable omental nodules but no grossly detectable tumoral deposits; no peritoneal organ invasion;
- $T_2$ = histologically detectable omental nodes but no other peritoneal organ invasion;
- $T_3$ = histologically detectable omental masses; at least one other peritoneal organ other than the omentum invaded;
- $T_4$ = grossly detectable omental masses and/or two or more peritoneal organs involved according to histologic evaluation.

For each group the median pathologic score from five mice was calculated.
Statistical Analysis

VEGF expression was described with standard summary statistics. The correlation between VEGF levels in plasma and ascites volume and the pathologic score was assessed using the Spearman correlation coefficient. Changes in ascites, VEGF levels and pathologic scores for vehicles and treated mice were compared with the nonparametric Wilcoxon test. Differences in survival were analyzed by the log-rank test.

Results

VEGF Expression in 1A9 Tumor Cell Variants

The human ovarian carcinoma cell line A2780/1A9 was stably transfected with the VEGF121 cDNA plasmid in both the sense and antisense orientations. The 1A9-VS-1 clone expressed VEGF mRNA levels three times higher than the parental 1A9, whereas in the 1A9-VAS-3 clone the levels were at least 5-fold lower than in the parental cell line (Fig. 1A).

Accordingly, the level of VEGF secreted in the culture supernatant was higher for 1A9-VS-1 cells than for 1A9 or 1A9-VAS-3 (Fig. 1B). However, in the 1A9-VAS-3 model, the VEGF121 mRNA levels were not completely abolished; thus, cells were able to produce an amount of VEGF that, due to the method sensitivity, seems to be comparable to that of the 1A9 parental cell line.

At 24 hours after seeding VEGF levels were (± SD) 12,790 ± 697.2, 3701.9 ± 289.8, and 3224.9 ± 6.8 pg/mL for 1A9-VS-1, 1A9, and 1A9-VAS-3, respectively. The release of VEGF by 1A9-VS-1 was time dependent, indicating its association with tumor cell growth. In vitro cell proliferation was not significantly different for the three variants. In a typical experiment, the doubling time (interpreted from the graph) was (± SD) 30.17 ± 4.7, 26.09 ± 0.24, and 27.38 ± 0.45 hours for 1A9, 1A9-VAS-3, and 1A9-VS-1, respectively.

Tumor Growth of 1A9-VS-1 Xenografts

The three 1A9 variants produced tumors in all the transplanted nude mice. Figure 2A shows the similar kinetics of growth for the three xenograft variants, the doubling time being 1.55 days for 1A9, 1.66 days for 1A9-VAS-3, and 1.54 days for 1A9-VS-1.

The expression of VEGF in the tumor xenografts was studied by real-time RT-PCR (Fig. 2B). Specific primers and Taqman probes were used to discriminate between human (tumor derived) and murine (host derived) isoforms of VEGF. Human VEGF mRNA was markedly increased in 1A9-VS-1 xenografts (five times higher than 1A9-VAS-3 or 1A9 parental biopsies; Fig. 2B), whereas mRNA levels for the endogenous mouse VEGF were comparable. These data were confirmed by visualizing on agarose gel the products from RT-PCR analysis done to discriminate the different VEGF isoforms (data not shown). No differences in gene expression were found for the other VEGF isoforms (VEGF165, VEGF189, and VEGF206) among the three tumor xenografts (data not shown).

Accordingly, only mice bearing 1A9-VS-1 had detectable VEGF in their plasma, the level correlated with tumor weight (Fig. 2C). VEGF in plasma was already detectable in mice with a 200-mg tumor (mean 566.3 pg/mL) and was as high as 21,771.2 pg/mL in mice bearing 1.5-g tumors (Fig. 2C).

Histologically, the three variants showed no significant differences, with the features of poorly differentiated ovarian carcinoma, with a solid pattern and multifocal to coalescing areas of necrosis. However, the 1A9-VS-1 specimen showed a wide range of blood vessel sizes and frequently marked dilation of the vessels (Fig. 3A–C), confirmed by digital image analysis (Fig. 3D). The microvessel density was not increased in 1A9-VS-1 xenografts at any time during tumor growth (data not shown).

In vivo Angiogenic Activity of 1A9-VS-1 Xenografts

To assess whether VEGF121 transfection was responsible for higher angiogenic activity in vivo, biotic fragments from 1A9-VS-1, 1A9 and 1A9-VAS-3 tumor xenografts were tested on day 8 of incubation for their angiogenic activity using the chick chorioallantoic membrane assay. They were examined grossly on day 12 of incubation. Numerous allantoic vessels were seen, converging radially in a “spoked wheel” pattern toward the implant; they
were significantly more numerous in the chorioallantoic membranes treated with bioptic specimens from 1A9-VS-1 than 1A9 and 1A9-VAS-3 (mean, 31 ± 4, 22 ± 3 and 15 ± 4; \( P < 0.001 \), Fig. 4). The angiogenic response was comparable to that obtained with gelatin sponges loaded with VEGF121 (mean, 33 ± 5, not shown) and significantly higher than with gelatin sponges loaded with vehicle alone (mean, 8 ± 2; \( P < 0.001 \) versus 1A9-VS-1 and 1A9 xenografts, not shown).

**1A9-VS-1 Grows Orthotopically and Releases VEGF in the Plasma**

When the 1A9, 1A9-VS-1, and 1A9-VAS-3 variants were transplanted i.p., they all produced tumors in the mouse peritoneal cavity resembling ovarian cancer in humans. Overall analysis of survival indicated that 1A9-VS-1 grew significantly faster \( [P < 0.001; \text{mean survival time (MST), 26 days; range, 21–35 days}] \) than 1A9 (MST, 31 days; range, 27–52 days), or 1A9-VAS-3 (MST, 31 days; range, 26–58 days) (Fig. 5A). Histopathologic examination at various postinoculation times showed similar degrees of local invasion and metastatic spread in the three variants (Figs. 5B and 6A–D). Tumor cells spread by direct seeding in the body cavity and peritoneal surface. This is characteristic of carcinoma from the ovary (29). A time-related increase in tumor spread and metastasis volume was seen in all groups (Fig. 5B). However, only 1A9-VS-1-bearing mice accumulated ascites in the peritoneal cavity (median, 3.4 mL; range, 1.8–5.0; 21 days after tumor implantation, Fig. 5C). Interestingly, only this group had high level of VEGF in plasma (median, 13,109 pg/mL; range, 9,532–20,349; Fig. 5D) and in ascites (median, 3,311 pg/mL; range, 2,930–4,476; Fig. 5E) 21 days after tumor implantation. There was a time-related increase in peritoneal fluid accumulation and VEGF levels in plasma and ascites (Fig. 4C–E). Seven days after tumor implantation, plasma VEGF was already measurable (median, 214 pg/mL; range, 0–1,689). Interestingly, this preceded the appearance of macroscopic tumor nodules and ascites in the peritoneal cavity, indicating the potential of this parameter as a marker of tumor dissemination (Fig. 5B and D).

Only the ovaries of 1A9-VS-1 tumor-bearing mice (80% of mice) presented severe dilation of vessels (angiectasis). The lesion involved networks of dilated channels lined by a single layer of flattened spindle-shaped or pleomorphic endothelial cells with plump nuclei (Fig. 6E and F). Ovarian angiectasis correlated with the high VEGF plasma levels (data not shown).

Table 1 shows that plasma and ascites VEGF, ascitic volume, and tumor burden (pathologic score) in mice bearing 1A9-VS-1 tumor in the peritoneal cavity were significantly correlated, as assessed by the nonparametric Spearman correlation coefficient. Therefore, VEGF in plasma might be useful as a marker of tumor progression in this ovarian carcinoma model.

**Plasma VEGF Correlates to Response to Chemotherapy**

To study whether VEGF plasma levels could serve as a marker of response to therapy, mice bearing 1A9-VS-1 were treated with cDDP and an interim analysis was done after treatment (Table 2). At the beginning of treatment (day 7) VEGF level in plasma reflected the low tumor burden.
At day 20 (24 hours after last treatment), plasma VEGF levels in vehicle-treated mice were increased, paralleling the growth of the tumor (median, 18,197 pg/mL; range, 11,524–24,630). On the same day (24 hours after the last treatment) the plasma VEGF levels of mice treated with cDDP were roughly halved compared with vehicle-treated animals (median, 10,301 pg/mL; range, 1,139–19,609, \( P < 0.0273 \)). There was a parallel reduction of the ascitic volume in cDDP-treated mice compared with vehicle-treated mice (\( P < 0.0024 \)). Tumor dissemination in the peritoneal cavity of cDDP-treated mice also decreased.

**Discussion**

The properties of VEGF as an angiogenic and permeability factor in ovarian cancer have been widely described (8, 30). Several studies have shown high levels of VEGF in plasma and ascites of patients with advanced disease (4, 15). Therefore, circulating VEGF might be important as a marker of tumor progression and overall survival. We propose a human ovarian carcinoma xenograft model with stable overexpression of VEGF121 growing orthotopically in the peritoneal cavity of nude mice, in which the level of plasma VEGF correlated with tumor progression and production of ascites.
The rationale for choosing VEGF121 was its secretory nature and the evidence that it is responsible for angiogenesis and permeability in tumors (30). Our xenograft model stably overexpressed human VEGF, but not murine VEGF, and produced elevated levels of VEGF protein in plasma and ascites compared with the parental xenograft or with the control transfected with antisense VEGF121. The model recapitulates human ovarian carcinoma in several aspects. It grew orthotopically and disseminated onto the peritoneal viscera, with preferential involvement of the pancreas, omentum, diaphragm, and ovaries. The amount of VEGF released in ascites falls within the range detected in patients with advanced ovarian carcinoma (4).

Other experimental tumor models overexpressing VEGF (31), including ovarian carcinomas, have been described. However, plasma VEGF was rarely detectable and only in mice with advanced tumors (32). The unique feature of our model is the plasma VEGF detectable at an early stage, before there is any evidence of tumor nodules, and the correlation of its levels with tumor burden. Plasma levels continued to increase as the tumors grew; on the other hand, they correlated with the decrease of tumor burden in response to therapy (Tables 1 and 2).

Cisplatin-based chemotherapy is routinely used for ovarian cancers (33). We have previously shown a correlation between tumor burden, ascites formation, and
survival and response to cisplatin in ovarian carcinoma xenografts (34). Here, we have found in mice bearing 1A9-VS-1 that the decrease in plasma VEGF at the end of the treatment correlated with decreases in ascites and tumor burden compared with vehicle-treated mice. We are aware that in our animal model the values of plasma VEGF might be somewhat too high with respect to those found in the plasma of patients with cancer. However, this model, using plasma VEGF as a marker to explore the response after or during chemotherapy (or other treatments), could help avoid invasive analysis and the need for long-term survival experiments as large tumors develop in mice.

Grafting tumors onto the chorioallantoic membrane permit a study of the morphologic aspects of tumor interactions with the host’s blood vessels and the identity of the vessels that supply the grafts (21). Our results indicate that the angiogenic response is significantly higher with the VEGF_{121}-overexpressing tumor specimens implanted onto the chorioallantoic membrane than with nonamplified ones, in line with previously published data showing that tumor cell lines overexpressing VEGF induce a vasoproliferative response when adsorbed on gelatin sponges implanted on the chorioallantoic membrane (35).

In line with their angiogenic properties, VEGF_{121}-transfected cells produced s.c. tumors with strikingly different vascular patterns from parental or antisense-transfected tumors, showing severe angiectasis and wide heterogeneity in vessel caliber (Fig. 3). The induction of angiectasis by the VEGF_{121} isoform has been previously reported (36, 37). However, we noted no difference in microvessel density in the three xenograft variants and at different tumor weights (data not shown); this agrees with the previously described independence of blood vessel architectural properties from the size and rate of tumor growth (38). This is supported by other studies showing that VEGF_{121} is an efficient inducer of endothelial cell proliferation in the absence of sprouting angiogenesis (39).

Figure 6. Histological analysis of 1A9-VS-1 in peritoneal organs. Peritoneal surfaces are coated with a heavy layer of cancerous cells. The tumor may remain confined to the surface of the organs (A, omentum; B, diaphragm) or invade the parenchyma (C, pancreas; D, ovarian parenchyma). A typical vascular lesion involved severe angiectasis and parenchymal atrophy in the ovary of 1A9-VS-1 mice (E) compared with the 1A9 mice (F). Figures are representative of histologic analysis on organs from mice at day 21, as described in Fig. 5. Original magnification, ×100.
In contrast to other xenograft models in which the overexpression of VEGF resulted in increased vascularization and accelerated tumor growth (31), the growth rate of 1A9-VAS-1 xenografts transplanted s.c. was comparable to the controls. Tumor angiogenesis is regulated by a complex network of cytokine and growth factors. Therefore, angiogenic growth factors other than VEGF might be compensatorily up-regulated under VEGF deprivation. It is also possible that endogenous VEGF released by 1A9 tumor cells is enough to induce angiogenesis sprout and tumor growth. Alternatively, stroma-derived VEGF could also be implicated in tumor angiogenesis in this tumor model (40, 41). The three tumor cell variants did not differ significantly in growth in vitro either.

The worse survival of mice bearing 1A9-VS-1 i.p. was quite likely due to the accumulation of ascites as a consequence of the vascular permeability proprieties of VEGF (2). In ovarian cancer, the circulation of peritoneal fluid facilitates the dissemination of tumor cells onto the peritoneal surfaces (1), and in preclinical models of ovarian carcinoma, inhibitors of VEGF activities reduced the formation of malignant ascites, resulting in longer survival of the mice (5, 6).

A time-related increase in tumor spread was seen in mice bearing 1A9-VS-1, but, surprisingly, we found no striking vascular change in the peritoneal masses analyzed from these mice (data not shown). It has been suggested that in a more natural setting (such as the peritoneal cavity for ovarian carcinoma), tumor cells might co-opt existing mesenteric blood vessels (42). As described by Kusters et al. (39), we found that the growth of omental masses was accompanied by regression of vessels and tumor coagulative necrosis. Necrotic areas varied in size and distribution. Surviving vessels were sparse and uniform in caliber and frequently were centrally located in respect to the surrounding cuffs of viable tumor cells. We found no dilated vessels in the peritumoral zone. These features characterized all three xenograft variants (data not shown). Thus, in a peritoneal microenvironment, tumor masses consisting of several hundreds of cells may grow without inducing sprouting angiogenesis even in the presence of high levels of VEGF. That only VEGF121-overexpressing cells were associated with ascites production confirms once again that VEGF overproduction is directed to the peritoneal and mesenteric vessels, causing hyperpermeabilization and accumulation of abdominal fluid (43).

It is worth noting the typical vascular lesions in the ovary, found in mice bearing 1A9-VS-1 and associated with elevated plasma VEGF levels. VEGF can induce the growth of aberrant blood vessels and hemangiomas (44), depending on the microenvironmental VEGF levels (45). Follicular growth is dependent on the proliferation of new capillary vessels (46). Several studies have shown that VEGF expression is temporally and spatially related to the proliferation of blood vessels in the ovary (47, 48) and that VEGF inhibitors delay follicular development in rodents and primates (49). These findings suggest that the excessive tumor-produced VEGF could play a role in the etiology of ovarian lesions, which correlated with the increase in VEGF plasma levels.

In conclusion, this model of ovarian carcinoma recapitulates the behavior of ovarian cancer in patients by growing in the peritoneal cavity, where it is highly malignant and produces ascites. This xenograft model induces a high level of plasma VEGF associated with

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NOTE: The experiment was done as described in Fig. 5. VEGF levels in plasma, ascites, volume of ascitic fluid, and pathologic score were correlated between them. The correlations were evaluated using the nonparametric Spearman correlation coefficient.

\(^1\)Significance.

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<th>Table 2. Response to cisplatin (cDDP) correlates with VEGF levels and ascites volume</th>
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NOTE: Mice bearing 1A9-VS-1 xenografts growing in the peritoneal cavity were treated with cDDP (4 mg/kg i.v., days 7, 11, 15, and 19) and autopsied (\(n = 5\)) 24 hours after the last injection (day 20) for pathologic and biochemical analysis. Results are the median (range). Data are representative of two independent experiments.

Abbreviation: NS, not significant.
alterations in the vascular pattern. Because of the correlation between plasma VEGF and tumor burden, this model may serve to monitor tumor progression and the response to treatments.

Acknowledgments

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Molecular Cancer Therapeutics

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