Vascular Endothelial Growth Factor Stimulates Organ-Specific Host Matrix Metalloproteinase-9 Expression and Ovarian Cancer Invasion

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Abstract
Vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP) regulate each other, contributing to tumor progression. We have previously reported that MMP9 induces the release of tumor VEGF, promoting ascites formation in human ovarian carcinoma xenografts. The aim of this study was to investigate whether tumor-derived VEGF regulated the expression of gelatinase by the stroma, influencing the invasive properties of ovarian tumors. Tumor variants derived from 1A9 human ovarian carcinoma, stably expressing VEGF121 in the sense (1A9-VS-1) and antisense orientations (1A9-VAS-3), were used. In vivo, zymographic analysis of tumors from 1A9-VS-1 implanted in the peritoneal cavity of nude mice showed higher levels of gelatinases, particularly murine MMP9, indicating that VEGF stimulates host expression of the matrix-degrading enzyme. Murine MMP9 expression was also high in the ovaries of mice bearing 1A9-VS-1 tumors. The effect on host MMP9 activity was organ-specific. The levels of host pro-MMP9 in ovaries correlated with the plasma levels of tumor VEGF and with the selective invasion of the ovaries. Induction of host MMP9 expression in tumors and ovaries was independent of the site of tumor growth as it was seen in mice carrying both intraperitoneal and subcutaneous tumors. The anti-VEGF antibody bevacizumab (Avastin) inhibited MMP9 expression and tumor invasion in the ovaries of mice bearing 1A9-VS-1 tumors. These findings point to a complex cross-talk between VEGF and MMPs in the progression of ovarian tumor and suggest the possibility of using VEGF inhibitors to affect MMP-dependent tumor invasion.


Introduction
Ovarian carcinoma is the fourth leading cause of cancer death in women (1). The high mortality from this disease reflects the fact that it is often at an advanced stage when diagnosed. Usually, symptoms become detectable when the disease extends outside the pelvis, but by this time, treatment and prognosis may be seriously compromised (2). The accumulation of ascites in the peritoneal cavity facilitates multifocal dissemination of ovarian tumor cells onto the intraperitoneal surface, with variable degrees of stromal invasion, and may directly influence the clinical course.

Pelvic organs, such as the uterus and fallopian tubes, are probably invaded by direct extension, whereas the mechanism behind tumor spread to other abdominal structures is less clear (3). Distant lymphatic and/or hematogenous spread of ovarian carcinoma can involve any organ, including the brain (4) and the liver (5), although this is much less frequent. The pleural cavity is the most common site for stage IV disease (6).

Tumor cell invasion and metastatic spread largely depend on the tumor’s interaction with its microenvironment. Cancer cells themselves can alter the stroma to form a permissive and supportive environment for tumor progression and direct metastasis to a designated organ by producing stroma-modulating growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, and basic fibroblast growth factor (7). These disrupt normal tissue homeostasis, induce stromal reactions including angiogenesis and inflammation, and activate stromal cell types such as endothelial cells, fibroblasts, smooth muscle cells, and adipocytes, leading to the secretion of additional growth factors and proteases. In addition, growth factors mobilize normal bone marrow cells, causing them to migrate to particular regions and change the local environment so as to attract and support a developing metastasis (8, 9).

VEGF, the most important proangiogenic factor, is primarily thought to contribute to ovarian tumor progression not only on account of its proangiogenic activity, but also through its ability to increase the permeability of diaphragmatic and tumor-associated vasculature, contributing to the formation of ascites. Previous studies have reported high levels of VEGF in the plasma and ascites of patients with ovarian carcinoma (10). VEGF levels correlate with the progression of the disease, and with response to chemotherapy in human ovarian carcinoma xenografts (11, 12). VEGF inhibitors reduce ascites formation and dissemination in experimental ovarian tumor models (13).
MMP9 expression in the two tumor variants, suggesting that enzyme (Fig. 1). In contrast, there were no differences in human tumor cells stimulated host expression of the matrix-degrading murine origin of the enzyme, showing that VEGF produced by molecular weight of the pro-MMP9 detected indicated the higher levels of pro-MMP9, activated MMP9, and pro-MMP2 than 1A9-VAS-3 intraperitoneal tumors. Interestingly, the overexpression of 1A9-VS-1–bearing mice accumulated ascites in the peritoneal cavity and had high levels of MMP9 and MMP2 in cell invasion, 1A9-VS-1–conditioned medium induced more endothelial cell invasion and motility in human umbilical vein endothelial cells (HUVEC; Fig. 2A).

Several findings suggest an interplay between VEGF and matrix metalloproteinases (MMP) in ovarian cancer invasion and metastasis (14-17). We described a functional link between gelatinase activation, VEGF release, and ascites formation in human ovarian carcinoma models (11). VEGF, MMP2, and MMP9 are all expressed in the ascites and plasma of patients with ovarian cancer (10) and a subset of MMPs causes the proteolytic cleavage of VEGF in ascites from such patients (18).

There is thus a possible relationship between VEGF, matrix-degrading enzymes, and cancer progression. However, there is only scant evidence to indicate precisely how VEGF contributes to creating a permissive microenvironment for ovarian tumor cell invasiveness. Therefore, using a model of VEGF-overexpressing human ovarian carcinoma, we investigated whether tumor VEGF affects organ-specific proteolytic activity and promotes ovarian tumor cell invasiveness by inducing stromal gelatinase expression.

Results
Host MMP9 Is Increased in Tumors Overexpressing VEGF
To assess the role of tumor VEGF in the regulation of MMP activity, we used a model of human ovarian carcinoma consisting of two cell variants derived from the 1A9 line, stably transfected with VEGF121 cDNA in the sense (VEGF-overexpressing 1A9-VS-1) and antisense orientations (1A9-VAS-3; ref. 12). 1A9-VS-1 or 1A9-VAS-3 cells transplanted in the peritoneal cavity of nude mice produced tumors resembling human ovarian cancer. Only 1A9-VS-1–bearing mice accumulated ascites in the peritoneal cavity and had high levels of VEGF in the plasma and ascites. Three weeks after tumor transplantation, the presence of gelatinases in the intraperitoneal tumor masses was analyzed by gelatin zymography. The VEGF-overexpressing 1A9-VS-1 intraperitoneal tumors had higher levels of pro-MMP9, activated MMP9, and pro-MMP2 than 1A9-VAS-3 intraperitoneal tumors. Interestingly, the molecular weight of the pro-MMP9 detected indicated the murine origin of the enzyme, showing that VEGF produced by tumor cells stimulated host expression of the matrix-degrading enzyme (Fig. 1). In contrast, there were no differences in human MMP9 expression in the two tumor variants, suggesting that VEGF overproduction does not affect MMP9 expression by the tumor cells themselves.

In vitro experiments confirmed that tumor-derived VEGF had no autocrine effect because neither gelatinase production nor cell invasiveness and motility differed significantly in the two ovarian cell variants (data not shown).

Tumor-Derived VEGF Influences Gelatinase Expression in Endothelial Cells In vitro
In vitro VEGF released by 1A9-VS-1 cells stimulated the proteolytic activity of endothelial cells, potential targets of the angiogenic factor. Conditioned medium of 1A9-VS-1 cells induced greater gelatinase expression in the endothelium compared with 1A9-VAS-3–conditioned medium. Pro-MMP9 expression in particular was increased in murine endothelial cells from the lung capillaries (1G11) and aorta (MAE), whereas both pro-MMP9 and pro-MMP2 were increased in human umbilical vein endothelial cells (HUVEC; Fig. 2A).

SU5416, a small molecule inhibiting VEGF receptor-2 (VEGFR-2; KDR/flk), and DC101 or 1121b, two monoclonal antibodies, respectively, antimurine and human VEGFR-2, were used to prove the specificity of the effect. Figure 2B illustrates the results in HUVEC. SU5416 and the antihuman VEGFR-2 monoclonal antibody 1121b stopped gelatinase expression stimulation induced by 1A9-VS-1–conditioned medium. Results were similar with the murine endothelial cells 1G11 and MAE, using the antimurine VEGFR-2 monoclonal antibody DC101 (data not shown). Reflecting the role of MMP9 and MMP2 in cell invasion, 1A9-VS-1–conditioned medium induced more endothelial cell invasion and motility in the Boyden chamber than 1A9-VAS-3–conditioned medium. Invasion and motility were both blocked by SU5416, 1121b, or DC101 (data not shown).

We next investigated whether this increased production of MMP9 by the host cells might in turn increase the invasive capabilities of 1A9-VS-1 cells. Conditioned medium of HUVEC stimulated by 1A9-VS-1 and containing higher amounts of pro-MMP9 and pro-MMP2 induced greater 1A9-VS-1 cell invasion in the Boyden chamber than conditioned medium of HUVEC untreated or stimulated by 1A9-VAS-3 (Fig. 2C). The specificity of the effect was verified using recombinant MMP9 (10 ng/mL) as a chemoattractant (Fig. 2D).
Host MMP9 Is Increased in the Ovaries of 1A9-VS-1 Transplanted Mice and Is Inhibited by Bevacizumab (Avastin)

We next investigated whether VEGF’s effect on the proteolytic activity of host cells extended beyond the tumor site, and also occurred at distant organs. Three weeks after intraperitoneal 1A9-VS-1 or 1A9-VAS-3 tumor cell transplantation, zymographic analysis was done on protein extracts from liver, lung, spleen, uterus, kidney, and pancreas of mice with tumors in the peritoneal cavity, or in healthy mice. The organs of mice with tumors in the peritoneal cavity could generally be divided into two types according to their gelatinolytic profile: liver, lung, spleen, and uterus had higher levels of gelatinases than healthy mice independent of VEGF production (this was observed in both 1A9-VS-1– and 1A9-VAS-3–bearing mice), whereas kidney and pancreas had gelatinase levels similar to healthy mice (Fig. 3). Zymography of protein extracts from lung and spleen of mice injected with 1A9-VAS-3 showed a gelatinolytic band with an apparent molecular weight of 130 kDa, possibly representing a homodimer of the 68 kDa spontaneously truncated pro-MMP9 (19), or a complex of MMP9 with α2-microglobulin–related protein (20) or lipocalin (21). The ovaries of tumor-bearing mice had a peculiar gelatinolytic profile. Zymographic analysis indicated that murine pro-MMP9 and its activated form were higher in mice transplanted with 1A9-VS-1 than in 1A9-VAS-3 and healthy mice (Fig. 4A). Interestingly, murine pro-MMP9 expression in ovaries of 1A9-VS-1–bearing mice significantly correlated with the levels of human VEGF in plasma ($P < 0.0001$, $R^2 = 0.829$; Fig. 4B). Immunohistochemical analysis were done in order to verify the cellular source of MMP9 in the ovaries. Ovaries of 1A9-VS-1–transplanted mice showed a strong staining for MMP9 in inflammatory cells, morphologically referable to neutrophils, and in the granulosa cells of growing follicles (Fig. 5A). The infiltration of inflammatory cells was more pronounced in ovaries of 1A9-VS-1–bearing mice compared with ovaries of 1A9-VAS-3–bearing mice. Ovaries of 1A9-VAS-3–injected mice presented a faint immunostaining for MMP9 mostly in granulosa cells of growing follicles (Fig. 5B).

Ovaries of 1A9-VS-1, but not 1A9-VAS-3 tumor–bearing mice, presented severe dilation of vessels (angiectasis) that correlated with the high VEGF plasma levels (12) and with the presence of inflammatory cells in the ovaries (Fig. 5A).

To verify whether the site of tumor growth influenced host MMP9 induction, 1A9-VS-1 or 1A9-VAS-3 cells were injected subcutaneously in nude mice, where they produced tumors. As in the peritoneal tumor masses, host MMP9 expression was also increased in subcutaneous 1A9-VS-1 tumors 3 weeks after injection (Fig. 6), suggesting that the induction of host MMP9 by tumor VEGF did not depend on the site of tumor growth. Host MMP9 expression also increased in the ovaries of these mice (data not shown), indicating that tumor VEGF can affect host proteolytic activity even at distant sites.

FIGURE 2. Effect of VEGF-overexpressing cell-conditioned medium on endothelial cell gelatinase production. Murine capillary endothelial cells from the lung (1G11), murine aortic endothelial cells (MAE), and HUVEC were incubated with conditioned medium of 1A9-VS-1 and 1A9-VAS-3 cells depleted of endogenous gelatinases, VEGF (10 ng/mL), or control medium. A. After 24 h of incubation, gelatinase expression in endothelial cells was evaluated. B. Effect of SU5416 (5 μmol/L) and 1121b (1 μg/mL) on HUVEC gelatinase expression. C. Effect of conditioned medium of untreated HUVEC (striped column) stimulated by 1A9-VAS-3 (white column) or 1A9-VS-1 (black column) on 1A9-VS-1 cell invasion. Columns, mean number of cells which migrated in 10 high-power fields; bars, SD; *, $P = 0.01$, compared with untreated cells (ANOVA followed by Bonferroni Dunn post hoc test). D. Effect of recombinant MMP9 (10 ng/mL) used as the attractant on 1A9-VS-1 cell invasion. Columns, mean number of cells which migrated in 10 high-power fields; bars, SD; *, $P = 0.004$, compared with control cells (Student’s $t$ test). Results are representative of at least three independent experiments.
To prove the specificity of the effect, we investigated whether inhibiting VEGF had an effect on host MMP9 expression. Nude mice were injected subcutaneously with 1A9-VS-1 cells and treated with bevacizumab. Bevacizumab (150 μg/200 μL) or vehicle (saline) was injected intraperitoneally every 3 days for 4 weeks, starting 2 days after tumor cell transplantation. This treatment resulted in a marginal (treated versus control, 80-90%) effect on subcutaneous tumor growth. Mice were killed 24 hours after the last injection and the ovaries were collected and processed for zymographic analysis, as described above. Mice treated with bevacizumab showed a strong drop in host MMP9 expression in the ovaries compared with vehicle-treated mice (Fig. 7). Weak MMP2 inhibition was also evident, indicating that VEGF has only a limited effect on the expression of this gelatinase.

The Selective Invasion of the Ovaries of Mice Bearing 1A9-VS-1 Is Inhibited by Bevacizumab

The 1A9-VS-1 tumor model sporadically invades the ovaries after intraperitoneal transplantation. Histologic analysis of intraperitoneal organs showed selective invasion of the ovarian parenchyma in 30% of mice (11/36 mice with invaded ovaries in three separate experiments). In contrast, only 7% of mice bearing 1A9-VAS-3 cells presented ovary invasion (1/15 mice with invaded ovaries in two experiments; Fig. 8A and B). This suggests that the increased proteolytic activity in the ovaries caused by tumor VEGF might favor the invasion of this organ by tumor cells.

To confirm this hypothesis, we investigated whether inhibition of VEGF could prevent the invasion of the ovary in mice bearing 1A9-VS-1. Nude mice were injected intraperitoneally with 1A9-VS-1 cells and treated with bevacizumab. Bevacizumab (150 μg/200 μL) or vehicle (saline) were injected intraperitoneally every 3 days for 4 weeks, starting 2 days after tumor cell transplantation. Mice were killed 24 hours after the last injection and the ovaries and adjacent tumor masses were collected and processed for histologic analysis as described above. After 4 weeks, bevacizumab significantly reduced tumor burden in the peritoneal cavity (P = 0.0241; Fig. 8C) and completely abolished invasion of the ovaries (0/10 mice with invaded ovaries), supporting our hypothesis that circulating VEGF is responsible for the induction of ovarian invasion in 1A9-VS-1 tumor–bearing mice.

These findings further confirm the role of VEGF in the pattern of ovarian cancer spread by inducing stromal MMP9 expression, and indicate the possibility of using VEGF inhibitors to affect MMP-dependent tumor invasion.

Discussion

Epithelial ovarian cancer invasion requires a series of coordinated events depending on the communication of epithelial cells with the underlying ovarian stroma and the peritoneal microenvironment. We have shown that ovarian cancer cells overexpressing VEGF121, injected intraperitoneally in nude mice, induced host MMP9 expression specifically in the ovaries and in tumor masses. In addition, host pro-MMP9 levels in ovaries correlated with VEGF levels in plasma. These findings point to an additional important role for VEGF in ovarian cancer invasion. VEGF plays a critical role in ovarian cancer progression by enhancing vascular permeability, promoting new vessel growth and inducing ascites formation, and

![Figure 3](https://mcr.aacrjournals.org/ijc/article-pdf/6/4/528/409721/mcr-2008-0372-D-00002.pdf)
hence, tumor cell dissemination (22). In addition, this study shows that by promoting host MMP9 expression, tumor VEGF enhances the tumor cell’s invasive potential. Local tissue environmental changes such as effects on protease and growth factors, matrix remodeling, and inflammatory response may be critical to tissue-specific invasion (23, 24).

Our results, showing the murine origin of MMP9 in tumor masses and in ovaries of mice injected with 1A9-VS-1 cells, are in agreement with the previous identification of host cells as the source of MMP9 and is responsible for promoting the growth of ovarian cancer (17). We also noted an increase of MMP2 levels in intraperitoneal tumor extracts, although zymographic analysis did not distinguish between human MMP2 secreted by tumor cells xenografted in nude mice and murine MMP2 derived from host cells. However, because in vitro VEGF did not affect MMP2 expression in 1A9-VS-1 cells, it is unlikely that tumor MMP2 influences ovarian tumor progression.

Other studies have shown that in vitro VEGF-regulated ovarian cancer invasion involves the expression and activation of tumor MMPs via VEGFR-1 and VEGFR-2 (25). However, in vitro, VEGF released by ovarian tumor cells had no direct influence on either tumor cell invasion or tumor gelatinase expression. In addition, 1A9-VS1 and 1A9-VAS-3 cells did not express VEGFR-1 and VEGFR-2, and their expression was not induced when tumor cells were injected in nude mice (data not shown). In our model, therefore, VEGF seemed to have no autocrine effect. Tumor VEGF, however, stimulated invasion, motility, and gelatinase release by murine and human endothelial cells used as a prototype of VEGF-responsive host cells. Besides endothelial cells, other cells can express MMP9, including pericytes, macrophages, or hematopoietic cells (26).

Indeed, immunohistochemical analysis revealed that the cells mostly responsible for the production of host MMP9 in the ovaries of 1A9-VS-1 transplanted mice were inflammatory cells morphologically referable to neutrophils. Accordingly, the higher expression of murine pro-MMP9 detected by zymography correlated with an increased infiltration of MMP9-positive inflammatory cells in ovaries of 1A9-VS-1-bearing mice compared with 1A9-VAS-3-bearing mice. These data indicate that, similar to what is observed in other tumor models (27), VEGF promotes neutrophil recruitment to the ovary leading to increased host MMP9 secretion.

We and others have shown that metalloproteinases, particularly MMP9, regulate VEGF bioavailability in ovarian carcinoma xenografts (11, 18). The fact that tumor VEGF induces the expression of stromal MMP9 in nude mice transplanted with ovarian tumor xenografts suggests a positive loop between VEGF and MMP9 in ovarian cancer progression. MMP9 regulates VEGF bioavailability, and in turn, VEGF stimulates MMP9 expression. The high levels of VEGF and MMP9 found in the plasma and ascites of patients with ovarian carcinoma (10), and in the plasma and ascites of nude mice with human ovarian carcinoma xenografts (11), seems to confirm the importance of this mutual regulation in ovarian cancer progression.

We have shown that VEGF stimulation of host MMP9 expression is specific for the ovaries of nude mice bearing 1A9-VS-1 tumors overexpressing VEGF. The involvement of VEGF-induced MMP9 in ovarian tumor invasion is sustained.

FIGURE 4. Gelatinase expression in the ovaries of 1A9-VS-1–transplanted and 1A9-VAS-3–transplanted mice. Nude mice were transplanted intraperitoneally with 1A9-VS-1 and 1A9-VAS-3 cells. A. Gelatinase expression in ovaries was evaluated by zymography 3 wk after tumor transplantation. Two mice per group, representative of eight tested. Conditioned medium of HT1080 fibrosarcoma cells and NIH3T3 fibroblasts was used as reference for human and murine MMP9 and MMP2, respectively. B. Murine pro-MMP9 expression levels in ovaries (n = 16) and VEGF levels in plasma were correlated using linear regression analysis (P = 0.01, R^2 = 0.829).
by histologic analysis, which showed that the ovaries of mice transplanted with ovarian tumor cells overexpressing VEGF were invaded more than those of mice transplanted with ovarian tumor cells releasing low levels of VEGF. Interestingly, despite similar degrees of intraperitoneal metastatic spread, ovaries were the only organs invaded differently by the tumor variants releasing high and low levels of VEGF. In addition, the anti-VEGF antibody bevacizumab inhibited both MMP9 expression and tumor invasion in the ovaries of mice bearing 1A9-VS-1 tumors.

The organ specificity of host MMP9 up-regulation and of vessel dilation induced by VEGF is probably due to the physiology of the ovary, which makes it more responsive to VEGF stimulation. Corpus luteum development and regression is a very useful model for studying VEGF/MMP relationships. During the very early luteal phase, high MMP activities coupled with high VEGF levels drive the tissue to an angiogenic phenotype, enabling the corpus luteum to grow under luteinizing hormone stimulus. In the late luteal phase, low VEGF and high MMP levels play a role in apoptotic tissue and extracellular matrix remodeling during structural luteolysis (28, 29). It is conceivable that the ovary’s physiologic responsiveness to VEGF creates an environment in which tumor progression is supported by high proteolysis and vascularization.

We have shown that other organs of tumor-bearing mice can be divided into two types in relation to their proteolytic activity: (a) organs that present a general increase in gelatinases compared with organs of healthy mice (liver, lung, spleen, and uterus), but independently from the levels of VEGF production, and (b) organs that have similar gelatinase levels (kidney and pancreas) to healthy mice. In our model, VEGF-dependent proteolytic activity did not seem to play a major role in all of these organs. The VEGF-independent increase of gelatinases in the lungs of tumor-bearing mice compared with healthy mice, in our model, is worth noting. This can be explained by the fact that ovarian tumors secrete several other factors besides VEGF, which can induce the production of host proteolytic enzymes (3), and the pleural cavity is the most common extraperitoneal site to which stage IV epithelial ovarian cancer preferentially metastasizes (6). Increased host proteolytic activity would therefore endorse pleural cavity colonization by ovarian tumor cells.

Bevacizumab has been used to treat ovarian carcinoma xenografts (30, 44). There are several reports of single-agent bevacizumab having considerable activity as a second-line therapy of ovarian cancer (~15% objective response rate; ref. 31). In addition, a number of novel combinations with other biological therapeutics targeting epidermal growth factors/receptors and platelet-derived growth factors/receptors, metronomic cytotoxic chemotherapy and immunotherapeutics are being developed in patients with recurrent ovarian cancer (32). However, the mechanism by which bevacizumab influences ovarian carcinoma progression remains to be clarified.

We have shown that, besides inhibiting ascitic liquid formation and intraperitoneal tumor burden, bevacizumab affects VEGF-induced MMP9 expression. On the basis of our results, suggesting an interplay between VEGF and MMP9 in ovarian cancer invasion, three classes of ovarian cancer therapeutic targets could therefore be envisioned: metalloproteinases, host cells, and VEGF. We and others have already reported that batimastat (BB-94), a MMP inhibitor that inhibits both MMP2 and MMP9, affected ascites formation and delayed ovarian tumor growth in mice, prolonging their survival (33, 34). However, clinical trials with MMP inhibitor have thus far given disappointing results, highlighting the need for a better understanding of the mechanisms by which this family of multifunctional enzymes contributes to tumor progression (35).

Our findings suggest the possibility of using VEGF inhibitors in an adjuvant setting to prevent invasion. Further studies are planned to investigate whether VEGF/VEGFR inhibition complements the activity of specific MMP inhibitors in ovarian cancer treatment.

**Materials and Methods**

**Cells**

1A9-VS-1 and 1A9-VAS-3 clones, generated by stable transfection of the 1A9 human ovarian carcinoma cell variant derived from A2780 (36) with human VEGF121 cDNA in the sense (1A9-VS-1) and antisense (1A9-VAS-3) orientations (12),...
were cultured in RPMI 1640 supplemented with 10% FCS (Life Technologies, Inc.), 1% glutamine, and 500 μg/mL of G418. HUVEC were isolated from umbilical cord veins and grown as described previously (37). Cells were used between the third and fifth passage. Murine aortic endothelial cells (MAE; ref. 38) and murine capillary endothelial cells from the lung (1G11; ref. 39) were maintained in DMEM supplemented with 20% FCS (Life Technologies, Inc.).

For preparation of the conditioned medium, 2 × 10^6 tumor cells were seeded in T25 flasks in culture medium for 48 h. Subconfluent cultures were washed thrice with saline and incubated with serum-free medium for 2 h. After one more wash, cells were incubated for 48 h in serum-free medium. Conditioned medium was collected, centrifuged, and used to measure either tumor gelatinase contents or to stimulate endothelial cell gelatinase expression and invasion as described (11). When used to stimulate endothelial cells, tumor cell–conditioned medium was incubated overnight with gelatin Sepharose (Amersham Bioscience) at 4°C to deplete gelatinases released by tumor cells.

SU5416, an inhibitor of VEGFR-2 (KDR/flk; ref. 40), and DC101 and 1121b, antimurine and human VEGFR-2 monoclonal antibodies, respectively (both supplied by ImClone Systems Incorporated; refs. 41, 42), were incubated with murine or human endothelial cells 2 h before and throughout the stimulation with tumor-conditioned medium. SU5416 was dissolved in DMSO (stock solution, 4 × 10^-2 mol/L) and diluted with medium before use.

**Zymographic Analysis**

Zymography was done using SDS-polyacrylamide (8%) gels copolymerized with 1 mg/mL of gelatin (Sigma Chemical Co.), as previously described (11): 30 μg of protein were loaded for all samples. Conditioned medium of HT1080 fibrosarcoma cells and NIH3T3 fibroblasts were used as reference standards, respectively, for human and murine MMP9 and MMP2 (11).

**VEGF Assay (ELISA)**

Human VEGF was measured by ELISA (Quantikine; R&D Systems) according to the manufacturer’s instructions. This assay specifically recognizes human VEGF121 and VEGF165. The sensitivity of the assay was 9.0 pg/mL. Each sample was analyzed in duplicate.

**Animals**

Female NCr-nu/nu mice were obtained from the animal production colony of the National Cancer Institute (National Cancer Institute-BTB-Developmental Therapeutics Program), Frederick Cancer Research and Development Center (Frederick, MD). Mice were used at 6 to 8 weeks of age. They were 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 (D.L. no. 116, G.U., Suppl. 40, Feb. 18, 1992; Circolare no. 8, G.U., July, 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358. 1, Dec. 12, 1987; Standards for the Care and Use of Laboratory Animals, United States National Research Council, Statement of Compliance A5023-01; November 6, 1998).

**Human Ovarian Carcinoma Xenografts**

Nude mice were injected intraperitoneally (orthotopic tumor growth) or subcutaneously (subcutaneous tumor growth) with 10^6 1A9-VS-1 or 1A9-VAS-3 cells. Mice with tumors in the peritoneal cavity (n = 8-10) were euthanized by carbon dioxide inhalation 3 weeks after tumor cell injection and...
autopsied. Peripheral venous blood was collected 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 min. Ascites was harvested, centrifuged at 1,200 rpm for 10 min, and the fluid volume was recorded after subtracting the pellet volume. Plasma and ascitic fluid were aliquoted and stored at −80°C until further processing. The ovaries, uterus, pancreas, liver, kidney, spleen, lung, and tumor masses were collected and either lysed in lysis buffer [1 mol/L Tris-HCl (pH 8), 500 mmol/L EDTA, 1% Triton X-100, 2% sodium azide] at 4°C for zymographic analysis, or fixed in 10% phosphate-buffered formalin and processed for histologic analysis. Tumors growing subcutaneously (n = 10-16) were harvested 3 weeks after tumor cell injection and processed for zymographic analysis as above.

Immunohistochemistry

Serial sections (5 μm) were obtained from ovaries of 1-A9-VS-1 and 1-A9-VAS-3 tumor–bearing mice and processed with the avidin-biotin complex peroxidase method (43) with a commercial immunoperoxidase kit (Vectastain Standard Elite; Vector Laboratories). Section were dewaxed and incubated with 0.5% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity and rehydrated. The primary antibody used for this study was a mouse anti-human monoclonal antibody directed against MMP9 (AF909; R&D systems) at a dilution of 1:100 in Tris buffer. The unmasking procedure was done before the application of primary antibody, boiling thesections in citrate buffer (pH 6) for 20 min. Sections were then incubated overnight at 4°C. After washing thrice in Tris buffer, the sections were incubated with horse anti-mouse biotinylated immunoglobulin for 30 min at 1:200 dilution in Tris buffer (Vector). Afterwards, the sections were incubated with the avidin-biotin complex peroxidase (Vectastain Elite, Vector Labs, Inc.) at a 1:100 dilution for 30 min at room temperature and developed in red stain with 3-amino 9-ethyl carbazole (Vector). The sections were then counterstained with Mayer’s hematoxylin. Negative control slides were incubated with secondary antibody only, replacing the primary antibodies with normal horse serum.

Drug and Treatment Evaluation

Tumor invasion and gelatinase inhibition were evaluated in nude mice bearing 1-A9-VS-1 cells intraperitoneally (n = 10) or subcutaneously (n = 10) and treated intraperitoneally with the anti-VEGF antibody bevacizumab (Avastin, Roche; ref. 44). Bevacizumab (150 μg/200 μL) or vehicle (saline) were injected intraperitoneally every 3 days for 4 weeks, starting 2 days after

FIGURE 8. Ovary invasion by 1A9-VS-1 tumors. Nude mice were transplanted intraperitoneally with 1A9-VS-1 or 1A9-VAS-3 cells. A, Selective ovarian tropism is detectable in mice 3 wk after transplantation with 1A9-VS-1 cells. Ovaries are completely effaced by a dense mass of ovarian carcinoma cells. The residual ovarian parenchyma was infiltrated and ovarian follicles were compressed by neoplastic cells (*). B, No invasion was observed in the ovaries of mice transplanted with 1A9-VAS-3 cells. Arrows, growing follicles. Original magnification, ×100. Results are representative of two independent experiments. C, Decreased 1A9-VS-1 invasion upon inhibition of VEGF by bevacizumab. Mice were injected intraperitoneally with 1A9-VS-1 cells and treated with bevacizumab (150 μg/200 μL; n = 10) or vehicle (saline; n = 9) intraperitoneally every 3 d for 4 wk, starting 2 d after tumor cell transplantation. Mice were killed 24 h after the last injection and the ovaries and the mesentery were collected and processed for histologic analysis. Data are expressed as pathologic score (see Materials and Methods). Bars, median; P = 0.02 compared with controls (Mann-Whitney U test).
tumor cell transplantation. Mice were killed 24 h after the last injection and the ovaries were collected and processed for zymographic and histologic analysis, as described above. H&E-stained sections of the ovaries and mesentery of mice bearing intraperitoneal tumors were analyzed and tumor invasion was graded according to the size of the tumor lesions. Each lesion was scored as: 1, small nodules (<10 tumor cells); 2, nodules (10-100 tumor cells); 3, nodes (>100 tumor cells); or 4, masses (macroscopically detectable nodular lesions of at least 1 mm). For each mouse, the resulting sum was recorded as the final score.

Statistical Analysis
Statistical significance was determined by the Mann-Whitney U test or the Student’s t test or by ANOVA followed by Bonferroni/Dunn post hoc test, as detailed in the figure legends. The limit of statistical significance was P < 0.05. The correlation between VEGF levels in plasma and pro-MMP9 expression in ovaries was assessed by linear regression analysis.

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