Clinical and Biological Significance of Vascular Endothelial Growth Factor in Endometrial Cancer

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Abstract

Purpose: Vascular endothelial growth factor (VEGF) is critical for angiogenesis and tumor progression; however, its role in endometrial cancer is not fully known. Therefore, we examined the clinical and therapeutic significance of VEGF in endometrial carcinoma using patient samples and an endometrioid orthotopic mouse model.

Experimental Design: Following Institutional Review Board approval, VEGF expression and microvessel density (MVD) counts were evaluated using immunohistochemistry in 111 invasive endometrioid endometrial cancers by two independent investigators. Results were correlated with clinicopathologic characteristics. For the animal model, Ishikawa or Hec-1A cancer cell lines were injected directly into the uterine horn. Therapy experiments with bevacizumab alone or in combination with docetaxel were done and samples were analyzed for markers of angiogenesis and proliferation.

Results: Of 111 endometrial cancers, high expression of VEGF was seen in 56% of tumors. There was a strong correlation between VEGF expression and MVD ($P < 0.001$). On multivariate analysis, stage ($P = 0.04$), grade ($P = 0.003$), VEGF levels ($P = 0.03$), and MVD ($P = 0.037$) were independent predictors of shorter disease-specific survival. In the murine model, whereas docetaxel and bevacizumab alone resulted in 61% to 77% tumor growth inhibition over controls, combination therapy had the greatest efficacy (85-97% inhibition over controls; $P < 0.01$) in both models. In treated tumors, combination therapy significantly reduced MVD counts (50-70% reduction over controls; $P < 0.01$) and percent proliferation (39% reduction over controls; $P < 0.001$).

Conclusions: Increased levels of VEGF and angiogenic markers are associated with poor outcome in endometrioid endometrial cancer patients. Using a novel orthotopic model of endometrioid endometrial cancer, we showed that combination of antivascular therapy with docetaxel is highly efficacious and should be considered for future clinical trials.

Endometrial cancer is the most common cancer of the female genital tract and is estimated to account for 39,080 new cases and 7,400 deaths in 2007 (1). Besides established prognostic factors in endometrial cancer, such as histologic grade, stage, depth of myometrial invasion, and pelvic lymph node metastasis, angiogenesis has also been associated with survival (2). Angiogenesis is critical for the continuous growth of tumors and the development of metastases (3). The initiation of angiogenesis is tightly regulated by the balance between proangiogenic and antiangiogenic molecules and is an early and essential event in tumor development and progression (4, 5). Among angiogenic factors, vascular endothelial growth factor (VEGF)-A plays a pivotal role in endothelial cell proliferation and increased permeability of tumor-associated blood vessels and is the focus of this article (6, 7). VEGF is expressed by a wide variety of cells, including primate and rodent endometrium. The effects of VEGF are mediated by two high-affinity transmembrane tyrosine kinase receptors, VEGFR-1 and VEGFR-2, which are expressed on vascular endothelium (8–10). Overexpression of VEGF in tumor cells enhances tumor growth and metastasis in several malignancies, including colorectal, head and neck, ovarian, and endometrial cancer (11).

In 1971, Folkman proposed that attacking the blood supply of tumors could be an effective therapeutic strategy (12). Since then, advances in the mechanistic understanding of tumor vasculature have led to antiangiogenesis strategies that are starting to show promise. For example, the Food and Drug

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Administration has recently approved an anti-VEGF antibody, bevacizumab, based on its efficacy in metastatic colorectal cancer (13). Despite these encouraging data, there is limited information about tissue-based angiogenic markers and their prognostic importance in endometrial cancer. In a small cohort of patients, Sivridis et al. found VEGF to be an independent prognostic factor for women with stage I endometrial cancer (14). Similarly, Chen et al. evaluated 53 women with endometrial cancer and found histologic grade and VEGF expression to be independent predictors of clinical outcome (15). There are some reports that show reduced survival in patients whose tumors have high microvessel density (MVD; ref. 15), but other aspects of the angiogenic phenotype of these tumors, such as correlation with VEGF expression, are lacking (16).

In the present study, we sought to evaluate the clinical relevance of MVD and VEGF-A expression in endometrial carcinoma. Additionally, we established and used an orthotopic mouse model of uterine carcinoma to investigate the therapeutic efficacy of targeting VEGF using bevacizumab alone and in combination with taxane chemotherapy.

Materials and Methods

Samples for immunostaining. Following Institutional Review Board approval, archived formalin-fixed, paraffin-embedded samples were obtained from 111 patients with primary endometrioid endometrial carcinoma who were surgically treated at the University of Texas M. D. Anderson Cancer Center between 2000 and 2004. All patients were surgically staged based on the International Federation of Gynecology and Obstetrics staging system.

Immunohistochemical staining for VEGF, CD31, proliferating cell nuclear antigen, and angiogenic markers. Human CD31 staining was done on paraffin-embedded slides. Slides were heated at 55°C on a hot plate for 4 h, deparaffinized, and hydrated to PBS. Antigen retrieval for human CD31 was done with citrate buffer (pH 6.0) in microwave at 98°C for 5 min. After PBS wash, slides were incubated in 3% H_2O_2 for endogenous blocking, washed with PBS, blocked with 5% normal horse serum and 1% normal goat serum for 20 min at room temperature, and then incubated with anti-human CD31 antibody M-0823 clone JC/70A (1:20; Dako) overnight at 4°C. The following day, slides were washed and incubated with biotinylated horse anti-mouse secondary antibody (Biocare) for 30 min at room temperature, amplified with streptavidin-horseradish peroxidase for 30 min at room temperature, detected with 3,3′-diaminobenzidine, and counterstained with hematoxylin for 35 to 60 s.

Expression of VEGF, interleukin-8, and basic fibroblast growth factor was assessed in paraffin-embedded slides. Primary antibody incubation was done with rabbit polyclonal anti-VEGF (1:50; Santa Cruz Biotechnology), interleukin-8 (1:25; Biosource International), and basic fibroblast growth factor (1:1,000; Sigma) at 4°C overnight. Appropriate horseradish peroxidase secondary antibody was applied for 1 h at room temperature, incubated with 3,3′-diaminobenzidine (Phoenix Biotechnologies) for 7 to 10 min for detection, and counterstained with Gill's no. 3 hematoxylin (Sigma) for 20 to 30 s. CD31 staining for orthotopic tumors from therapy experiments was done on frozen tissue samples as previously described (17). Slides were incubated with rat anti-mouse primary antibody m-CD31 (1:800; BD Bioscience PharMingen) overnight at 4°C and then appropriate secondary antibody for 1 h at room temperature. Proliferating cell nuclear antigen (PCNA) staining was done as previously described (17). Slides were initially incubated with fragment blocker (1:10; Jackson ImmunoResearch Laboratories) overnight at 4°C followed by primary anti-PCNA-PC10 (1:50; Dako) incubation for 3 to 4 h at room temperature and appropriate secondary antibody for 1 h at room temperature. Quantification of PCNA was done as previously described (17).

Analysis of immunohistochemical staining and MVD. VEGF expression in tumor and stromal cells was scored by two independent investigators (A.A.K. and W.M.M.), who were blinded to the clinical outcome of patients. Semiquantitative assessment of VEGF expression was done as previously described (18) by assessing the percentage of stained tumor cells and staining intensity. Briefly, the percentage of positively stained cells was rated as follows: 0 point, 0% to 5%; 2 points, 6% to 50%; and 3 points, >50%. The staining intensity was rated in the following manner: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for the intensity and percentage of positive cells were added, and a score index (SI; 0-3) was assigned. Tumors were categorized into four groups based on the SI: negative expression (SI, 0 or <5% cells stained regardless of intensity); weak expression (SI, 1), 1 to 2 points; moderate expression (SI, 2), 3 to 4 points; and strong expression (SI, 3), 5 to 6 points. For statistical analysis, the patients were dichotomized into two groups: low expression (SI, 0 or 1) included those with negative or weak expression and high expression (SI, 2 or 3) included those with moderate or strong expression. The independent scores from both investigators were consolidated into a final score, which is reported in this study. Any differences in the scores were adjudicated following discussion between the two investigators.

For CD31 staining, a vessel was defined as an open lumen with one or more CD31-positive cells adjacent to the lumen. The average derived from scores of both investigators was used in the final analysis. Using a receiver operator characteristic curve, MVD of 13.7 was used as a cutoff and predicted the greatest hazard for death due to disease. Patients were dichotomized into two groups: low MVD (<13.7) and high MVD ($\geq$13.7).

Clinicopathologic analysis. All patients underwent surgical exploration and primary surgical staging as the initial treatment. The treating gynecologic oncologist determined the adjuvant therapy. The pathologic diagnosis was verified by the pathology reports. A gynecologic pathologist (D.C.) reviewed all the H&E slides to confirm the histopathologic diagnosis and tumor grading. Based on International Federation of Gynecology and Obstetrics stage, patients were divided into two groups: low stage (International Federation of Gynecology and Obstetrics stages I and II, $n = 77$) and high stage (International Federation of Gynecology and Obstetrics stages III and IV, $n = 34$). A clinical remission was defined as no evidence of disease based on physical examination and/or imaging studies. Disease-specific survival (DSS) was defined as the time from treatment completion until the date of death or the date of last contact.

Cell line and cultures. Endometrial cell lines Ishikawa (19) and Hec-1A (20) were maintained and propagated in MEM (Ishikawa) and McCoy's 5A (Hec-1A) supplemented with 10% fetal bovine serum and 1% penicillin (Life Technologies) at 37°C. The Ishikawa and Hec-1A cell lines were obtained from Dr. Russell Broaddus (M. D. Anderson Cancer Center, Houston, TX). All experiments were conducted at 60% to 80% confluent cultures. Before *in vivo* injection, cells were trypsinized, centrifuged at 1,000 rpm for 7 min at 4°C, washed twice with HBSS, and resuspended in HBSS for intrauterine injections. Both cell lines were tested for murine antigen reactivity and *Mycoplasma* species before injection into mice.

Cell viability assay. To test the sensitivity of both endometrial cell lines to docetaxel, 2,000 cells per well were plated into a 96-well plate, allowed to adhere overnight, and treated in triplicate with varying concentrations of docetaxel (0.001-1,000 nmol/L) in serum-containing medium. After 5 days, cell viability was assessed by adding 50 μL of 0.15% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) to each well. After 2 h of incubation at 37°C, medium/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was removed, 200 μL DMSO (Sigma) was then added to each well, and the absorbance at 570 nm was recorded using a Falcon plate reader

(Becton Dickinson Labware). The IC_{50} was determined by calculating the mean absorbance at 570 nm [(max absorbance - min absorbance) / 2 + min absorbance] and then determining the dose of docetaxel at which this absorbance reading intersected with the dose-response curve.

Development of orthotopic uterine model. Female athymic nude mice were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. They were cared for in accordance with the guidelines set forth by the American Association for Accreditation for Laboratory Animal Care and the USPHS Policy on Human Care and Use of Laboratory Animals, and all studies were approved and supervised by the M. D. Anderson Cancer Center Institutional Animal Care and Use Committee. Before injection, mice were anesthetized with 200 μL nembutal i.p. and a 0.5-cm incision was made in the right lower flank to optimize exposure to the right uterine horn. The distal portion of the horn was then identified and pulled to the incision for exposure. A single-cell suspension of 50 μL was then injected into the lumen of the uterine horn. The injection site was closely monitored during and following injection to ensure no spillage into the peritoneal cavity occurred. The incision was then closed with staples.

To characterize tumor growth kinetics, varying concentrations of cell suspensions (1×10^6 to 4×10^6 cells/mL) were injected into the right uterine horn of nude mice. Tumor growth was assessed by palpation and mice were sacrificed when tumors were >1 cm or mice seemed moribund. At the completion of the experiment, tumor growth patterns and animal survival were recorded for each cell line. Histologic confirmation of tumor was done with H&E staining.

In vivo *bioluminescence imaging*. To ensure that there was no spillage of tumor cells during the intrauterine injection, luciferase-tagged human cancer cells were injected into the right uterine horn as described previously (21). For longitudinal assessment, bioluminescence imaging (22) was conducted 2 and 14 days after tumor cell injection. Fifteen minutes before imaging, mice were injected i.p. with 15 mg/mL of luciferin potassium salt in PBS at a dose of 150 mg/kg. Tumor volume and locations were assessed.

Antivascular therapy of endometrial tumors in nude mice. Therapy experiments were designed using human endometrial cancer cell lines, Ishikawa and Hec-1A. For these experiments, 4×10^6 cells were used because this was the lowest concentration required for consistent tumor growth. Following cell line injection, mice were randomized into four treatment groups: (*a*) control, 200 μL PBS (i.p., twice weekly); (*b*) bevacizumab, 5 mg/kg in 200 μL PBS (i.p., twice weekly); (*c*) docetaxel, 2.5 mg/kg in 200 μL PBS (i.p., weekly); and (*d*) bevacizumab and docetaxel (both drugs given at doses and frequency described above for each drug alone). Therapy was initiated 2 weeks following cell line injection. Mice were monitored for adverse effects and sacrificed by cervical dislocation 6 to 7 weeks following initiation of treatment. At the completion of each experiment, mouse weights, tumor weights, tumor location, number of tumor nodules, and presence of ascites were recorded for each treatment group. Tumor specimens were processed for further analysis by preservation in OCT (Miles, Inc.) medium (for frozen slides) as well as fixed in formalin (for paraffin slides).

Effects on tumor vascular perfusion following bevacizumab therapy. To assess the effects of bevacizumab therapy on the perfusion of endometrial tumors, mice with formed Ishikawa tumors were treated with bevacizumab (5 mg/kg, i.p., every 3 days) for three doses. Before sacrifice, mice were anesthetized and injected i.v. with 100 μL of fluorescein *Lycopersicon esculentum* lectin (Vector Laboratories; ref. 22). Ten minutes later, the abdominal and thoracic cavities were exposed and the ascending aorta was infused with 20 mL of 4% paraformaldehyde (Poly Scientific) over a 2-min interval. During the perfusion, the portal vein was isolated and transected to facilitate adequate perfusion. Tumors were then harvested and placed immediately into

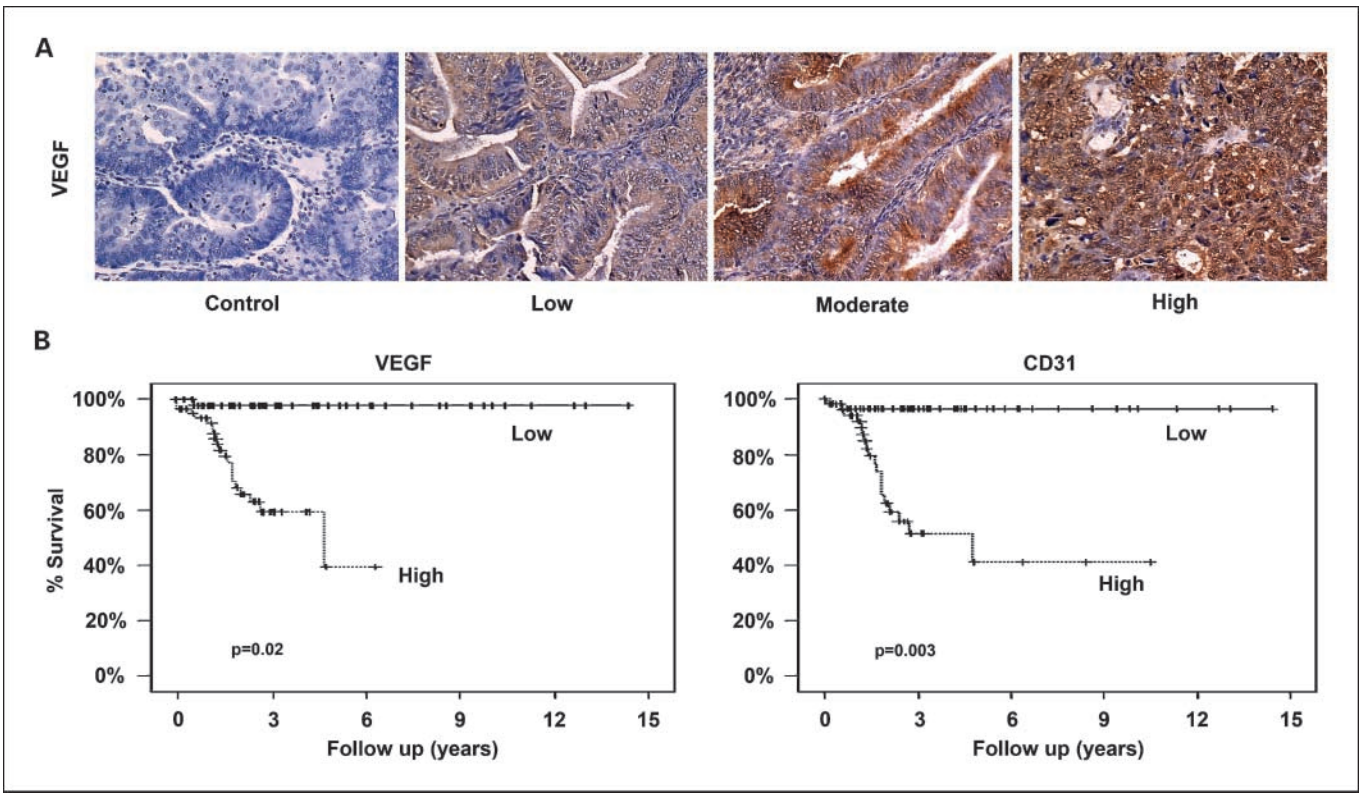

Fig. 1. Immunohistochemical analysis of VEGF and CD31 expression. *A,* immunohistochemical expression pattern of VEGF in human endometrial adenocarcinoma specimens showing negative control, low, moderate, and high expression. Original magnification, ×100. *B,* Kaplan-Meier survival curves for VEGF expression (*left*) and CD31 (*right*). High levels of VEGF and high MVD were both significantly associated with decreased DSS.

Table 1. Association of clinicopathologic variables with VEGF expression and MVD counts

Variable	VEGF expression			MVD		
	Low	High	*P*	Low	High	*P*
Stage						
Low (I/II)	41	36	0.004	46	31	
High (III/IV)	8	26		13	21	0.03
Grade						
1 or 2	42	46		51	37	
3	7	16	0.11	8	15	0.04
Depth of invasion						
≤1/2	36	42		42	36	
>1/2	13	20	0.51	17	16	0.82
MVD						
Low (<13.7/HPF)	39	20				
High (≥13.7/HPF)	10	42	<0.001			

Abbreviation: HPF, high-power field.

4% paraformaldehyde for 2 h at room temperature and then transferred to 30% sucrose and stored overnight at 4°C. The following day, tumors were embedded in OCT medium and stored at -80°C for 24 to 48 h. Tissue sections (30 μm) were prepared and analyzed by confocal fluorescence microscopy.

Statistical analysis. For human samples, χ^2 or Fisher's exact tests were used, as appropriate, to test for the association in the proportions across levels of a single covariate factor and VEGF expression or MVD. Patients who were alive at last follow-up or died from causes other than uterine cancer were censored at the date of last follow-up. DSS was estimated using the Kaplan-Meier product limit method. A two-sided log-rank test was used to test for differences between survival curves. DSS was assessed using both univariate and multivariate Cox proportional hazards regression. Continuous variables were compared with the Student's *t* test (between two groups) or ANOVA (for all groups) if normally distributed and the Mann-Whitney rank sum test or Kruskal-Wallis test (for all groups) if nonparametric. For *in vivo* therapy experiments, 10 mice in each group were used, as directed by a power analysis to detect a 50% reduction in tumor size (β error 0.2). $P < 0.05$ on two-tailed testing was considered significant.

Results

Association of VEGF expression and MVD with clinical outcome. Given the paucity of information about the clinical significance of VEGF expression in human endometrial cancers, we first examined 111 samples stained for VEGF and CD31 expression (Fig. 1A). Mean age of the patients was 63.9 years (range, 39-91). Thirty-four percent of patients had advanced-stage (III and IV) disease, and 90% of tumors were grade 2 or 3. All tumors were of endometrioid histology. Low VEGF expression was present in 44% of samples, whereas 56% had high positivity. The presence of high VEGF expression was significantly associated with high stage ($P = 0.004$) and high MVD counts ($P < 0.001$). There was a trend toward an association with grade 3 tumors ($P = 0.11$; Table 1). The mean MVD was 16.3/high-power field ± 4.1 (range, 5.5-53). High MVD was associated with high-stage and grade 3 tumors ($P = 0.03$ and 0.04, respectively; Table 1). VEGF expression and MVD were not associated with depth of myometrial invasion.

Before testing the prognostic relevance of VEGF and MVD, we first did univariate analyses of traditional clinical variables for DSS. As expected, high-stage ($P < 0.001$) and grade 3 tumors ($P < 0.001$) both had a 5.5- to 7-fold increased risk of death due to disease (Table 2). Patients with tumors exhibiting high VEGF expression ($P = 0.002$) had an ~19-fold higher risk of death compared with those with low expression. Similarly, individuals with high MVD ($P = 0.001$) had a 13-fold increased risk of death compared with those with low MVD. Figure 1 depicts the Kaplan-Meier survival curves for VEGF and MVD (Fig. 1B). To assess whether there was an independent association between any of the clinicopathologic variables and DSS, we did a multivariate Cox proportional hazards model. This model included multiple prognostic variables, such as stage, grade, depth of myometrial invasion, and expression levels for VEGF and MVD counts. Advanced tumor stage ($P = 0.04$), high grade ($P = 0.003$), high VEGF levels ($P = 0.03$), and high MVD ($P = 0.037$) were found to be independent predictors of shorter survival.

Characterization of orthotopic endometrial cancer model. An *in vivo* model that recapitulates the pattern of uterine cancer growth and metastasis would be useful for the development of novel therapeutic agents. However, most of the preclinical studies in endometrial cancer to date have used s.c. or i.p. xenograft models. Given the importance of the microenvironment in cancer pathogenesis, we developed and characterized an orthotopic model of uterine carcinoma (Fig. 2).

Table 2. Univariate survival analysis of prognostic variables for DSS

Variable	Median survival (y)	HR (95% CI)	*P*
Stage			
Low (I/II)	NR		
High (III/IV)	4.81	5.53 (2.22-13.79)	<0.001
Grade			
Low (1 or 2)	NR		
High (3)	1.80	6.94 (3.27-19.27)	<0.001
MVD			
Low (<13.7/HPF)	NR		
High (≥13.7/HPF)	4.73	13.15 (3.50-42.49)	0.001
VEGF expression			
Low	NR		
High	4.76	19.34 (3.03-49.76)	0.002

Abbreviation: NR, not reached.

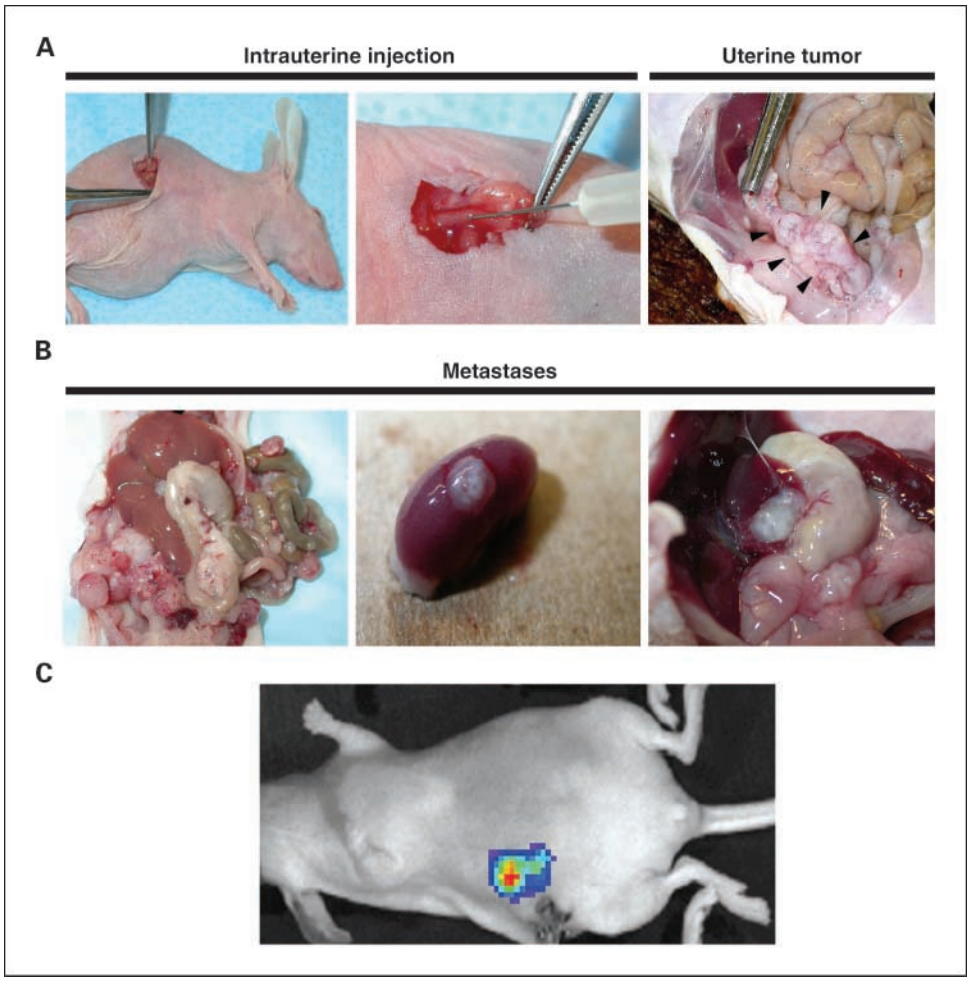

Fig. 2. Development and characterization of an orthotopic endometrial cancer model. *A,* a 0.5-cm incision was made in the right lower flank and the distal portion of the uterine horn was identified and pulled to the incision for exposure. Development of an intrauterine tumor can be visually seen. *B,* metastatic tumor implants in the bowel mesentery, kidney, and liver. *C,* imaging for luciferase activity 2 d following tumor cell injection confirmed orthotopic implantation of tumor cells at the injection site of the uterine horn.

Initial experiments were done to determine the growth kinetics of Ishikawa and Hec-1A cell lines. Tumor growth patterns and time to moribund state were recorded following injection of 1, 2, or 4 million cells per animal. Mice bearing Ishikawa tumors became moribund around day 44 and developed tumors ranging from 0.25 ± 0.13 g to 1.37 ± 0.58 g (mean ± SE) depending on the number of cells injected. Furthermore, Hec-1A–injected mice became moribund around day 54 with tumors ranging from 0.28 ± 0.05 g to 1.21 ± 0.30 g. Tumor confirmation within the uterine horn was confirmed by visual and histologic inspection (Fig. 2A). Moreover, both models produced metastatic implants to the peritoneum (>75%), bowel mesentery (50-57% of animals), lymph nodes (43-63%), kidney (<10%), and liver (<10%) depending on the cell line (Fig. 2B). Minimal ascites was noted in tumor-bearing mice. To confirm that metastases were not related to tumor cell spillage at the time of intrauterine injection, we used *in vivo* bioluminescence imaging. Luciferase-tagged tumor cells were injected into the right uterine horn of nude mice ($n = 5$) followed by *in vivo* bioluminescence imaging at 2 and 14 days following tumor cell injection. At these early time points, all animals had tumor growth confined to the uterine horn (Fig. 2C). In addition, tumor localization to the uterine horn was confirmed by necropsy at 2 weeks following injection of the Ishikawa cells.

Effects of antivascular therapy in an orthotopic endometrial cancer model. After designing and characterizing the orthotopic model, we first determined the expression of angiogenic factors in tumors from the models described above. Immunohistochemical staining showed overexpression of VEGF, interleukin-8, and basic fibroblast growth factor in both models (Fig. 3A). Antivascular therapy has shown promising results among many tumor types. However, little is known about the effects of targeting angiogenic factors in endometrial carcinoma. Before initiating *in vivo* therapy experiments, we also assessed whether Ishikawa and Hec-1A cell lines were sensitive to docetaxel using *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The IC_{50} of Ishikawa and Hec-1A cell lines was 5 and 3 nmol/L, respectively. Based on the clinical significance of high VEGF expression in endometrial carcinoma, we focused on targeting VEGF in the orthotopic model using bevacizumab. The Ishikawa and Hec-1A cell lines were injected into the uterine horn of nude mice as previously described. Two weeks after cell line injection, therapy was initiated according to the following four groups: PBS, i.p. twice weekly; bevacizumab, i.p. twice weekly; docetaxel, i.p. weekly; and bevacizumab and docetaxel. Mice were sacrificed following 6 to 7 weeks of therapy. Tumor weight and distribution in both models is listed in Fig. 3B. In the Ishikawa model, single-agent bevacizumab, docetaxel alone, and combination therapy

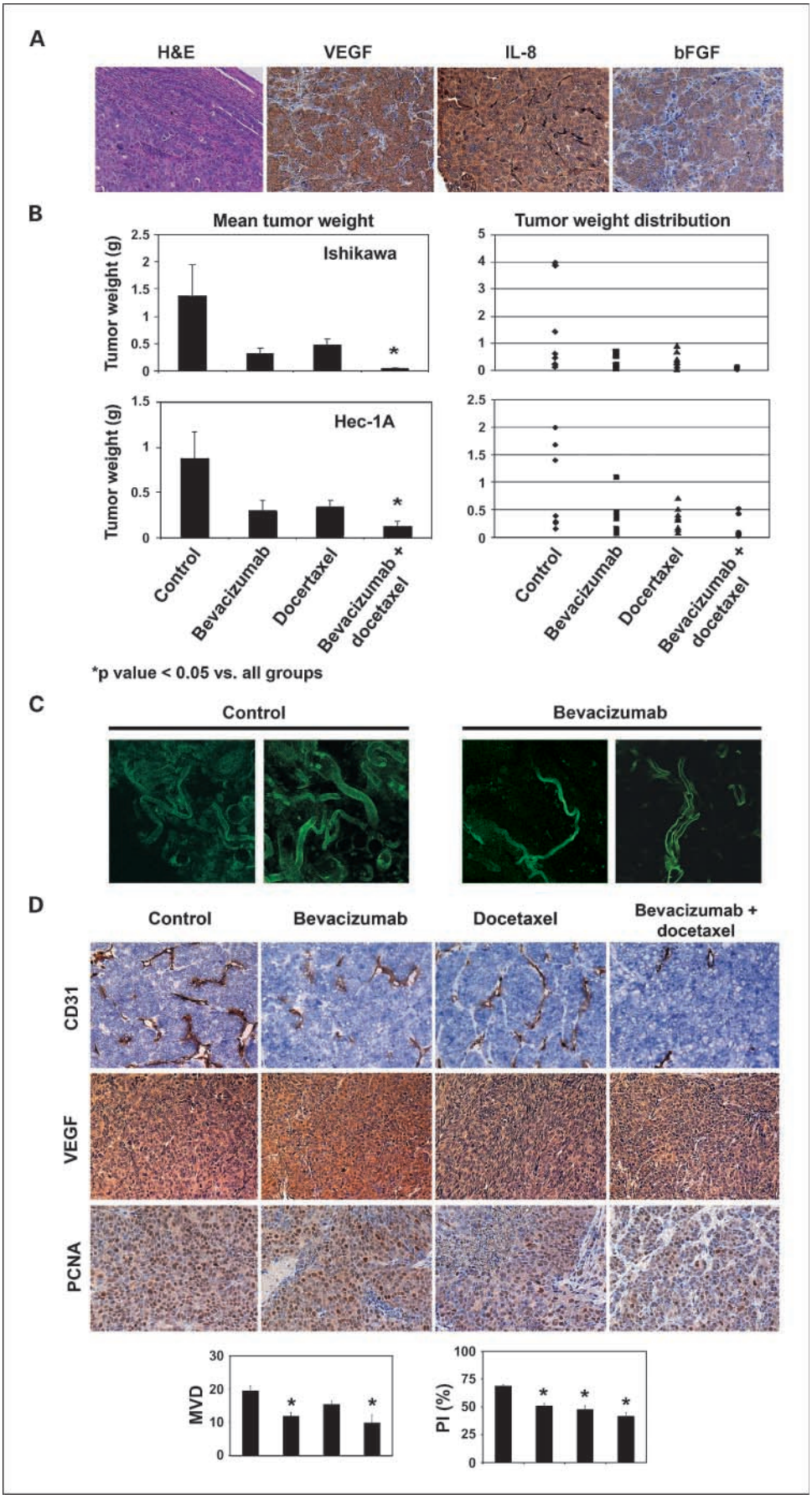

Fig. 3. Evaluation of angiogenesis in the endometrial cancer model. *A,* representative images of orthotopic tumors. Magnification, ×100. H&E staining (*left*) and immunohistochemical expression of angiogenic factors VEGF (*middle left*), interleukin-8 (*IL-8*; *middle right*), and basic fibroblast growth factor (*bFGF*; *right*). All three factors are strongly expressed in the orthotopic tumors. *B,* mice were injected with Ishikawa or Hec-1A cells and treated with PBS, bevacizumab, docetaxel, or the combination. When control mice were moribund (6-7 wk after cell injection), animals in all groups were sacrificed, tumors were excised, and tumor weights were recorded. Left, columns, mean weights; bars, SD. Right, individual weights from these experiments. *, $P < 0.05$, compared with controls. *C,* representative images of tumor vascular perfusion (×400 magnification) in PBS-treated tumors (*left two panels*) and bevacizumab-treated tumor (*right two panels*). Treatment with bevacizumab resulted in decreased tortuosity and leakiness of the vessels. *D,* Hec-1A and Ishikawa tumors collected at the conclusion of therapy experiments were stained for CD31, VEGF, and PCNA. Representative sections (final magnification, ×100) are shown for the various treatment groups (Ishikawa). A graphic representation of the average number of CD31-positive vessels per field and mean percent PCNA positive cells [proliferative index (*PI*)] are shown in the adjoining graphs (bars in graphs correspond to the columns in the figure). Five fields per slide, and at least five slides per group, were examined and compared with Student's *t* test and ANOVA. *, $P < 0.01$.

reduced mean tumor weight by 77% ($P = 0.15$), 66% ($P = 0.46$), and 97% ($P < 0.001$), respectively. Compared with single-agent treatment groups, combination therapy showed a significant effect toward tumor growth inhibition ($P < 0.01$ for both groups). Similarly, in the Hec-1A tumor-bearing mice, bevacizumab (66%; $P = 0.09$), docetaxel (61%; $P = 0.37$), and their combination (85%; $P < 0.01$) each resulted in tumor growth inhibition compared with control mice, with combination therapy having the greatest efficacy over single-agent arms ($P < 0.05$ for both groups). Overall tumor incidence, tumor nodules, and metastatic spread were also recorded for each treatment group (Table 3). The overall tumor incidence observed between treatment groups was not significantly different. However, the number of tumor nodules was significantly decreased in response to treatment in both models (Table 3). The most prevalent sites of tumor spread in both therapy experiments included the mesentery, peritoneum, and lymph nodes. Among mice that developed tumors, in the Ishikawa model, metastatic spread was as follows: controls (100%), bevacizumab (71%), docetaxel (88%), and combination (62%). Similarly, among Hec-1A animals that developed tumors, 100% of controls, 89% in the bevacizumab group, 89% in the docetaxel group, and 50% in the combination group had metastatic spread beyond the uterine horn.

To assess potential toxic effects of antivascular therapy in our murine orthotopic endometrial cancer model, we observed daily eating/drinking habits, activity levels, and mouse weights at necropsy. Based on assessments by ourselves and animal care technicians, no obvious differences were recorded among treatment groups throughout. In addition, among all treatment groups, mouse weights at the completion of each experiment were not statistically different (data not shown).

Effect of antivascular therapy on angiogenesis and proliferation. VEGF is known to exert direct proangiogenic effects on the tumor microenvironment. Therefore, we first examined the effects of short-term anti-VEGF therapy on vessel architecture using confocal microscopy. Compared with controls, tumor-associated blood vessels from treated animals were less dilated and tortuous following bevacizumab therapy (Fig. 3C).

We also assessed the long-term effects of anti-VEGF therapy on tumor vasculature by MVD analysis with CD31 staining of tumors (Fig. 3D). Compared with controls, bevacizumab therapy alone reduced MVD in the Ishikawa and Hec-1A tumors by 38% to 40% ($P < 0.01$, both cell lines). However, the most significant reduction in tumor vascularity compared with controls was observed in tumors from animals treated with combination bevacizumab and docetaxel therapy (50-70%; $P < 0.01$, both cell lines). Moreover, compared with docetaxel alone, combination therapy was more effective in decreasing tumor vascularity in both models ($P < 0.01$). There was no difference in VEGF expression between the therapy groups (Fig. 3D). Next, we examined the effects of anti-VEGF therapy on tumor cell proliferation by PCNA staining (Fig. 3D). Compared with controls, bevacizumab therapy alone and docetaxel alone decreased proliferation by 26% ($P < 0.001$) and 30% ($P < 0.001$), respectively. However, the most significant effect on proliferation was seen in the combination treatment group, which showed a 39% reduction ($P < 0.001$) in proliferation compared with controls (Fig. 3D).

Discussion

The key findings of the present study are that high expression of angiogenic markers, such as VEGF-A and MVD, is an independent predictor of poor prognosis in patients with endometrioid endometrial cancer. In addition, we developed and characterized a novel orthotopic model for endometrial cancer that recapitulates the pattern of spread seen in endometrial cancer patients. Using this model, we investigated the effect of anti-VEGF therapy using bevacizumab alone and in combination with taxane chemotherapy. Long-term therapy with bevacizumab alone and especially in combination with docetaxel was highly effective for inhibiting tumor growth and metastasis. These effects seem to be mediated in part by the inhibition of tumor vascularity. These results provide a solid foundation to test these agents in clinical trials for patients with endometrial cancer.

VEGF represents a family with multiple functions that affect tumor growth and metastasis. Specifically, VEGF-A is crucial for tumor angiogenesis and plays a key role in endothelial cell proliferation, survival, and permeability (2–8). Other isoforms of VEGF, such as VEGF-C and VEGF-D, are more important in lymphatic spread of metastases and were not evaluated in the current study (2–8). We have shown that high VEGF-A expression is associated with multiple aggressive features in uterine carcinoma. These findings are supported by a limited number of previous smaller studies. Holland et al. showed VEGF expression in 100% of the endometrial cancer specimens examined using *in situ* hybridization (23). Furthermore, they found no expression of VEGF in benign endometrial tissue and in only 20% of tissue samples with atypical hyperplasia. Immunohistochemical expression of VEGF in tumor specimens

Table 3. Characteristics of tumors after bevacizumab with or without chemotherapy

Cell line	Group	Overall tumor incidence (%)	No. nodules (mean ± SE)	*P*
Ishikawa	Control	80	19.2 ± 9.4	
	Bevacizumab	70	8.8 ± 3.8	0.64
	Docetaxel	90	2.12 ± 0.35	0.02
	Combination*	100	2 ± 0.37	0.02
Hec-1A	Control	70	10.1 ± 3.6	
	Bevacizumab	90	5.3 ± 1.53	0.56
	Docetaxel	90	3.1 ± 0.48	0.24
	Combination*	100	1.8 ± 0.32	0.028

*Combination = bevacizumab + docetaxel.

has been correlated with higher histologic grade (24), greater depth of myometrial invasion, lymphvascular space invasion, lymph node metastasis, and shorter disease-free survival (25). Another commonly used histologic marker of angiogenesis is MVD as described by Weidner et al. (26). Whereas initial studies used factor VIII–related antigen as a marker for MVD, most recent reports have used CD31 staining to assess tumor vascularity (2). Interestingly, there was a high correlation between MVD counts and VEGF expression in our samples, thus supporting a biological basis for these results. Kaku et al. have reported decreased progression-free and overall survival in endometrial cancer patients with high MVD counts by both univariate and multivariate analysis (27). Additionally, comparing patients with recurrent and nonrecurrent disease, low MVD counts were associated with a mean survival of 123 months compared with high counts (75 months; ref. 26). Even after recurrence, patients with low MVD counts fared better than those with high MVD counts (65 versus 45 months; ref. 28). In our patients, high MVD was significantly associated with DSS in multivariate analysis. Patients with high-stage disease had higher levels of VEGF expression. Among those with early-stage disease, 47% showed high VEGF expression and may represent a high-risk group that would benefit from adjuvant therapy with antiangiogenic agents or chemotherapy. There are recent data that suggest that the vascular proliferation index, which is a measure of "active" angiogenesis, may be a more sensitive marker of angiogenesis than MVD alone and is also independently related to survival in patients with endometrial cancer (29).

Tumor blood vessels are morphologically distinct from their normal counterparts; they are irregularly shaped, dilated, and tortuous and can have dead ends (4). Tumor vasculature is often leaky and hemorrhagic, which is in part due to overproduction of VEGF (30). Additionally, tumors induce capillary inflow from preexisting host vessels and are thin walled, probably due to a lack of a competent extracellular matrix due to the migratory endothelium (31). Stefansson et al. have shown that structural changes in tumor vessels indicated by reduced pericyte coverage were associated with increased frequency of invasion by tumor cells and decreased survival in patients with endometrial cancer (29). In our murine model, short-term treatment with bevacizumab resulted in less tortuosity and permeability of vessels compared with controls. Moreover, anti-VEGF therapy alone and in combination with taxane chemotherapy significantly reduced tumor vascularity, thereby resulting in growth inhibition as well as less extensive metastases. Although treated tumors did show decreased cellular proliferation, this effect was likely secondary to inhibition of angiogenesis because biological agents, such as bevacizumab, have limited effect on cellular proliferation directly but do inhibit metastatic tumor spread (26, 27).

Most preclinical studies in endometrial cancer have used either transgenic or s.c. xenograft models to study therapeutic efficacy of various drugs (32–34). Although transgenic models are useful for studying the chemopreventive effect of drugs, tumors that develop in these animals are frequently due to single gene mutation (e.g., *PTEN*) and are not aneuploid, which is not the case with human tumors (34, 35). Additionally, these models display variable penetrance, making them unreliable for therapy experiments. A major drawback of using s.c. xenograft models is that tumors grown in the s.c. region do not truly represent the characteristics of intrauterine tumors due to differences in the microenvironment (34, 35). These differences are even more relevant when evaluating the efficacy of antiangiogenic agents. The data about orthotopic endometrial cancer models are limited. Berry et al. reported the development of an orthotopic endometrial cancer model; however, they injected and studied endometrial cancer cell lines of serous histology (36). Indeed, 20% to 30% of endometrial cancers are serous and known for aggressive features; however, the majority of cases are of endometrioid histology. Therefore, in the present study, we have developed and characterized an orthotopic model for endometrial cancer using two cell lines derived from patients with endometrioid adenocarcinomas of the uterus. Additionally, the pattern of spread was representative of the natural progression of human endometrial carcinoma, making this a useful model for preclinical studies. In the present study, although the depth of tumor invasion into the myometrium could not be assessed, mice treated with bevacizumab had significantly lower incidence of metastatic spread. Furthermore, the human xenografts maintained a high level of expression of proangiogenic proteins, such as VEGF, basic fibroblast growth factor, and interleukin-8, as seen in human uterine carcinoma (23–26).

Although there are several antiangiogenic drugs in development, bevacizumab was the first antiangiogenic drug to receive Food and Drug Administration approval for the up-front treatment of patients with advanced colorectal cancer in conjunction with i.v. 5-fluorouracil–based chemotherapy (13). This approval was based on significant improvement in both progression-free and overall survival. Recently, the Gynecologic Oncology Group conducted a phase II trial of single-agent bevacizumab in patients with recurrent ovarian cancer and reported an overall response rate of 18% (37). In a retrospective clinical report, a significant improvement in progression-free survival was seen with bevacizumab after multiple cytotoxic chemotherapeutic regimens in patients with recurrent ovarian cancer ($P < 0.001$; ref. 38). Currently, bevacizumab is undergoing phase II investigation in the Gynecologic Oncology Group (protocol 229-E) as a single agent in patients with recurrent epithelial endometrial cancer. Future studies with VEGF Trap and sorafenib are planned, highlighting the interest and importance of this pathway in endometrial cancer. Based on the data presented here, bevacizumab alone or in combination with cytotoxic chemotherapy is a promising therapeutic option for patients with endometrial cancer and merits evaluation in clinical trials.

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