Expression of Angiogenesis-Related Genes and Progression of Human Ovarian Carcinomas in Nude Mice

Junya Yoneda, Hiroki Kuniyasu, Marta A. Crispens, Janet E. Price, Corazon D. Bucana, Isaiah J. Fidler*

Background: By the time patients are diagnosed with ovarian carcinoma, peritoneal dissemination of the tumor often has occurred. The progressive growth and spread of ovarian carcinoma depend, in part, on the formation of an adequate blood supply. We determined whether the expression of genes that regulate distinct steps in angiogenesis (i.e., the formation of new blood vessels) was associated with the pattern and progressive growth of human ovarian carcinomas implanted in the peritoneal cavity of nude mice. Methods: Five different human ovarian carcinomas were injected individually into the peritoneal cavity of female NCr-nu/nu nude mice. The expression of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), interleukin 8 (IL-8), and collagenase type IV (MMP-2 [matrix metalloproteinase-2] and MMP-9) was determined by northern blot analysis, in situ hybridization of messenger RNA, and immunohistochemical analysis. Blood vessel distribution and density, macrophage infiltration pattern, and stromal reaction were determined by immunohistochemical analysis with specific antibodies. Results: Three of the carcinomas produced both solid lesions and ascitic tumors, whereas the remaining two produced only solid lesions. Two of the carcinomas produced rapidly progressive disease, two produced slow disease, and one produced intermediate disease. The formation of ascites was directly associated with expression of VEGF/VPF, and survival was inversely associated with expression of IL-8. In rapidly growing tumors, the number of blood vessels was high throughout the lesion; in contrast, in slow-growing tumors, most vessels (and infiltrating macrophages) were located at the periphery. Conclusions: The expression of various genes that regulate angiogenesis in human ovarian carcinomas is associated with the pattern of the disease and its progression. Therefore, targeting specific genes that regulate angiogenesis could offer new approaches to the treatment of ovarian cancer. [J Natl Cancer Inst 1998;90:447–54]

More than 26,000 new cases of ovarian cancer were diagnosed in the United States in 1996 (1), and the disease has a mortality rate of about 50%, making it the leading cause of death from gynecologic cancer (1,2). By the time of diagnosis, metastasis has occurred within the peritoneum of the majority of patients (3,4). The most common route of spread is by direct extension, and metastatic lesions may develop on any peritoneal surface, including the omentum (2). The lesions can give rise to ascitic tumor cells growing in plasma-derived exudate. Both tumor size and the accumulation of ascites are inversely associated with survival (1–4).

The growth and spread of neoplasms depend, in part, on the formation of adequate vascular support, i.e., angiogenesis (5,6), the extent of which is determined by the balance between positive and negative regulatory molecules (7–10). The process of angiogenesis can be divided into four distinct phases: 1) the degradation of extracellular matrix, 2) cell migration, 3) cell proliferation, and 4) structural reorganization (6,11,12). These distinct steps are likely to be differentially regulated. During the last decade, many angiogenic molecules have been identified (6–8,11,12). Among the major molecules identified is basic fibroblast growth factor (bFGF), which induces the proliferation, migration, proteolytic activity, and differentiation of endothelial cells (8,12–17). Vascular permeability factor (VPF)—also called vascular endothelial growth factor (VEGF)—has been shown to induce the proliferation of endothelial cells, to increase the cells’ vascular permeability, and to induce the production of plasminogen activator by these cells (18–26). Interleukin 8 (IL-8), a chemoattractant cytokine produced by a variety of tissues and blood cells, has been shown both to attract and activate neutrophils in inflammatory regions and to be angiogenic (27–29). The levels of matrix metalloproteinase-2 (MMP-2) and MMP-9 have been associated with active neovascularization because these MMPs can degrade the extracellular matrix, an essential step in the process of angiogenesis (30,31). As is true for other solid cancers (32), the extent of angiogenesis is inversely associated with prognosis in patients with ovarian cancer (33). The participation of the different angiogenic molecules in the progression of the disease, however, is only now being defined. Indeed, most correlative studies have reached the conclusion that the expression of an angiogenesis-related gene is necessary—but insufficient—to account for the multistep process of angiogenesis (10).

Because the biologic behavior of vascular endothelial cells can be regulated by a number of independent genes (10), we investigated the relationship between the
expression of various angiogenic molecules and the pathologic behavior of five different human ovarian cancers growing in an orthotopic site (the peritoneal cavity) of nude mice.

**Materials and Methods**

**Human Ovarian Carcinoma Cell Lines**

The five tumor cell lines used were SKOV3, SKOV3ip.1, 2774-C10, HEY-A8, and OCCI. The SKOV3 cell line was obtained from the American Type Culture Collection (Rockville, MD). The SKOV3ip.1 variant was derived from ascites arising in a nude mouse given an intraperitoneal injection of SKOV3 cells (35). The 2774-C10 cell line was a single-cell clone isolated from the 2774 cell line provided by Dr. R. S. Freedman (Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston) (36). HEY-A8 (37) and OCCI (38) cells were obtained from Dr. R. Bast and Dr. G. B. Mills (Division of Medicine, The University of Texas M. D. Anderson Cancer Center). All of the cell lines were established from ascites fluids, with the exception of the HEY-A8 cell line, which originated from a xenograft of a peritoneal deposit of a cystadenocarcinoma of the ovary.

**In Vitro Conditions**

All of the tumor cell lines were maintained as monolayer cultures in Dulbecco’s modified Eagle medium supplemented with 5% fetal bovine serum, 1× sodium pyruvate, 1× nonessential amino acids, 1× L-glutamine, and 2× vitamin solution (Life Technologies, Inc., Gaithersburg, MD). The cell cultures were maintained as monolayers on plastic petri dishes and were incubated in 5% CO2 – 95% air at 37°C. The cultures were free of Mycoplasma and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler’s encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioprocess, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

**Animals and Production of Tumors**

Female NCr-mu/nu nude mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were maintained under specific-pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the National Institutes of Health. The mice were used according to institutional guidelines when they were 8 weeks old. For the production of tumors, the cells growing in culture were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. A single-cell suspension of 1 × 10⁶ cells with a viability of greater than 95% was injected into the peritoneal cavity of the mice. The mice were monitored daily for evidence of disease (abdominal swelling, hunched posture, and listlessness) and were killed when moribund or by day 92. Values in column 1×L of Table 1 are the median (range) of survival times for mice, including all mice, with and without tumor.

**Northern Blot Analysis**

Poly(A)+ messenger RNA (mRNA) was extracted from 1 × 10⁶ tumor cells growing as 70% confluent cultures and the mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA was subjected to electrophoresis on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 mA to GeneScreen nylon membrane (Du Pont NEN, Boston, MA), and UV cross-linked with 120 000 rad. The nylon filters were hybridized with poly d(T) 20 staining defined as 100. Values in columns ‡ of Table 1 are the median (range) of survival times for mice, including all mice, with and without tumor.

### Table 1. Biologic behavior associated with messenger RNA (mRNA) expression level of angiogenesis-related genes of human ovarian carcinoma cells as measured by in situ hybridization

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Tumorigenicity*</th>
<th>Peritoneal disease</th>
<th>Survival † (days)</th>
<th>Median vessel density §</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo</td>
<td>In vitro</td>
<td>In vivo</td>
<td>In vitro</td>
</tr>
<tr>
<td>HEY-A8</td>
<td>99</td>
<td>Solid</td>
<td>27 (26–40)</td>
<td>30.6</td>
</tr>
<tr>
<td>OCCI</td>
<td>8/9</td>
<td>Solid</td>
<td>61 (53–91)</td>
<td>23.5</td>
</tr>
<tr>
<td>SKOV3</td>
<td>6/10</td>
<td>Solid/ascites</td>
<td>92 (28–92)</td>
<td>4.6</td>
</tr>
<tr>
<td>SKOV3ip.1</td>
<td>10/10</td>
<td>Ascites/solid</td>
<td>40 (35–51)</td>
<td>44.0</td>
</tr>
<tr>
<td>2774-C10</td>
<td>8/10</td>
<td>Ascites/solid</td>
<td>88 (35–92)</td>
<td>4.6</td>
</tr>
</tbody>
</table>

*Nude mice were given an intraperitoneal injection of 1 × 10⁶ viable cells. The mice were killed when moribund or by day 92. Values in column 1×L of Table 1 are the median (range) of survival times for mice, including all mice, with and without tumor.

†Intensity of cytoplasmic staining was quantified by an image analyzer and compared with the intensity of poly d(T)20 staining defined as 100. Values in columns ‡ of Table 1 are the median (range) of survival times for mice, including all mice, with and without tumor.

‡Vessel counts were assessed both at the center and at the periphery of the tumors by light microscopy after staining for CD31. Areas containing the highest number of capillaries and small vessels were identified by scanning at low power, and individual vessel counts were performed at ×200 magnification (32).

448 REPORTS

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tially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCC, Madison, WI) based on the FastA algorithm (47); these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by northern blot analysis (see below). A poly d(T)20 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3’ end via direct coupling with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, AL) (48). The lyophilized probes were reconstituted to a stock solution at 1 µg/µL in 10 mmol/L Tris (pH 7.6) and 1 mmol/L EDTA. Immediately before use, the stock solution was diluted with probe diluent (Research Genetics).

Preparation of Samples for In Situ Hybridization

Cells were plated onto sterilized ProbeOn Plus slides (49,50) (Fisher Scientific Co., Pittsburgh, PA) and allowed to attach with 70%–90% confluence over a period of 3 days. The cultures were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 minutes. They were then rinsed with ribonuclease (RNase)-free PBS and stored at 4°C if the procedure could not be finished on the same day. Fixed slides were treated with 1% Triton X-100, rinsed with 0.2 N HCl for 3 minutes at 99°C, and then hybridized as described below.

Tissue sections (4 µm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific Co.). The slides were placed in the Microprobe (Fisher Scientific Co.) slide holder, dewaxed, and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), digested with pepsin (45), and then hybridized as described below.

In Situ mRNA Hybridization

In situ mRNA hybridization was performed as described previously (49,50) with minor modifications. This procedure was carried out by use of the Microprobe manual staining system (Fisher Scientific Co.) (51). The probes were hybridized for 45 minutes at 45°C, and the samples were then washed three times for 2 minutes each time with 2X standard saline citrate (SSC) at 45°C. RNase-free water was used to make up Tris buffer and 2X SSC solutions. The samples were then incubated with alkaline phosphatase-labeled avidin for 30 minutes at 45°C, rinsed in 50 mmol/L Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 minute, and incubated with a chromogen substrate for 15 minutes at 45°C. Additional incubation with fresh chromogen substrate was done if it was necessary to enhance a weak reaction (52). A positive enzymatic reaction in this assay stained red. Known positive controls were used in each hybridization reaction. Controls for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and use of chromogen alone.

Image Analysis to Quantify Intensity of Color Reaction

Stained sections were examined in a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip, charge-coupled device color camera (model DXC-960 MD; Sony Corp., Tokyo, Japan). The images were analyzed with the use of Optimas software (Optimas Corp., Bothell, WA). The slides were prescreened by one of the investigators to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue modes of the color camera. All subsequent images were quantified on the basis of this threshold. The integrated optical density of the selected fields was determined on the basis of its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained, so the optical density was due solely to the product of the in situ mRNA hybridization reaction. Five different fields in each sample were quantified to derive an average value. The intensity of staining following enzymatic reactions in tissue sections is not linearly associated with mRNA quantity; therefore, to ascertain differences in mRNA expression among different samples, we determined the intensity of staining by subtracting the background optical density level and standardizing the value with the integrated optical density of poly d(T)20, which was set at 100.

Histology and Immunohistochemistry

Tumors harvested from the peritoneal cavity of nude mice at autopsy were divided into 5-mm3 fragments and placed in 10% (vol/vol) neutral formalin for 24 hours before use, the stock solution was diluted with probe diluent. A 1 : 200 dilution of rabbit polyclonal anti-bFGF antibody (Santa Cruz Biotech, Santa Cruz, CA), a 1 : 70 dilution of mouse monoclonal anti-CD31 antibody (Pharmingen, San Diego, CA), a 1 : 50 appropriate dilution (1 : 100) of monoclonal rat anti-CD3 antibody (Pharmingen, San Diego, CA), a 1 : 50 dilution of a rabbit polyclonal anti-IL-8 antibody (BioSource International, Camarillo, CA), a 1 : 70 dilution of a rat polyclonal anti-savenger receptor antibody (Santa Cruz Biotech, Santa Cruz, CA). The samples were then rinsed four times with PBS and incubated for 60 minutes at room temperature with the appropriate dilution of peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-rat IgG. The slides were rinsed with PBS and incubated for 5 minutes with diaminobenzidine (Research Genetics). The sections were then washed three times with distilled water, counterstained with Mayer’s hematoxylin (Biogenex Laboratories, San Ramon, CA), washed once with distilled water and once with PBS, and rinsed again with distilled water. The slides were mounted with a Universal mount (Research Genetics) and examined in a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Sections (5 µm thick) of formalin-fixed, paraffin-embedded tumors were also stained with hematoxylin–eosin for routine histologic examination and with Gomori’s trichrome stain for identification of collagen (53,54).

Vascular Density

Blood vessels in solid human ovarian carcinomas growing in the peritoneal cavities of nude mice were counted under a light microscope after immune staining of sections with anti-CD31 antibodies (55). Areas containing the highest number of capillaries and small venules were identified by scanning the tumor sections at low power (×40 and ×100). After the areas of high vascular density were identified, individual vessels were counted in ×200 fields (×20 objective and ×10 ocular [0.739-mm2/field]) (56). On the basis of criteria described by Weidner et al. (57), observation of a vessel lumen was not required for a structure to be classified as a vessel.

Statistical Analysis

The Mann–Whitney test was used to compare the level of gene expression among the different cell lines (univariate analyses). The ovarian cancer cell lines were classified as high (HEY-A8 and SKOV3.ip.1) or low (SKOV3 and 2274-C10) expressers of IL-8 and high (SKOV3, SKOV3.ip.1, and 2274-C10) or low (HEY-A8 and OCCI) expressers of VEGF/VPF. Survival analysis was computed by the Kaplan–Meier method and compared by the log-rank test (58). Significance was defined as a two-sided P<0.05.

Results

Tumorigenicity and Pattern of Disease

The median survival times of the mice were not associated with the production of ascites, being 27 and 61 days for those tumors that did not produce ascites and 40, 88, and 92 days for those that did (Table 1). In particular, mice receiving an intraperitoneal injection of HEY-A8 cells (median survival, 27 days; range, 26–40 days) developed large (up to 10 mm in diameter) peritoneal lesions arising predominantly from the anterior and lateral peritoneal surfaces, with five to 10 discrete lesions per mouse and no ascites. Mice given an intraperitoneal injection of OCCI cells (median survival, 61 days; range, 53–91 days) developed discrete
peritoneal lesions of 5–10 mm in diameter, up to 20 lesions per mouse, without evidence of ascites. Injection of the other three ovarian cancer cell lines produced a combination of solid tumor lesions, predominantly in the upper abdomen (omental and perisplenic masses), peritoneal carcinomatosis, and ascitic fluid containing tumor cells, leukocytes, and red blood cells. Of 10 mice given an injection of SKOV3 cells (median survival, 92 days; range, 28–92 days), six developed abdominal disease comprising a tumor mass in the upper abdomen and multiple, small (1- to 2-mm) tumors attached to the mesentery, diaphragm, and peritoneum. In contrast, all 10 mice given an injection of the variant cell line SKOV3ip.1 developed rapidly progressive disease, manifested as more than 200 small peritoneal lesions attached to the mesentery, uterus, horns, diaphragm, and other structures. Some lesions exceeded 5 mm in diameter, although most were 1–2 mm in diameter. All mice receiving SKOV3ip.1 cells developed ascites, 3–5 mL in volume, that contained numerous tumor cells, leukocytes, and red blood cells. As reported previously (35), the median survival time of the mice given an injection of this selected variant was 40 days (range, 35–51 days), as opposed to 92 days (range, 28–92 days) for the original SKOV3 cells. Eight of 10 mice given an injection of 2774-C10 cells (median survival, 88 days; range, 35–92 days) developed more than 200 small (1- to 2-mm) lesions adherent to the mesentery and posterior peritoneum, in addition to large upper abdominal masses (10–15 mm in diameter). In some moribund mice, a large volume of ascitic fluid containing tumor cells, leukocytes, and red blood cells.

**Constitutive Expression of Angiogenesis-Related Genes in Human Ovarian Carcinoma Cells Growing in Culture**

In the next set of experiments, we examined the constitutive expression of several angiogenesis-related genes (VEGF/VPF, IL-8, bFGF, MMP-2, and MMP-9) in the five different human ovarian carcinoma cell lines growing in culture. The results of northern blot analysis are shown in Fig. 1. For densitometric quantitation, the ratio of the area between the specific transcripts was compared with that of 1.3-kb GAPDH internal control. HEY-A8 cells and OCCI cells (solid tumors without ascites) expressed IL-8, bFGF, and MMP-2 mRNA transcripts but not VEGF/VPF mRNA. SKOV3, SKOV3ip.1, and 2774-C10 cells, which produced solid tumors and carcinomatosis (Table 1), all expressed IL-8, bFGF, and VEGF/VPF mRNA but did not express MMP-2 mRNA. Expression of MMP-9 transcripts was found only in 2774-C10 cells (Fig. 1).

To detect mRNA expression in individual cells, we performed in situ mRNA hybridization analyses with probes specific for the mRNA transcripts, and we quantified the intensity of cytoplasmic staining by an image analyzer (Table 1). We used a poly d(T)20 probe to verify the integrity of the mRNA in the samples. An intense histochemical reaction in all samples indicated that the mRNA was not degraded. All cells expressed similar levels of bFGF mRNA. Although northern blot analysis had detected expression of MMP-2 only in HEY-A8 and OCCI cells, in situ mRNA hybridization detected it in all cell lines, perhaps because the cultures were sparse (59). The 2774-C10 cell line expressed a higher level of MMP-9 mRNA than the other four cell lines.

The major differences in gene expression were found for IL-8 and VEGF/VPF. The expression level of IL-8 was inversely associated with survival (P<.0001, logrank test) (Table 1), whereas the expression level of VEGF/VPF was directly associated with production of ascites (P<.0001, Mann–Whitney test).

**Expression Level of Angiogenesis-Related Genes in Implanted Tumors**

Since the angiogenic behavior of neoplasms is influenced by the organ environment (60), we next examined the expression level of IL-8, bFGF, VEGF/VPF, MMP-2, and MMP-9 transcripts in human ovarian cancer cells growing as solid lesions in the peritoneal cavity of nude mice. We did not analyze ascitic fluid because it contained a large number of leukocytes and red blood cells. Before treating tissue sections with the specific probes, we verified the integrity of the mRNA in each sample by using a poly d(T)20 probe.

Tumor cells growing in the peritoneal cavity of nude mice expressed higher levels of MMP-2 and MMP-9 mRNA than did their in vitro controls, suggesting that the expression of MMP-2 and MMP-9 genes can be induced by the organ environment (61). No significant differences were found in the expression level of bFGF mRNA among the five different tumors, suggesting that the expression level of bFGF is not associated with the pattern of disease (solid and/or ascites) or with disease progression (survival). The mean IL-8 mRNA level differed significantly between the high (HEY-A8 and SKOV3ip.1) and low (SKOV3 and 2774-C10) expressing tumors (P<.0001, Mann–Whitney test). All mice implanted with high IL-8-expressing tumors died by day 51, whereas 12 of 20 mice implanted with low IL-8-expressing tumors were alive on days 88–92 (P<.0001, logrank test). The OCCI tumors expressed intermediate lev-
els of IL-8 mRNA, and mice given an injection of these cells also had an intermediate median survival time.

The in vivo expression level of VEGF/VPF mRNA was directly associated with the production of ascites: HEY-A8 and OCCI tumors (solid) expressed the lowest levels, and SKOV3, SKOV3ip.1, and 2774-C10 tumors (ascites/solid) expressed the highest levels \( P < .0001 \) (Mann–Whitney test). The 50% survival periods for the high and low VEGF/VPF-expressing tumors were 48 and 88 days, respectively \( (P = .0071) \), (logrank test).

### Immunohistochemical Analyses of Angiogenesis-Related Genes in Implanted Tumors

In the next set of studies, tissue sections obtained from solid human ovarian carcinomas in the peritoneal cavity of nude mice were analyzed for expression of bFGF, VEGF/VPF, and IL-8. The results closely agreed with those obtained by in situ mRNA hybridization. All tumors were stained with the rabbit polyclonal anti-bFGF antibody. HEY-A8 and OCCI tumors did not stain with the rabbit polyclonal anti-VEGF/VPF antibody, whereas SKOV3, SKOV3ip.1, and 2774-C10 tumors did. Staining with the rabbit polyclonal anti-IL-8 antibody was observed for the HEY-A8, OCCI, and SKOV3ip.1 tumors, but minimal reaction was found with the slow-growing SKOV3 and 2774-C10 tumors. To show the distinctions clearly, we present representative staining of HEY-A8 and 2774-C10 tumors in Fig. 2.

### Histologic Features and Vascular Density

The number of blood vessels was determined at the center and periphery of the five ovarian carcinomas (all lesions were 3–5 mm in diameter) subsequent to immunohistochemical analysis with the use of anti-CD31 antibodies \( (55) \). The median number of blood vessels in the center of the lesions was higher in the faster growing HEY-A8, OCCI, and SKOV3ip.1 tumors than in the more slowly growing SKOV3 and 2774-C10 lesions (Table 1).

Since host-infiltrating lymphoid cells have been shown to augment angiogenesis of human neoplasms \( (62–65) \), we also stained the tissues with antibodies against

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Fig. 2. Representative immunohistochemical staining of HEY-A8 (HEY) and 2774-C10 human ovarian carcinomas, growing in the peritoneal cavity of nude mice, for expression of basic fibroblast growth factor (bFGF), vascular epithelial growth factor/vascular permeability factor (VEGF/VPF), and interleukin 8 (IL-8). Tissue sections were stained with rabbit polyclonal anti-bFGF antibodies, rabbit polyclonal anti-VEGF/VPF antibodies, and rabbit polyclonal anti-IL-8 antibodies.

Fig. 3. Representative immunohistochemical staining of HEY-A8 (HEY) and 2774-C10 human ovarian carcinomas growing in the peritoneal cavity of nude mice. Tissue sections were stained with a rat polyclonal anti-scavenger receptor, i.e., Scavenger-R (to show macrophages), a rat monoclonal anti-CD31 antibody (to show endothelial cells), and Gomori’s trichrome stain (to show formed collagen).
the macrophage scavenger receptor (66). The distribution of the macrophages shown in Fig. 3 paralleled that of the blood vessels. In the rapidly growing HEY-A8 tumors (and in the unshown OCCI and SKOV3ip.1 tumors), macrophages were found throughout the lesions, whereas in the more slowly growing 2774-C10 tumors (and in the unshown SKOV3 tumors), most macrophages were found at the periphery.

To determine whether the distribution and extent of connective tissue (extracellular matrix) differed among the tumors, we stained the tissue sections with Gomori’s trichrome stain (53). The rapidly growing HEY-A8 (and the unshown OCCI and SKOV3ip.1) tumors contained many thin septa of collagen trabecula, whereas the more slowly growing 2774-C10 (and the unshown SKOV3) tumors contained a stroma consisting of thick collagen fibers (Fig. 3).

Discussion

Our results demonstrate that the biologic behavior of human ovarian cancer cells in the peritoneal cavity of nude mice is associated with the expression of genes that regulate various steps of angiogenesis. We studied the pathobiology of five different human ovarian cancers. Three of the tumors grew both as solid nodules and in ascites, whereas two cell lines produced only solid nodules. Three tumors produced rapidly progressive disease (one with ascites; two without ascites). The production of ascites was directly associated with the expression of VEGF/VPF, whereas survival was inversely associated with the expression of IL-8. The distribution of blood vessels also varied among the tumors. In rapidly growing tumors, blood vessels were distributed throughout the lesions; in contrast, in the more slowly growing tumors, blood vessels were found mainly at the periphery.

To produce a blood vessel, endothelial cells must divide, migrate, degrade the extracellular matrix, differentiate, and survive (5,6,11). This multistep process is controlled by a large number of distinct positive and negative regulatory molecules. One of these molecules, bFGF, is a potent mitogen for endothelial cells. In agreement with previously published reports (13–17), all of the ovarian carcinomas expressed high levels of bFGF, suggesting that this molecule is associated with tumorigenicity and angiogenesis. Another molecule, VEGF/VPF, was discovered because of its ability to increase vascular permeability. The factor was therefore called VPF (18,19). The VPF gene was subsequently cloned, shown to be a growth factor, and named VEGF (20). Local administration of VEGF/VPF has been shown to transiently increase vascular permeability (67) through activation of vesicular-vascular organelles present in the cytoplasm of endothelial cells (68) and through the induction of interendothelial cell gaps and endothelial fenestration (69). Increased permeability of blood vessels facilitates the extravasation of proteins and, thus, the formation of ascites (18,19,70,71).

In agreement with previous reports (70–72), the expression level of VEGF/VPF was directly associated with production of ascites and carcinomatosis. The administration of VEGF/VPF monoclonal antibodies or antiserum to mice given an injection of human ovarian carcinoma cells inhibits formation of ascites and growth of solid peritoneal lesions (73–75). Our finding that the most aggressive ovarian carcinoma (HEY-A8 cell line) did not express high levels of VEGF/VPF indicates that expression of VEGF/VPF is not associated with survival.

Although bFGF does not modify vessel permeability in acute experimental conditions, it has recently been shown that the new blood vessels formed in response to either bFGF or VEGF are hyperpermeable to albumin (76). This finding suggests either that there are additional mechanisms responsible for this hyperpermeability or that bFGF induces the expression of VEGF (77), which then increases vessel permeability. Increased proteolytic activity is critical for angiogenesis because degradation of extracellular matrix provides a permissive microenvironment in which activated vascular cells can proliferate, invade, and migrate away from the pre-existing parental vessel (29,30). The expression of MMP-2 and MMP-9 is cell density dependent (59) and is found on the leading invasive edge of neoplasms (78). These data may explain why we detected the expression of MMP-2 and MMP-9 in the ovarian carcinomas by in situ mRNA hybridization but not by northern blotting. The expression level of MMP-2 and MMP-9 was higher in the HEY-A8 and OCCI solid lesions. These data agree with those obtained in a previous report (79) showing that ascitic tumor cells that do not secrete type IV collagenase fail to produce solid tumors even after subcutaneous implantation.

The incidence of metastasis has been associated with the number and density of blood vessels in a variety of human cancers (56,57). In our study, the distribution of blood vessels in solid lesions varied among the ovarian carcinomas. The rapidly growing HEY-A8, OCCI, and SKOV3ip.1 tumors had a homogeneous distribution of blood vessels, whereas in the slow-growing SKOV3 and 2774-C10 tumors, the blood vessels were concentrated at the periphery.

Infiltrating lymphoid cells can produce multiple angiogenic molecules and hence can contribute to accelerated angiogenesis (62–65). Our data show that the number and distribution of scavenger receptor+ macrophages paralleled those of the blood vessels (CD31+ cells). Whether the increased number of macrophages in the rapidly growing lesions was due to an increased number of blood vessels or vice versa is unclear at present.

IL-8 is a multifunctional cytokine that can regulate migration of leukocytes (80) and tumor cells (81). It can also induce the proliferation of keratinocytes (82), some melanoma cells (83), and endothelial cells (27–29). In our study, the expression of IL-8 was directly associated with rapid growth of the human ovarian carcinoma cells and, hence, was inversely associated with survival of the injected nude mice. Increased expression of IL-8 has been reported in aggressive cases of ovarian cancer (29,84) and in ovarian carcinoma cells that resisted exposure to paclitaxel (85). The possibility that the expression of IL-8, which can be studied by in situ mRNA hybridization (61), could predict disease outcome in primary human ovarian carcinomas is intriguing and under active investigation.

In summary, we show that the expression of different genes that regulate angiogenesis in human ovarian carcinomas is associated with the pattern of the disease and its progression: Expression of bFGF reflects tumorigenicity, expression of VEGF/VPF is directly associated with the development of peritoneal ascites and carcinomatosis, and expression of IL-8 is inversely associated with survival. Tar-
getting specific genes such as these that regulate angiogenesis could offer new approaches to the therapy of ovarian cancer.

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Notes
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