Angiogenic Protein Expression in Advanced Epithelial Ovarian Cancer

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ABSTRACT

We set out to determine whether advanced epithelial ovarian cancer (EOC) is associated with elevated serum and ascitic concentrations of the angiogenic factors angiogenin (ANG), basic fibroblastic growth factor (bFGF), and vascular endothelial growth factor (VEGF), and whether the expression of angiogenic factors was associated with tumor vascularity. Serum and ascitic samples were collected from previously untreated patients with FIGO stage III and IV EOC and stored at −70°C. Levels of the three factors were determined by enzyme-linked immunoassay. Histological sections from paraffin blocks of ovarian cancers were stained immunohistochemically for factor VIII, CD34, and VEGF. Thirty-nine patients were studied, although not all had paired serum and ascitic samples. For each angiogenic factor, the following findings were noted: (a) there was a wide range in serum and ascitic fluid concentrations; (b) the mean serum concentration was higher (P < 0.05) than the mean concentration in normal serum; (c) the mean serum concentration was lower (P < 0.05) than the mean ascitic concentration. Overall, the most consistent pattern of elevated serum and ascitic concentrations was with bFGF. With serum samples, 38.9% of patients had a normal VEGF concentration, as did 15.3% for ANG and 7.7% for bFGF. In ascites, the VEGF concentration was in the range for normal serum in 24.5% of samples, compared to 39.4% for ANG and 28.0% for bFGF. In paired samples, both VEGF and bFGF showed higher ascitic concentrations in 100 and 88.3% of samples, compared to 53.3% for ANG. There was no correlation between the serum and/or ascitic concentration of one factor and that of another, suggesting that these factors are independently regulated. Staining with anti-CD34 was more sensitive and reliable than with anti-factor VIII. VEGF staining was most prominent in poorly differentiated tumors and was observed only on tumor cells. There was no correlation between the serum or ascitic concentrations of angiogenic factors and tumor vascularity. Advanced EOC is associated with raised serum and ascitic bFGF concentrations and with markedly elevated ascitic VEGF in most cases. Serum VEGF and serum and ascitic ANG are less often elevated. There was no correlation between the angiogenic profile in serum and ascites and tumor vascularity.

INTRODUCTION

The propensity for malignant tumors to increase in size, to invade locally, and to metastasize is dependent, in part, on angiogenesis. Both angiogenic factors and inhibitors (angiostatins) have been identified, and in normal tissues these are in equilibrium (1, 2). If tumors are to enlarge beyond 2–3 mm³, then, because the nutritional and metabolic needs of cells cannot be satisfied by diffusion in larger volumes, angiogenesis has to be established (2–4). During carcinogenesis, some in situ carcinomas, such as those of the breast, pancreas, and cervix, are angiogenic before they become invasive (5). Interactions between tumor cells and cells in the ECM, including inflammatory cells, may also release angiogenic factors, as macrophages have been shown to secrete angiogenic factors (6, 7). The establishment of a new network of blood vessels supports tumor growth, but because these vessels are highly permeable and have supportive connective tissue that is less well developed, they also provide a means for tumor dissemination (8).

Of the angiogenic factors described thus far, we chose to look at three of the better characterized factors: bFGF, ANG, and VEGF (also known as VPF). bFGF, also called heparin-binding growth factor 2, has been isolated from many tissues and is synthesized by a variety of normal cells and tumor cells in culture. It has an apparent molecular weight of 18,000, but it also exists in heavier forms. It does not have a classic signal peptide sequence and was thought to be released only following cell damage. However, studies on bovine papilloma virus 1-transfected murine cells showed that bFGF was released from transformed cells (9, 10). It is one member of a family of seven sequence-related growth factors and has chemotactic and mitogenic properties on endothelial cells in vitro (11, 12).

ANG was initially discovered as a factor released from the human colonic adenocarcinoma cell line HT29 that promoted vascularization in the chicken chorioallantoic membrane (13). It...
has a molecular weight of about 14,000, and unlike other angiogenic factors, it does not bind heparin, nor does it share sequence homology with the FGF family. Because it is not mitogenic for endothelial cells in culture, it is often considered an indirect angiogenic factor. It is found in tumor cells, is involved in early angiogenesis by activating capillary endothelial cells, and can be detected in normal plasma and normal tissues at the mRNA level (14, 15). ANG has been shown in vitro to support endothelial and fibroblast cell adhesion and spreading by mechanisms that depend on direct interaction of ANG with the endothelial cells (16).

VEGF is a M, 32,000–42,000 protein that binds heparin and has potent effects on endothelial cells and on blood vessels. It is widely expressed in normal and cancerous cells (8). The gene for VEGF is organized into eight exons, and as a result of alternative splicing, there are four transcripts containing from 121 to 206 amino acid residues (17). The transcripts VEGF121, and VEGF165 are diffusible proteins, whereas VEGF189 and VEGF206 have high heparin affinity and remain mostly bound to heparin-containing proteoglycans in the ECM (18). Tumor-associated endothelial cells, in contrast to normal endothelial cells, express the VEGF receptor fbl but have not been found to produce VEGF (19, 20). As its name suggests, VEGF (or VPF) increases vascular permeability and acts directly on endothelial cells, especially tumor-associated blood vessels, causing proteins such as fibrinogen to leak from the vascular system into the ECM. This vascular hyperpermeability is an important component of tumor-associated angiogenesis (20, 21).

Aside from angiogenic factor expression, there has been increasing evidence that tumor vascularity is an independent prognostic factor in different tumor types. Using conventional histology, new blood vessels are often not apparent or are collapsed. Immunohistochemical staining using anti-factor VIII and anti-CD34 improves detection and visualization of blood vessels in tumors. For example, in invasive breast cancer, it was reported that angiogenesis, as determined by vessel density in histological sections, correlated with metastases, and moreover, microvessel density was the best predictor of prognosis in node-negative breast cancer patients (22, 23).

Ovarian cancer, a major cause of gynecological cancer deaths, is characterized by late stage of presentation and by i.p. carcinomatosis and ascites. Our hypotheses were that advanced EOC would be associated with elevated serum and ascitic concentrations of the angiogenic factors ANG, bFGF, and VEGF. Because ovarian cancer characteristically remains confined to the peritoneal cavity, it was also hypothesized that there would be preferential expression in ascites and that the expression of angiogenic factors would not be associated with tumor vascularity.

MATERIALS AND METHODS

Patients with FIGO stage III (n = 35) or IV (n = 4) EOC were eligible for study. All patients were treatment naive.

Serum and Ascitic Samples. Before the exploratory laparotomy, a 10-ml peripheral blood sample was drawn under sterile conditions and centrifuged at 400 × g for 10 min, the supernatant was collected and recentrifuged at 400 × g, and the serum was separated. Aliquots of 1 ml were then stored at −70°C. During surgery, after the abdomen was opened, ascitic fluid (up to 500 ml) was collected into sterile containers without heparin or fixatives. Neither peritoneal washings nor obviously blood-stained ascitic fluid were used in this study (as a result, not all patients had paired serum and ascitic samples). The ascitic samples were centrifuged at 400 × g for 10 min, the supernatant was collected and recentrifuged, and the supernatant was passed through a 0.22 μm filter (Costar, Cambridge, MA) and stored at −70°C in 1 ml aliquots. Control serum samples from 11 normal adult female volunteers were also assayed. We did not have access to peritoneal fluid from healthy women.

Assays of Angiogenic Factors. Each serum and ascitic sample was subjected to one freeze-thaw cycle only. All assays were performed in duplicate on microtiter plates by ELISA (R&D Systems, Minneapolis, MN). The working range in the ELISA for bFGF assays was 0–320 pg/ml. When necessary, dilutions of the serum and ascitic samples were made. In practice, many of the ascitic samples required dilution to 1:10. In the normal controls, in some cases there was no detectable factor. In these cases, the assay was repeated in further samples from the same control.

Tumor Samples. Tissue sections were obtained from two paraffin blocks from the primary ovarian tumor and subjected to immunohistochemical studies using the avidin-biotin complex method, essentially as described previously (24). Sections (5 μm thick) were cut and mounted on noprone (polychloroprene)-coated slides (Aldrich Chemical Company, Milwaukee, WI) and air dried overnight. Slides were then run on an automated staining instrument (Code-On, Bathyal Solution, Santa Barbara, CA). The slides were then deparaffinized and blocked for endogenous peroxidase with 1.75% hydrogen peroxide in methanol for 20 min. The internal control for the immunohistochemical technique was vimentin (murine monoclonal IgG1, DAKO Corp., Carpinteria, CA). The negative controls used were sections from the same tissue block in which the primary antibody was omitted. Those slides stained with anti-CD34 (murine monoclonal IgG1, Immunotech, West Brook, ME) were incubated in a 90°C water bath for 90 min with Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA), and then allowed to cool to room temperature. For anti-factor VIII (murine monoclonal IgG1, DAKO Corp.), slides were pretreated with 0.1% pepsin. Staining for VEGF (using a rabbit antihuman antibody kindly provided by Dr. Donald Senger, Dept. of Pathology, Harvard Medical School, Boston, MA), CD34, and factor VIII was performed using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). Slides were blocked with normal horse serum for 5 min at 42°C. Slides treated with anti-CD34 and anti-factor VIII antibodies were incubated for 30 min at 30°C, whereas slides treated with anti-VEGF were incubated overnight (16 h) at room temperature. Species-specific secondary labeling was performed for 15 min at 30°C. The slides were stained with the chromogen 3,3-diaminobenzidine (Scytek Laboratories, Logan, UT) for 10 min at room temperature and then counterstained with hematoxylin, dehydrated, and coverslipped.

The slides were examined under low power (×40–×100)
to determine the region of greatest vascularity. Because staining with anti-CD34 was more sensitive and specific than with anti-factor VIII, results from anti-CD34 were used to assess tumor vascularity. The vessel count (stained single cells and stained cell clusters without a lumen were counted as vessels but larger vessels with obvious muscular coats were excluded) was determined at ×200 on sections stained with anti-CD34. Sections were interpreted blinded to clinical details and to the assay results for bFGF, ANG, and VEGF.

Statistical Analysis. The serum and ascitic levels were tested for normality (Bartlett’s test for homogeneity of variance) and analyzed using Student t test and nonparametric Kruskal-Wallis analysis. Results are expressed as mean ± SE. The level of significance was $P < 0.05$.

RESULTS

Serum Concentrations of bFGF, ANG, and VEGF. In the assays for the serum concentrations in normal controls, in some cases, the angiogenic factor was undetectable. All serum concentrations of bFGF were <10 pg/ml, and the mean concentrations in normal serum of ANG and VEGF were 327.3 ng/ml (range, 92–472 ng/ml) and 253 pg/ml (range, 42–578 pg/ml), respectively. The mean serum concentrations of bFGF, ANG, and VEGF ([bFGF]$_s$, [ANG]$_s$, and [VEGF]$_s$) in EOC patients were $318.6 \pm 104.9$ pg/ml, $643.3 \pm 49.6$ ng/ml, and $1167 \pm 227.1$ pg/ml, respectively; these concentrations were significantly elevated ($P < 0.05$) compared to normal serum concentrations (Fig. 1, a–c). In a small group of patients, the serum
bFGF (7.7%) and the serum ANG (15.3%) were normal, and none of the patients with a normal serum bFGF also had a normal serum ANG. However, for VEGF, 38.9% of patients had a normal serum level. There was a wide range in the serum concentrations for each of these factors, with no apparent association between the serum concentration of one factor and another (Fig. 1d).

**Ascitic Concentration of bFGF, ANG, and VEGF.** For bFGF, in the 36 samples tested, the mean ascitic concentration [bFGF]a was greater than the mean serum level (P < 0.001). On the other hand, for ANG, although the mean ascitic concentration of ANG ([ANG]a) exceeded the mean [ANG]s (P < 0.05), in 17 of 36 ascitic samples tested the ascitic concentration was less than the mean normal serum level of ANG (Fig. 2a and b). Although in all cases [VEGF]a exceeded the [VEGF]s, in 8 of 33 ascitic samples the [VEGF]a was within the range for normal serum, and in the remaining 25 cases the [VEGF]a was markedly elevated (P < 0.001; Fig. 2c). As with the serum samples, for each of the angiogenic factors, there was a wide range of values in the ascitic samples, and there was no pattern to the ascitic levels of bFGF, ANG, and VEGF in individual cases (Fig. 2d).

**Paired Serum and Ascitic Concentrations of bFGF, ANG, and VEGF.** There was no correlation between the serum and ascitic concentrations of any of the three factors (Fig. 3). Whereas the ascitic bFGF level exceeded the paired serum level in 88.3% of cases, this pattern was observed in only 53.3% of cases for ANG. In contrast, in paired VEGF levels, the ascitic level always exceeded the serum level, although as noted above, the ascitic level was within the range for normal serum in 24.5% of cases. No conclusion can be made about the levels of the factors bFGF, ANG, and VEGF in relation to volume of ascites or the volume of the tumor burden because it is not possible to assess either of these accurately. Because most patients were diagnosed and treated no more than 3 years ago, no conclusion...
can be made on the levels of bFGF, ANG or VEGF and prognosis.

**Immunohistochemical Studies.** Staining with anti-CD34 was more sensitive than with anti-factor VIII (Fig. 4). The staining with anti-VEGF varied from almost none to strong, and poorly differentiated tumors stained positive for VEGF. The staining for VEGF was observed exclusively on tumor cells and not on blood vessels within tumors or in the immediate vicinity of tumor cells. There was considerable intertumor and intratumor variation in vessel density, and no pattern between the level of angiogenic protein expression in serum or ascites and tumor vessel density was identified.

**DISCUSSION**

This is the first study to report on the serum and ascitic concentrations of angiogenic factors in advanced EOC. Because local tumor invasion and metastatic spread are angiogenic dependent, it was hypothesized that advanced malignancies would be associated with up-regulation of angiogenic factors (25). Our data indicate that the only clear pattern to emerge is with bFGF, where in the majority of cases both the serum and the ascitic level were elevated, and where the ascitic level exceeded the paired serum level. With VEGF, many patients (38.9%) had normal serum concentrations, and although most patients had very elevated ascitic concentrations, in 24.5% of patients the [VEGFI] was with normal serum levels. Similarly, for ANG, in almost half of the cases the ascitic concentration was less than the paired serum concentration, in contrast to the pattern observed for paired bFGF and VEGF samples. The finding of higher levels of angiogenic factors in ascites is consistent with the disease distribution in ovarian cancer. Because the patterns of expression of one factor were not mirrored in the other factors, there was no distinct angiogenic factor profile. These data suggest that these factors are independently regulated. The significance of these angiogenic profiles is not yet known.

Ovarian cancer is an unusual solid malignancy in that despite an often massive tumor burden, metastatic disease outside the peritoneal cavity is uncommon. This feature would suggest that the ascitic concentrations of angiogenic factors should exceed the serum concentration. Furthermore, because the majority of ovarian cancers are believed to arise from the surface epithelium, tumor cells can be rapidly shed from this surface into the peritoneal cavity, often in association with ascites. This dissemination of tumor cells can occur without invasion of ovarian stroma, as illustrated by the group of ovarian cancers classified as low malignant potential tumors, which are essentially intraepithelial tumors. By definition, these tumors do not invade the stroma, yet widespread intraperitoneal disease can be found (26). Invasive EOC are often enormous tumors; ultrasonically and clinically, many of these cancers are highly vascularized, as recently reported (27).

Recently, Shimoyama et al. (28) reported increased ANG mRNA and protein expression in a proportion of pancreatic cancer patients. ANG expression was associated with a poor prognosis, and serum ANG levels were not associated with clinical parameters, similar to the findings of this study. Fujimoto et al. (29) reported that the serum and urinary levels of bFGF were elevated in 16 of 31 patients with renal cell carci-
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1. There was some correlation between stage of disease, degree of tumor differentiation, and level of bFGF. In a similar model, Nguyen et al. (30) reported elevated levels of bFGF in the urine of patients with bladder cancer. Patients with metastatic disease had the highest levels, although not all patients had raised urinary bFGF. These data are similar to our data, in which levels were highest in the urine of patients with bladder cancer. Patients with metastatic disease had the highest levels, although not all patients had raised urinary bFGF. These data are similar to our data, in which levels were highest in the tumor compartment, but not all cases had elevated levels. In brain tumors, which are typically highly vascular, raised levels of bFGF in cerebrospinal fluid were found in only 62% of cases, and the authors reported that raised levels correlated with density of microvessels in histological sections (31).

2. Levels of VEGF (also called VPF), an angiogenic factor first identified from the ascitic samples of tumor-bearing rodents, might be expected to be elevated in ovarian cancer, in which ascites is a common finding (32). Yeo et al. (33) showed in an animal model that there was an association between increasing ascitic levels of VPF (VEGF) and increasing volume of ascites and tumor cell numbers, although, of interest, not all animals had increased levels of VPF (VEGF). However, they did not find increased serum levels in the serum or urine of these animals. The ascitic VPF levels did not correlate with the total protein content, but there was a strong correlation with the numbers of monocytes and macrophages in the effusion. Sheid (34) reported on the angiogenic effects of macrophages isolated from ovarian cancer ascitic samples, and although the growth factor(s) was not identified, VPF (VEGF) is a candidate. In more recent studies, however, VEGF has been detected in normal sera (35, 36). These apparent discrepancies in the detection of VEGF in serum might result from the different detection methods and different affinities of the antibodies used in the assays.

Olson et al. (37) reported that VPF121 and VPF165 transcripts were present in normal ovary, in normal and malignant ovarian epithelial cell lines, and in ovarian cancer cells cultured from ascitic samples and tumor samples. In an animal model of ascites-producing tumors, VEGF mRNA was localized to tumor cells and not to blood vessels, whereas VEGF protein was localized to tumor cells and to the endothelium of blood vessels in the immediate vicinity of tumor cells (20). Thus, in ovarian cancer, both the ascites-associated macrophages and the ovarian cancer cells can produce VEGF. Because the tumor remains

Fig. 4 a, sections from a poorly differentiated EOC tumor stained with H&E. The vascularity of the tumor was determined immunohistochemically using two antibodies, anti-CD34 (b) and anti-factor VIII (c), as described in “Materials and Methods.” From sections adjacent to that shown in a, there is superior immunohistochemical staining of blood vessels with anti-CD34 (b) compared with anti-factor VIII (c). d, VEGF expression in the same tumor detected with anti-VEGF showing that expression was found only on tumor cells.
confined to the peritoneal cavity, it might be expected that ascitic levels of angiogenic factors would exceed the serum levels. Similarly, in rheumatoid arthritis, the joint fluids contain high levels of VEGF, and the synovial lining macrophages express VEGF mRNA (38). The release of VEGF, in addition to promoting ascites, may also enhance metastatic potential, not only by circulating cancer cells throughout the peritoneal cavity but by an intrinsic mechanism, inasmuch as Ferrara et al. (39) reported that tumor cells transfected with VPF gene had increased ability to grow in nude mice. It seems likely that the presence of more than one angiogenic factor, as in the ascites of EOC patients, will lead to enhanced angiogenic activity because there is synergism between VEGF and bFGF (40, 41).

In a study on the distribution of VPF in solid tumors, VPF protein was expressed both in tumor cells and in those blood vessels not only within the tumor masses but immediately adjacent (within 0.5 mm) to the tumor cells (20). In breast cancer, VEGF protein expression was almost exclusively on tumor cells and not on adjacent endothelial cells (42). Similarly, we found that VEGF expression was most evident in poorly differentiated tumors and was exclusive to tumor cells. However, VPF mRNA has been detected only in the tumor cells, not in tumor-associated blood vessels, and was especially concentrated in zones of tumor necrosis. It has been suggested that hypoxia is a stimulus to VEGF (VPF) production (44). However, in renal cell cancers, mRNA expression was also noted in well-vascularized areas and not adjacent to areas of necrosis. Subsequently, it was shown that blood vessels in immediate proximity to the tumor expressed mRNA encoding for the VPF receptors flt-1 and KDR (KDR/Pk-1), whereas those vessels not immediately adjacent did not (42, 43). The implication is that tumor cells produce VPF, which may then induce VPF receptors on adjacent tumor-associated blood vessels (but not on normal endothelium), increasing their permeability and promoting the potential of tumor cells to invade and metastasize by establishing a tumor stroma (20). Recently, Boocock et al. (45) reported VEGF and KDR gene expression in ovarian cancer cells, and although protein expression was not studied, these data provide further evidence of an autocrine/paracrine angiogenic pathway in ovarian cancer growth.

A number of studies have indicated that tumor vascularity, assessed immunochemically, has prognostic value. Weidner et al. (22) reported an association between density of microvessels and axillary metastases in primary breast cancers. In contrast, Tanigawa et al. (46) did not find an association between microvessel density and node metastases in gastric cancer. Further studies in breast cancer reported a significant association between microvessel density (measured in neovascular “hot spots”), overall survival, and relapse-free survival in early-stage disease and that intratumoral microvessel density was an independent prognostic indicator in node-negative breast cancer (23, 47). In prostate cancer, microvessel density correlated with metastases (48). More recently, an association between microvessel counts in advanced ovarian cancer and disease-free survival but not overall prognosis was reported, for which the major predictive factor was stage of disease (28). However, the effect of surgery (optimal versus suboptimal cytoreduction) was not considered in this survival analysis. There has also been one report correlating raised levels of angiogenic factors with the degree of microvessel density determined by staining for factor VIII related antigen and CD31 (31). The same authors also showed a correlation between tumor microvessel density and recurrence. In contrast to these reports, our data did not show an association between tumor vascularity and levels of angiogenic factors. Whether the serum and/or ascitic angiogenic profile in advanced EOC is clinically useful and is of independent prognostic value remains to be determined.

REFERENCES


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