ABSTRACT

Epithelial ovarian cancer is an aggressive malignancy with a generally poor outcome. To improve survival, novel therapeutic strategies for this disease are needed and require elucidation of the biological events that underlie transformation and tumor growth. Vascular permeability factor (VPF), also known as vascular endothelial growth factor, is a homodimeric glycoprotein that acts on vascular endothelium as a potent permeability-inducing agent and mitogen. The present study demonstrates for the first time the constitutive gene expression of VPF in normal and neoplastic human ovaries. Abundant levels of VPF have been identified by an immunohistoassay in the ascites of patients with epithelial ovarian cancer (K-T. Yeo et al., Cancer Res., 53: 2912–2918, 1993). We have identified the malignant epithelium as one source of VPF in the ascites. Reverse transcription-polymerase chain reaction has demonstrated the expression of the two secreted isoforms, VPF121 and VPF165, in normal and neoplastic ovaries. Western blotting and an endothelial cell proliferation assay confirmed secretion of a biologically active product. VPF may be an important mediator of ascites formation and tumor metastasis observed in neoplastic conditions of the ovary.

INTRODUCTION

The clinical presentation of EOC is characterized commonly by the presence of intraabdominal disease and ascites. With accurate staging, more than 70% of patients will have advanced disease (Stage III or IV, disease beyond the pelvis) at the time of diagnosis (1, 2). Survival of this disease, like most malignancies, is directly related to stage (extent of disease), with a 5-yr survival of less than 20% in advanced cases (3). The events that contribute to malignant transformation and tumor cell growth and metastasis are under intense study. The role of growth factors, gene activation/inactivation, and chromosomal events are being investigated. There is increasing evidence that tumor-derived growth factors are important in the proliferation of malignant cells (4–6). Inappropriate production of growth factors along with the coexpression of growth factor receptors can result in autocrine stimulation. Additionally, tumor cells are known to produce paracrine factors which can influence the host cells to create a microenvironment ideally suited for tumor growth.

VPF, also known as vascular endothelial growth factor, is a 34- to 50-kDa dimeric, disulfide-linked glycoprotein synthesized by several human and animal cell types, including normal and neoplastic (7–12). This factor was first identified in the culture supernatant and the ascites of rodent tumors (13). More recently, VPF has been found to be present in the malignant effusions of human ovarian, breast, and lung tumors (14). Sequencing of the cDNA and genomic clones of VPF has revealed the existence of multiple isoforms containing 206-, 189-, 165-, and 121-amino acid residues (15–19). These different species which appear to have similar biological activities (in vitro) arise from alternative splicing of the primary gene transcript (15, 20). The smaller two isoforms, VPF165 and VPF121, are secreted proteins which may act as diffusible agents, whereas the larger two remain cell associated (20, 21).

VPF was first described as a potent inducer of vascular permeability in the Miles assay (13). By virtue of its permeability-inducing properties, a central role for VPF in tumor stroma generation has been advanced (22, 23). The extravasation of plasma proteins from the host vasculature results in the formation of a fibrin network which serves as a substrate for cell migration during angiogenesis. In addition, VPF was found to act as a specific mitogen for a variety of endothelial cells in vitro and as an angiogenic molecule in vivo (24–28). Transmembrane receptors with tyrosine kinase activity have recently been identified for VPF (29, 30). VPF is also able to stimulate interstitial collagenase synthesis and secretion by vascular endothelium (31). Degradation of extracellular matrix molecules is a critical component of the angiogenic response. These activities displayed by a single molecule commonly associated with malignant cell growth suggest a pivotal role in tumor angiogenesis, a prerequisite for tumor growth and metastasis.

The aim of this study is to examine human ovaries, normal and neoplastic, for VPF gene expression and secretion of a biologically active product. VPF mRNA has been identified in animal ovaries and implicated to have a physiological role in the ovarian cycle (9, 28). VPF is also present in the malignant effusions of several human tumors including the ascites of EOC (14). Animal studies with transplanted ascites-producing tumors displayed inhibition of peritoneal fluid accumulation following administration of anti-VPF antibodies (13). Similarly, rapid resolution of ascites is observed in patients with EOC following treatment with cytotoxic agents (32). It is plausible that this phenomenon is due to the reduction in volume of rapidly proliferating VPF-producing cells. Therefore, identification of the cellular origins of VPF in EOC may provide insights into the biological behavior of this tumor and help in the development of therapeutics.

MATERIALS AND METHODS

Materials. Reagents for total RNA extraction were bought from Stratagene (La Jolla, CA). PCR reagents were purchased from Perkin-Elmer Cetus (Norwalk, CT). Oligonucleotide primers were synthesized by Midland Certified Reagent Company (Midland, TX). PCR reactions were carried out in the PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). TE-70 Semiphor Semi-Dry Transfer Unit (Hoefer Scientific Instruments, San Francisco, CA) was used for transfer of protein from gel to nitrocellulose membranes. BioBlot-NC (0.45 μm) nitrocellulose was from Costar (Cambridge, MA). Rabbit antibody generated against the amino-terminal peptide fragment of human VPF was a generous gift of Dr. Donald Senger (33). Anti-rabbit IgG-alkaline phosphatase conjugate was bought from Sigma (St. Louis, MO). Alkaline phosphatase substrate was from Vector Laboratories (Burlingame, CA).

Cell Lines/Culture. U937, a human histiocytic lymphoma cell line and C166, a bovine pulmonary artery endothelial cell line; FBS, fetal bovine serum; cDNA, complementary DNA; RT, reverse transcription.

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The abbreviations used are: EOC, epithelial ovarian cancer; VPF, vascular permeability factor; PCR, polymerase chain reaction; C166, bovine pulmonary artery endothelial cell line; FBS, fetal bovine serum; cDNA, complementary DNA; RT, reverse transcription.
carcinoma of the ovary and was a generous gift from Dr. O. Martinez-Maza. OVCAR-3 and OC494 cells used in this study were obtained after I.p. passage in the athymic mouse. The above-mentioned cell lines were all maintained in RPMI 1640 with 15% FBS and antibiotics. MAR is human ovarian surface epithelium (normal) transfected with the SV40 large T-antigen and was provided by Dr. N. Auersperg. Malignant cells cultured from the tumor-associated carcinoma of the ovary and was a generous gift from Dr. O. Martinez-Maza. The above-mentioned cell lines were all maintained in RPMI 1640/15% FBS and antibiotics, is a primary peritoneal carcinoma (pathological diagnosis) obtained from a patient with histologically normal ovaries bilaterally. All primary tissue samples grown in culture were used for analysis between passages 1–3. Tumor samples were obtained from the Women’s Cancer Center, University of Minnesota, according to established protocols. Samples MA160 and MA990 were provided by Dr. Mark Moradi. The samples used in this study are summarized in Table 1. Normal ovarian tissue verified histographically was obtained at laparotomy from a variety of nonovarian conditions. CPAE cells were maintained in Dulbecco’s Modified Eagle’s Medium 20% FBS and antibiotics, insulin (10 μg/ml), and 1% of the following: sodium pyruvate, l-glutamine, and nonessential amino acids. To obtain serum-free culture supernatants, cells were washed three times with Hanks’ balanced salt solution, placed in serum-free culture medium and harvested at 72 h. Seeding density of U937 was at 5 x 10⁵ cells/ml. All other cells were seeded at 2.5 x 10⁵ cells/ml. Serum-free supernatants were concentrated at 72 h. Seeding density of U937 was at 5 x 10⁵ cells/ml. All other cells were seeded at 2.5 x 10⁵ cells/ml. Serum-free supernatants were concentrated by ultrafiltration (Amicon) and filter sterilized (Spin-X, 0.22-μm filter; Costar) before electrophoresis and use in the DNA synthesis assay.

RT-PCR. Total RNA was isolated from primary tissues and cultured cell lines by single step guanidinium thiocyanate A-phenol-chloroform extraction (34). Primary tissues obtained at the time of surgery were weighed and 300 mg of samples were used for RNA isolation. Samples were minced and homogenized manually in the presence of lysis buffer on ice. Cultured ascites cells used for RNA isolation were confirmed by immunohistochemistry to be of epithelial origin with positive staining for epithelial membrane antigen, cytokeratin, and OC125 (data not shown). Final RNA samples were suspended in diethyl pyrocarbonate treated water and quantitated by absorbance at 260 nm. RT-PCR was carried out according to the Perkin Elmer Cetus protocol for reverse transcription of RNA and amplification of cDNA. PCR primers were designed complementary to the coding sequences of the 5‘ and 3’ termini of human cDNA (16). The 37-mer primers synthesized that contain restriction sites for cloning are Downstream primer: for anti-sense strand synthesis

5’ CCTGCCCGCAGCTACGGCCTGCCTGTTGACA 3’

Upstream primer: for sense strand synthesis

5’ CTCGAGAAACCAATTCATGATCTGCTGCTTGG 3’

Antisense strand synthesis utilized 1.0 μg of RNA per sample for primer extension by reverse transcriptase at 42°C for 15 min. cDNA amplification was performed according to the following parameters: 94°C, denaturation; 52°C, annealing; 72°C, extension, all for 1 min and 10 cycles. This was followed by 20 cycles with an annealing temperature of 55°C. PCR products were run on a 1% agarose gel and visualized by ethidium bromide staining.

Western Blotting. Serum-free culture supernatants were run on a 12% sodium dodecyl sulfate-polyacrylamide gel under nonreducing conditions according to the method of Laemmli (35). Transfer of proteins to a nitrocellulose membrane was performed by a semidyamic sandwich technique. Transfer was completed with 10 mm 3-[cyclohexylamino]–1-propane sulfonic acid buffer at 100 mV for 30 min. The nitrocellulose membrane was then blocked in a buffer containing 1% bovine serum albumin, 10% dry milk, 0.1% Tween 20 in phosphate-buffered saline, pH 7.4, for 2–4 h at room temperature with gentle agitation. All washings of the membrane were carried out in phosphate-buffered saline/0.05% Tween 20. Primary and secondary antibody were diluted 1:200 in blocking buffer without Tween 20. Each antibody incubation was for 1 h at room temperature. Alkaline phosphatase substrate was added and the membrane was allowed to develop for 15–30 min.

DNA Synthesis Assay. CPAE cells were plated in complete tissue culture medium in 96-well culture plates at a cell density of 5 x 10⁴ cells/well. After a 6-h incubation at 37°C, concentrated serum-free culture supernatants (20% of total volume) or serum-free medium alone and 1.0 μCi of [3H]thymidine were added to each well. After an additional 24-h incubation, medium was gently aspirated and the cells were washed twice with serum-free medium. Cells were solubilized by addition of 1.5 n NaOH for 1 h at room temperature and absorbed with cotton wicks. The cell-associated radioactivity was determined by liquid scintillation counting.

RESULTS

Identification of VPF Transcripts. Mature VPF transcripts are known to arise from alternate splicing of the primary gene transcript (15, 20). All the splice variants, however, contain the same 5’ and 3’ terminus (15). Primers were designed to amplify all known splice variants that may be expressed in the samples studied. Furthermore, to identify the type of transcript, it was critical to analyze the PCR products using a positive control. For this purpose, we used U937 cells which are known to express VPF. RT-PCR products generated from the same set of primers from U937 cells were cloned and sequenced (data not shown). The cDNAs of VPF165 and VPF121 were used as standards for comparison of RT-PCR products from ovarian sources. DNA in Fig. 1 show the electrophoretic analysis of the PCR products. Both established ovarian cancer cell lines and EOC samples demonstrate two major amplified fragments. The relative mobility of the bands indicated that they correspond to the splice variants coding for VPF165 (slower migrating band, 606 base pairs) and VPF121 (faster migrating band, 474 base pairs). Malignant samples were processed as solid tumor masses (epithelium + stroma) and as free-floating ascites tumor. There were no differences in the amplified products between ascitic and solid tumor components of a given sample (data not shown). Ascites tumor cells were passaged in culture 1–3 times and then verified immunohistochemically to be of epithelial origin, free of stromal contamination. Concern that a 1% gel may not adequately resolve the 189- and 165-amino acid isoforms was addressed by running the amplified products on a high resolution gel (3% Nu-Sieve) with no change in the banding pattern. The appearance of intermediate size bands in samples MA155 and MA160 may represent an additional splice variant. Recently, a 145-amino acid isoform was described in a study of uterine carcinoma (36). Alternatively, these may represent inappropriately amplified sequences. Further sequencing data are necessary to identify these intermediate bands.

VGF gene expression was not restricted to the tumor cell population alone. RNA preparations from all five normal ovaries and a benign fibroma also showed the amplified bands corresponding to VPF165 and VPF121. Moreover, an immortalized normal ovarian epithelial cell line (MAR) was also found to be positive for VPF transcripts. In the present study, the primary peritoneal carcinoma was the only sample that RT-PCR failed to demonstrate the presence of VPF transcripts. Notably, no sample was positive for VPF109.

Secretion of VPF by EOC. The presence of transcripts need not always lead to the actual synthesis and secretion of a protein. Therefore, it was important to determine whether EOC produced VPF. Two different assays were used to verify VPF secretion. One assay used an

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (y)</th>
<th>Diagnosis/stage/ovarian status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO510</td>
<td>51</td>
<td>Adenocarcinoma cervix/IIIa/post</td>
</tr>
<tr>
<td>NO610</td>
<td>81</td>
<td>Adenocarcinoma uterus/I/post</td>
</tr>
<tr>
<td>NO720</td>
<td>70</td>
<td>Adenocarcinoma uterus/IIIa/post</td>
</tr>
<tr>
<td>NO450</td>
<td>45</td>
<td>Intraductal breast carcinoma/IVa/post</td>
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<td>NO500</td>
<td>50</td>
<td>Uterine leiomyoma/pre</td>
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<tr>
<td>MA155</td>
<td>65</td>
<td>Serous adenocarcinoma ovarvy/III-C/post</td>
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<tr>
<td>MA154</td>
<td>47</td>
<td>Mucinous adenocarcinoma ovarvy/III-C/post</td>
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<tr>
<td>MT155</td>
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<td>Serous adenocarcinoma ovarvy/III-C/post</td>
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<td>MT155</td>
<td>32</td>
<td>Mucinous adenocarcinoma ovarvy/III-C/post</td>
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<tr>
<td>MA160</td>
<td>55</td>
<td>Serous adenocarcinoma ovarvy/III-C/post</td>
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<tr>
<td>MT151</td>
<td>55</td>
<td>Fibroadenoma/post</td>
</tr>
<tr>
<td>MA990</td>
<td>72</td>
<td>Primary peritoneal carcinoma/post</td>
</tr>
</tbody>
</table>

a NO, normal ovary; MA, ascites (free-floating tumor cells); MT, tumor (solid tumor).
b Histologically normal ovary.
immunological method to characterize the secreted protein from representative EOC cells and the second method was a bioassay to detect VPF-like mitogen activity in the conditioned medium. Cells were grown in serum-free medium for the indicated time and then the conditioned media were collected. After concentration by ultrafiltration, samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, silver staining (data not shown), and immunoblotting (Fig. 2). A polyclonal antibody raised against a synthetic oligopeptide corresponding to the amino terminus of native VPF was used to identify the immunoreactivity of the secreted protein. Blots of conditioned media were also tested for biological activity as evidenced by VPF production. OVCAR-3- and OC494-established EOC cell lines were also found to be positive for VPF mRNA. Similar findings were also observed with normal ovarian tissues and cells. We did not have available fresh ovarian surface epithelium; however, MAR, an established cell line of normal ovarian epithelium, was positive for VPF expression as were all samples of normal ovarian tissue (pre- and postmenopausal).

Interestingly, VPFa65 and VPFb121, the secreted isoforms, were observed exclusively in these samples. The oligonucleotide primers were designed complementary to the 5’ and 3’ termini of human VPF cDNA. The known splice sites are internal to these sequences; therefore, all reported isoforms would be expected to be amplified if present (15). Although there are no apparent differences in the biological properties of the VPF isoforms, VPFa165/121 would be expected to be the isoforms of functional significance as paracrine mediators in physiological and pathological processes.

The expression of VPFb121 only in MA148 is distinct from the other malignant samples tested. It is noteworthy that this sample is highly tumorigenic, forming ascites and solid tumor within 3–4 weeks of i.p. transplantation in the nude mouse. In contrast, MA155 and MA160 grew satisfactorily in culture for several passages but did not grow after i.p. transplantation. Future studies will investigate the relationship of the pattern of isoform expression and tumorigenicity.

In addition to the PCR demonstration of the molecular species of VPF present, VPF was shown by immunoblotting to be secreted into the conditioned media of U937, OVCAR-3, and MA160. The conditioned media was also tested for biological activity as evidenced by identified VPF transcripts in samples from malignant and benign ovarian neoplasms and from normal ovaries. Positive expression in free-floating ascites tumor cells and solid tumor masses obtained from the same patient identifies the malignant epithelium as one source of VPF production. OVCAR-3- and OC494-established EOC cell lines were also found to be positive for VPF mRNA. Similar findings were also observed with normal ovarian tissues and cells. We did not have available fresh ovarian surface epithelium; however, MAR, an established cell line of normal ovarian epithelium, was positive for VPF expression as were all samples of normal ovarian tissue (pre- and postmenopausal).

DISCUSSION

This study systematically examined human tissues and cells of ovarian origin for the presence of VPF gene expression and the secretion of a biologically active protein product. RT-PCR analysis
stimulation of DNA synthesis of vascular endothelium. A modest stimulation of 150–250% of control was observed. Conceivably, there are other endothelial cell mitogens present in the conditioned media; however, conditioned media (serum-free) of OVCAR-3 and U937 displayed a single distinct band on silver staining corresponding to the band identified by immunoblotting using an anti-VPF antibody. VPF transcripts were absent in MA990 and therefore the conditioned media of this sample served as a negative control.

The development of ascites in patients with ovarian carcinoma contributes significantly to the morbidity of this disease. Mean survival after the discovery of ascites is 30–40 weeks (40). Our understanding of the mechanisms responsible for malignant ascites formation has evolved. Early work emphasized the importance of lymphatic obstruction, especially of the diaphragm, by tumor cells (41, 42). More recently, evidence for the participation of tumor-derived products has been provided (43). These investigations indicate that secreted products of tumor cells may act in a paracrine fashion by eliciting fluid leakage of the neighboring host vasculature. Accumulation of VPF in an animal model of ascites-producing tumors is found localized to the vessels of the tumor and the peritoneal surfaces (26, 44). Yeo et al. (14) have measured significantly elevated concentrations of VPF in the malignant ascites of patients with ovarian carcinoma. The cellular origin of VPF, however, was not studied. Our data identify the malignant epithelium as one of the sources. Activated peritoneal macrophages in these patients need to be studied as an additional site of production. Lastly, the neovascularure of the growing tumor is also thought to be intrinsically leaky, thereby providing an additional site for fluid extravasation from the vascular space (45). Thus, it appears that VPF may play a pivotal role in malignant ascites formation both as a potent inducer of vascular permeability and as an angiogenesis factor.

These findings may help explain the significant volume of ascites that is occasionally found in apparently early stage (I/II) EOC (32). Staging of EOC requires meticulous surgical exploration with the appropriate biopsies taken. Such staging commonly reveals advanced disease and results in upstaging (2, 3). Nevertheless, the intraabdominal tumor burden is relatively low in the presence of ascites accumulation. Interestingly, in MT161, the benign ovarian fibroma, 2 liters of ascites were drained at the time of surgical exploration. Of patients with benign ovarian fibromas greater than 10 cm, 10–15% develop ascites despite negative peritoneal cytology (46). The addition of pleural effusions to the above situation defines Meigs’ syndrome (47). These observations hint that a diffusible molecule with permeability-inducing properties such as VPF may be of greater relevance than lymphatic obstruction in the genesis of effusions, peritoneal and pleural, in these patients.

Solid tumor growth is dependent on the acquisition of a supporting vasculature (48, 49). Tumor neoangiogenesis (angiogenesis) is accompanied by a propensity to metastasize (50, 51). As noted earlier, VPF has been identified in several in vivo models as an angiogenesis factor. Ferrara et al. (52) transfected Chinese hamster ovary cells with the VPF gene and demonstrated the ability of the transfected cells, in contrast to parental cells, to grow s.c. in the nude mouse as small spherical tumor growth beyond a minimum size requires a neovascular network (27). Interestingly, in situ analysis of VPF mRNA in a malignant carcinoma sample which was characterized by growth in sheets and as ascites tumor. This possibility needs to be verified in additional cases of primary peritoneal carcinoma.

Studies examining the temporospatial expression of VPF in the normal ovarian and uterine cycles has provided important insights into regulation (9, 28). In situ analysis, VPF mRNA was identified in the cells of the developing ovarian follicle, corpus luteum, and the endometrium at times coincident with neovascularization of these structures. Addition of exogenous estrogen to uterine carcinoma cell lines in culture stimulated VPF mRNA severalfold (36). Therefore, differential expression of VPF in these cell types may be hormonally regulated by gonadotropin or steroid hormones. Recognizing the limitations of the following interpretation, it is interesting to note that the postmenopausal ovaries in this study, a condition characterized by elevated systemic levels of gonadotropins, displayed the most intense banding pattern by PCR analysis. If this correlation is borne out by quantitative analysis in current studies, the antitumor effects of gonadotropin-releasing hormone analogues in EOC may be explained by the inhibition of gonadotropic stimulation of VPF expression (55, 56). This would be an example of a novel method of antiangiogenesis therapy.

In summary, this study demonstrates the constitutive expression of VPF in normal and neoplastic human ovaries. Current investigations address the issue of quantitative differences in VPF expression and the regulatory elements involved.

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Vascular Permeability Factor Gene Expression in Normal and Neoplastic Human Ovaries

Timothy A. Olson, D. Mohanraj, Linda F. Carson, et al.


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