Expression of Endocrine Gland-derived Vascular Endothelial Growth Factor in Ovarian Carcinoma

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ABSTRACT

The first tissue-specific angiogenic molecule, endocrine gland-derived vascular endothelial growth factor (EG-VEGF), was identified recently in human ovary, raising hopes of developing tumor type-specific angiogenesis inhibitors. In the present study, we analyzed the expression of EG-VEGF mRNA in normal human tissues and ovarian neoplasms by quantitative real-time reverse transcription-PCR. EG-VEGF mRNA was expressed in all ovarian neoplasms examined. No significant difference was identified among benign, low malignant potential neoplasms or stage I ovarian cancer, all of which exhibited 2-fold lower mRNA levels compared with normal premenopausal ovaries. EG-VEGF mRNA levels further decreased in late stage compared with early stage carcinomas (P < 0.05) and were consistently lower in laser capture microdissected tumors islets compared with surrounding stroma. EG-VEGF was undetectable by reverse transcription-PCR in 17 established epithelial ovarian cancer cell lines or in cultured human ovarian surface epithelial cells, whereas it was detected in peripheral blood as well as tumor-infiltrating T lymphocytes. Finally, in contrast to VEGF, EG-VEGF mRNA levels did not correlate with clinical outcome in advanced ovarian carcinoma. These results suggest that EG-VEGF is most likely derived from nonepithelial components of ovarian carcinomas and may play a marginal role in promoting angiogenesis in advanced ovarian carcinoma. We postulate that EG-VEGF-targeted antiangiogenic therapy may prove useful in early stage but not in advanced stage ovarian carcinoma.

INTRODUCTION

Angiogenesis is one of the most critical steps in embryonic development, reproductive function, and tumorigenesis (1–3). The mechanisms underlying angiogenesis and vascular remodeling are revealed to be increasingly complex thanks to the recent identification of important regulatory molecules (4). VEGF is believed to be the most important proangiogenic factor (5, 6) and exhibits distinct isoforms in different tissues (7, 8) and developmental stages (7). VEGF may act as the earliest signal molecule to switch the angiogenic cascade (5), whereas angiopoietins and their receptor Tie-2 as well as ephrins and their receptors appear to act at a later stage of angiogenesis (1, 9). Importantly, the above angiogenic factors appear to be universal in their distribution and function, for they provide general signals promoting angiogenesis in most tissues (10, 11). Several experiments have suggested the existence of additional tissue-specific angiogenic factors, which locally regulate vascularization in different organs (10, 12–14).

EG-VEGF, the first tissue-specific angiogenic molecule, was identified recently in steroidogenic endocrine organs, including the ovary, testis, adrenal, and placenta (11, 15). Although EG-VEGF does not show any homology to the VEGF family (11), it possibly induces activation of mitogen-activated protein kinase p44/42 and phosphatidylinositol-3-kinase pathways and leads to proliferation, migration, and survival of endothelial cells (15), a role reminiscent of VEGF. EG-VEGF selectively induces proliferation, migration, and fenestration (the formation of membrane discontinuities) in capillary endothelial cells specifically derived from endocrine glands, suggesting the existence of tissue-restricted specific receptors (11). Importantly, EG-VEGF contributes to the endocrine function of these organs, as endothelial cell fenestration may accelerate the entry of hormones in the bloodstream (10, 11). Because angiogenic factors play a critical role in tumorigenesis, the discovery of tissue-specific angiogenic mechanisms raises hopes of developing tumor type-specific angiogenesis inhibitors (10, 11).
Angiogenesis mechanisms play a critical role in ovarian carcinogenesis. VEGF is overexpressed in ovarian carcinoma (16–19), where it exerts important biological functions, such as promoting tumor growth and the formation of ovarian cysts and ascites (20–23). Importantly, increased serum and/or tumor levels of VEGF have been associated with poor clinical outcome (24–28). To date, it is unknown whether EG-VEGF is expressed in ovarian carcinoma and whether it plays a role in supporting angiogenesis and tumor growth. In the present study, we analyzed the expression of EG-VEGF and VEGF mRNA in human ovarian neoplasms. We report for the first time that EG-VEGF is expressed in human ovarian carcinoma but most likely derives from nonepithelial components of the tumor, including stroma cells and tumor-infiltrating leukocytes.

MATERIALS AND METHODS

Patients and Specimens. Specimens from 50 ovarian neoplasms were collected between October 1991 and July 1999 at the University of Turin, Italy, following informed consent, from patients undergoing exploratory surgery for the presence of abnormal adnexal masses. Normal ovaries were collected from premenopausal patients undergoing elective pelvic surgery for nonovarian indications. Specimens were immediately snap frozen and stored at −80°C. Four tumors were benign, four were of low malignant potential, and 42 were malignant, including 9 stage I and 33 stage III carcinomas. Tumors were surgically staged and graded according to the International Federation of Gynecology and Obstetrics. All ovarian carcinoma specimens were collected from patients untreated previously during primary debulking surgery. Twenty-eight stage III tumors, which were collected from patients achieving complete response to chemotherapy, were used for outcome analysis. DFI was defined as the time between completion of chemotherapy and first recurrence. Six ovarian cancer specimens were collected fresh at the University of Pennsylvania Medical Center after obtaining appropriate informed consent under Institutional Review Board-approved protocols. Twelve normal human tissues were provided by the Cooperative Human Tissue Network.

Cell Lines and Cell Culture. A total of 17 human ovarian cancer cell lines (A2780, A2780/C30, A2780/C70, A2008, A1847, SKOV-3, PE01, PE04, OVCAR2, OVCAR3, OVCAR4, OVCAR5, OVCAR7, OVCAR8, OVCAR10, UPN251, and OAW42) was generously provided by Drs. Steven Johnson and Kang-Sheng Yao, Department of Cancer Pharmacology, University of Pennsylvania. All cell lines were cultured in 5% CO₂ atmosphere, at 37°C, and in DMEM medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin. HOE cells were isolated intraoperatively from normal ovaries by gently scraping the ovarian surface with a wooden spatula. Cells were collected in ice-cold PBS and immediately transported to the laboratory. Primary HOE cultures were established in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/ml penicillin. All reagents for cell culture were purchased from Invitrogen (Carlsbad, CA), unless stated otherwise.

PBLs were isolated by elutriation from the apheresis product of normal healthy donors and cultured at 1 × 10⁶ cells/ml in RPMI 1640 containing 10% human AB sera (BioWhittaker, Walkersville, MD) at 37°C and 5% CO₂. Cells stimulated by anti-CD3/anti-CD28-coated beads (29) were cultured at a 3:1 bead:cell ratio at a final concentration of 1 × 10⁶/ml for 48 h. Cells were then harvested, beads were removed by magnetic separation, and cells were subjected to RNA isolation.

Flow Cytometry. Single-cell suspension from fresh tumor nodules were generated as described previously (30). Briefly, fresh tumor specimens were mechanically dissected and enzymatically digested overnight at room temperature with type IV collagenase (0.1%; Sigma Chemical Co., St. Louis, MO), type IV DNase (30 units/ml; Sigma), and type V hyaluronidase (0.01%; Sigma). The tumor suspension was then filtered through a wire grid to yield a single-cell suspension. Cells were separated on a percoll (Pharmacia Biotech AB, Uppsala, Sweden) density gradient for 30 min at 1,500 × g at room temperature and subjected to four-color flow cytometry on a FACS-Calibur flow cytometer using CellQuest 3.2.1f1 software (Becton Dickinson, San Jose, CA), using monoclonal antibodies against CD45 (HI30), CD3 (UCHT-1), CD4 (RPA-T4), CD8 (RPA-T8), CD19 (HB19), CD57 (NK-1), IgG1, and IgG2a (BD PharMingen, San Diego, CA). Data representing 10,000–30,000 events were recorded and analyzed with CellQuest software (Becton Dickinson).

Isolation of Tumor-infiltrating Lymphocytes. Tumor-infiltrating lymphocytes were isolated as described previously (30). Briefly, single-cell suspensions obtained from dispersed tumor specimens, as described above, were separated on a percoll density gradient. The dense layer, enriched for lymphocytes, was collected. Tumor-infiltrating T lymphocytes were selected by fluorescence-activated cell sorting using monoclonal antibodies against CD4 and CD8 (BD PharMingen) and a MoFlo cell sorter (Cytomation, Fort Collins, CO) equipped with an argon laser beam.

Immunohistochemistry and Immunofluorescent Staining. Immunohistochemical staining was performed using the avidin-biotin-peroxidase method. Sections were fixed in cold acetone for 10 min, pretreated with 3% H₂O₂ for 20 min to block endogenous peroxidase activity, and incubated in normal serum (Vector, Burlingame, CA) for 30 min. After incubation with the primary antibody (Table 1) in a humid chamber for 1 h, the VECTASTAIN avidin-biotin complex method kit was applied as described by the manufacturer (Vector). Briefly, sections were incubated in biotin-labeled secondary antibody for 30 min, followed by incubation in biotin-peroxidase/avidin complex for 30 min. The immunoreactive sites were visualized with freshly prepared 3,3′-diaminobenzidine (Vector) containing H₂O₂. All staining steps were performed at room temperature. Sections were counterstained lightly with Gill’s hematoxylin (Vector). Images were acquired through Cool SNAP Pro color digital camera (Media Cybernetics, Carlsbad, CA). For immunofluorescent staining, sections were sequentially incubated in 5% normal serum, antihuman cytokerin antibody (DAKO, Carpinteria, CA) diluted at 1:500 for 1 h, and FITC-labeled antirabbit IgG (Vector) for 2 h.

LCM. Stage III ovarian cancer specimens were screened for LCM by immunohistochemical staining using a polyclonal antibody against cytokerin to select the ones allowing for clear identification of tumor islets and stroma (Fig. 2A). LCM was performed as described by others (31, 32), implementing minor
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lactating breast and skin (Fig. 1A). In contrast, VEGF mRNA was detected in all specimens, and no tissue-specific expression pattern was seen (data not shown). EG-VEGF mRNA was detected by RT-PCR both in resting as well as in PBLs activated ex vivo by CD3 cross-linking and CD28 costimulation, with slightly increased levels after costimulatory activation (Fig. 1B).

The identity of the PCR product isolated from PBLs with EG-VEGF primers was confirmed by sequencing.

EG-VEGF Expression in Ovarian Neoplasms. EG-VEGF mRNA was expressed in benign and low malignant potential neoplasms, as well as early stage (stage I) ovarian cancer by real-time RT-PCR. No significant difference was identified among these tumor types (P = 0.994), whereas 2-fold lower levels were observed in these tumors compared with normal premenopausal ovaries. EG-VEGF mRNA levels were significantly decreased in late stage (stage III) ovarian cancer compared with stage I carcinomas, low malignant potential, or benign tumors (P < 0.05; Fig. 1C). In contrast to EG-VEGF, malignant ovarian tumors significantly overexpressed VEGF mRNA compared with benign and low malignant potential neoplasms (P < 0.05; Fig. 1D). No significant difference was identified between stage I and III carcinomas (P = 0.866). Because EG-VEGF can be up-regulated by hypoxia via the same mechanism as VEGF (11), we analyzed the correlation between the EG-VEGF and VEGF mRNA in stage III tumors. No correlation was found between the mRNA levels of these two angiogenic molecules (P = 0.462; Fig. 1E).

EG-VEGF Expression in Stroma and Tumor Islets. EG-VEGF mRNA was quantified comparatively in tumor islets and peritumoral stroma. First, stage III tumor specimens were screened by immunohistochemistry using cytokeratin, an epithelial tumor marker, to identify tumors with clear architectural distinction between tumor islets and stroma. Only specimens with such distinction (Fig. 2A) were selected to perform LCM (Fig. 2B). By immunohistochemistry, we determined that as many as 70 to 80% of cells within tumor islets were cytokeratin-positive tumor cells, whereas no cytokeratin-positive cells could be seen in peritumoral stroma areas. EG-VEGF mRNA was detected in both stroma and tumor islets. Remarkably, mRNA levels were consistently higher in peritumoral stroma compared with tumor islets, with a ratio of stroma:islets ranging between 2 and 7 (Fig. 2C shows two representative cases).

EG-VEGF Expression in Established Ovarian Cancer Cell Lines. We investigated the constitutive expression of EG-VEGF by ovarian carcinoma cells in vitro. A total of 17 epithelial ovarian cancer cell lines was analyzed by RT-PCR. EG-VEGF was undetectable in all cell lines (Fig. 3A).
quality of cDNA was tested by analysis of housekeeping gene GAPDH, which was detected in all cell lines (data not shown). For comparison, VEGF mRNA expression was also tested in the same specimens. High levels of VEGF mRNA were found in all 17 ovarian cancer cell lines (Fig. 3A). To enhance the sensitivity of RT-PCR, we used nested RT-PCR. In 15 of 17 cell lines, a detectable band was observed at the expected molecular weight, the intensity of which depended on the amount of the first-round PCR product (Fig. 3B). Finally, no EG-VEGF could be detected by RT-PCR in cultured HOSE cells (data not shown). The above data indicate that EG-VEGF mRNA is present at extremely low levels in ovarian cancer cell lines in vitro.

**EG-VEGF Expression in Tumor-infiltrating Lymphocytes.** We sought to assess whether immune cells could contribute to EG-VEGF expression in ovarian carcinoma. First, we examined ovarian carcinomas for the presence of tumor-infiltrating immune cells. By immunohistochemistry, we found that ovarian carcinomas were heavily infiltrated by CD3+ T lymphocytes (Fig. 4A), including CD4+ and CD8+ subsets (data not shown) in >50% of patients. By contrast, other types of lymphocytes, such as CD19+ B cells (Fig. 4B) and CD57+ NK cells (Fig. 4C), were scarcely represented. By flow cytometry, we confirmed that CD45+ leukocytes represent ≤20–35% of cells isolated from mechanically dispersed and enzymatically di-

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**Fig. 2** EG-VEGF mRNA is higher in stroma compared with tumor islets in ovarian carcinoma. In A, cytokeratin immunostaining allows for the screening of ovarian cancers with clear distinction between tumor islets (green, FITC; inset: brown, 3,3'-diaminobenzidine) and surrounding stroma. B, representative serial fields of an ovarian tumor during LCM. After rapid H&E staining, highly pure samples of tumor islets or adjacent stroma were dissected separately from the same tissue sections by LCM. C, EG-VEGF mRNA quantified by real-time RT-PCR in matched tumor islets (black bars) and surrounding stroma (white bars) in two representative cases. The EG-VEGF mRNA level is higher in stroma compared with tumor islets.

**Fig. 3** EG-VEGF is minimally expressed in established ovarian cancer cell lines. In A, EG-VEGF mRNA cannot be detected by RT-PCR in 17 ovarian carcinoma cell lines. In contrast, all cell lines express high levels of VEGF. In B, EG-VEGF mRNA can be detected by nested RT-PCR in some cell lines. Abundance of transcript depends on the volume of the product of the first amplification used (0, 0.5, and 3 µl are shown).
gested ovarian carcinomas, whereas T cells were the main immune cell population encountered in ovarian carcinoma (Fig. 4), representing 35.1% of tumor-infiltrating leukocytes (Table 3). EG-VEGF expression was assessed in tumor-infiltrating T cells by RT-PCR. In all cases, EG-VEGF mRNA was expressed in tumor-infiltrating T lymphocytes (Fig. 4).

**EG-VEGF Expression and Patient Outcome.** VEGF overexpression has been associated with poor clinical outcome in ovarian carcinoma (24–28). To assess whether the expression of EG-VEGF also predicts clinical outcome, 28 specimens from stage III patients with complete clinical response to platinum-based chemotherapy and documented relapse were analyzed. Patients were stratified based on relative expression of EG-VEGF or VEGF mRNA below or above the median. VEGF overexpression (above median) was associated with significantly shorter DFI (P = 0.0027; Fig. 5B), but no difference in outcome was observed between patients with relatively high and low levels of EG-VEGF (P = 0.491; Fig. 5A).

**DISCUSSION**

EG-VEGF is the first tissue-specific angiogenic factor identified, reportedly being expressed only in steroidogenic endocrine tissues, including the ovaries (11). As such, EG-VEGF provides the first potential target for tissue-specific, and potentially tumor type-specific, antiangiogenic therapy. This study focused on characterizing the expression of EG-VEGF in ovarian carcinoma and assessing whether indeed this angiogenic factor should be considered as a potential therapeutic molecular target in this disease.

The normal ovary is formed by cells belonging to three distinct lineages: (a) the germ cell lineage, which comprises all eggs; (b) the sex cord-stroma lineage, which comprises the cortical stroma, the cumulus oophorus surrounding the eggs, the granulosa, and theca layers of follicles and interstitial stroma; and (c) the epithelial lineage, which comprises the surface epithelium (36–38). In the normal ovary, EG-VEGF mRNA is highly expressed in elements of the sex cord-stroma lineage, including the cortical stroma, the cumulus oophorus, as well as the granulosa and theca layers of developing follicles (11). Epithelial ovarian carcinomas, which represent >90% of ovarian cancers, likely derive from the epithelial lineage of the ovary (39–41). EG-VEGF was found to be expressed at the mRNA level in all human epithelial ovarian neoplasms, including benign cystadenomas, tumors of low malignant potential, and invasive carcinomas. However, the present data indicate that tumor cells are not an important source of EG-VEGF in ovarian cancer. In fact, EG-VEGF expression was lower in cystadenomas, tumors of low malignant potential, and early stage carcinomas to normal premenopausal ovary. In addition, through LCM and RT-PCR, we showed that EG-VEGF levels were consistently higher in tumor stroma compared with tumor islets. Finally, EG-VEGF mRNA was not detected in primary ovarian...
EG-VEGF levels, suggesting different mechanisms of regulation. Furthermore, no correlation could be found between VEGF and outcome in stage III ovarian carcinoma, such association was more readily established with advanced stage or poor prognosis. In fact, EG-VEGF expression decreased in late stage ovarian cancer compared with stage I tumors. Furthermore, no correlation could be found between VEGF and EG-VEGF levels, suggesting different mechanisms of regulation. Taken together, these data suggest that EG-VEGF likely plays a marginal role in promoting angiogenesis in advanced stage disease. Ovarian carcinomas may exhibit an abundant rich stroma component, which may be highly cellular. It has been postulated that stroma-epithelial cell interactions may inhibit the growth of tumorigenic surface epithelium in early stages of ovarian oncogenesis (44, 45). In a xenograft model, it was shown that normal ovarian stromal cells, when admixed with tumor cells, had the ability to inhibit the growth of ovarian carcinoma transplants but were gradually substituted by host (murine) stromal cells, which were recruited to the tumor and supported its growth (46). These experimental data are supported by histopathologic data; only 13% of ovarian cancer specimens examined in one series displayed evidence of stromal luteinization (47), whereas in another series, steroid cells were identified in <3% of advanced ovarian cancers, most of which were of the relatively uncommon mucinous histotype (48). It is possible that the overall influence of ovarian steroidogenic stroma on tumor development might be inhibitory, resulting in its progressive loss during malignant progression, which might provide an explanation for the decline of EG-VEGF expression.

In the present study, we found that EG-VEGF mRNA is dramatically overexpressed in two steroidogenic organs, ovary and testis, but is also expressed in other normal tissues at markedly lower levels. The difference in our results is likely attributable to the use of RT-PCR, which is more sensitive than Northern blot, and the tissue RNA array used in LeCoutur’s study (11). Additional studies need to be performed to assess whether EG-VEGF expression in nonsteroidogenic tissues has any physiological function. The most interesting finding in our present study was that EG-VEGF mRNA is also expressed in resting as well as activated PBLs, which is consistent with the detection of EG-VEGF mRNA in lymphoid tissues, such as spleen and thymus. Tumor-infiltrating T cells were also found to express EG-VEGF. This is the first evidence that peripheral and tumor-infiltrating T cells secrete EG-VEGF. T cells are known to secrete VEGF (49). Furthermore, tumor-infiltrating T cells may secrete VEGF and promote angiogenesis (50). The potential roles of EG-VEGF in tumor-infiltrating T cells remain to be elucidated.

In summary, in this study, we provide evidence indicating that EG-VEGF, a purported tissue-specific angiogenic factor, is expressed in ovarian carcinoma but is most likely derived from nontumor cells of the sex cord-stromal lineage, as well as tumor-infiltrating T lymphocytes. Furthermore, in contrast to VEGF, the expression of EG-VEGF in advanced stage tumors significantly declines compared with early stage cancers, low malignant potential tumors, or benign tumors and does not correlate with outcome in advanced stage disease. On the basis of these findings, we postulate that EG-VEGF-targeted antiangiogenesis therapy may prove useful in early stage but not in advanced stage ovarian carcinoma.

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