Paracrine VEGF/VE-Cadherin Action on Ovarian Cancer Permeability

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Ascites formation associated with neoplasms is most likely due to increased vascular permeability, a process in which vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) plays a pivotal role. We hypothesized that tumor-derived VEGF/VPF modulates ascites formation through a paracrine effect on both tumor and peritoneal vessels. We investigated human vascular endothelial permeability using a newly developed dual-chamber permeability assay by co-culturing human umbilical vein cells with and without ovarian cancer cell lines (OVCAR-3, Hey-A8, and OCC-1) in the presence or absence of a human VEGF monoclonal antibody and VE-cadherin function-blocking antibody. This method permits determination of mechanisms by which substances released from neoplasms and other sources of vascular endothelial cell secretagogues modulate vascular permeability and likely other pathologic states. Exp Biol Med 231:1646–1652, 2006

Key words: vascular permeability; ovarian cancer; VEGF; VE-cadherin

Introduction

An increase in vascular permeability is a hallmark of many neoplasms. In ovarian cancer, increased vascular permeability often leads to the formation of extensive ascites in the later stages of the disease. Several studies have indicated that vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is implicated in ascites formation because of its ability to increase vascular permeability (1, 2). In particular, VEGF/VPF plays an important role in ascites formation associated with ovarian cancer (1, 3, 4). However, the mechanisms by which VEGF/VPF increase vascular permeability in ovarian cancer have not been elucidated completely.

Recent studies indicate that VEGF acts on the adjacent vascular endothelium through paracrine mechanisms (5, 6). Vascular permeability is controlled, at least in part, at intercellular junctions (7). Vascular endothelial cadherin (VE-cadherin, cadherin-5) is a junctional protein located at intercellular junctions between endothelial cells. VE-cadherin is linked to catenins and the actin cytoskeleton (8). The cadherin-catenin complex is dynamic, and its composition changes rapidly in relation to the functional state of the cells (8). The location of VE-cadherin at endothelial cell junctions is also the major site of leakage in morphologic studies of tumor vessels (9). VEGF/VPF increases vascular permeability by rearrangement of VE-cadherin (10) and appears to increase permeability *in vitro* through mitogen-activated protein kinases (MAPKs) with a subsequent disruption of VE-cadherin from endothelial cell-cell junctions (10).

Because of the markedly altered nature of tumor vessels (9, 11) and the difficulty of culturing these vessels *in vitro*, regulation of tumor vasculature has been difficult to study. In an effort to simulate the unique tumor environment, we developed a dual-chamber assay for the specific quantification of vascular permeability in tumor cells *in vitro*, which has not been reported previously. We designed the present experiments with the underlying tenets that VEGF released from ovarian cancer cells plays a paracrine role in modulating VE-cadherin expression, which in turn affects endothelial permeability, and that decreased VE-cadherin plays a role in the increase in vascular permeability and development of ascites associated with ovarian cancer. To explore these tenets, we investigated vascular endothelial monolayer permeability, using a newly developed dual-chamber permeability assay. We co-cultured human umbilical vein cells (HUVECs) with and without ovarian cancer cell lines that do (OVCAR-3) and do not (HEY-A8 and OCC1) express large amounts of VEGF in the presence

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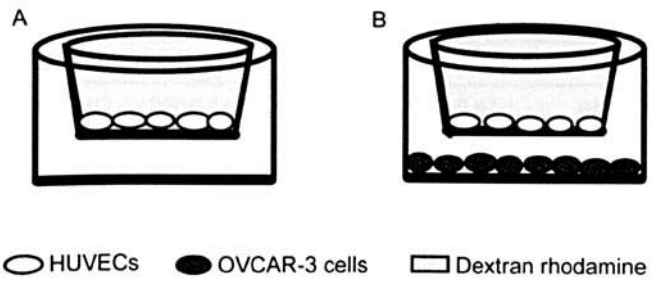

Figure 1. Schematic representation of the dual-chamber vascular permeability assay. (A) HUVECs cultured alone in the upper chamber insert. (B) HUVECs cultured on the upper chamber insert and OVCAR-3 cells cultured in the bottom chamber. Dextran-rhodamine was added to the upper chamber.

or absence of a human VEGF monoclonal antibody (mAb) and a VE-cadherin function–blocking antibody.

Here we demonstrate that VEGF/VPF derived from human ovarian cancer cells significantly induces increased vascular permeability concomitant with inhibition of VE-cadherin expression. These observations indicate that VEGF plays a paracrine role in modulating vascular permeability associated with ovarian neoplasms, at least in part by altering the expression of VE-cadherin. These studies also suggest that this novel dual-chamber co-culture permeability assay may serve as a useful, relatively rapid method for the study of vascular permeability in tumors and likely other tissues.

Materials and Methods

HUVECs were obtained from Clonetics (Walkersville, MD). The human OVCAR-3 cell line was kindly provided by Dr. T. Hamilton, Fox Chase Cancer Center, Philadelphia, PA. OCC-1 and HEY-8 were supplied by the University of California, San Francisco (UCSF) Ovarian Tumor Bank. Transwell inserts used for permeability studies were obtained from Becton Dickinson Labware (Franklin Lakes, NJ). Mouse anti-human VE-cadherin for immunostaining was obtained from Immunotech Inc. (Miami, FL). Mouse VE-cadherin blocking antibody (c175) was from Transduction Laboratories (Lexington, KY). Horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse mAb (A4.6.1) directed against human VEGF was used to neutralize VEGF activity in cell culture. Characterization of this antibody, including its high specificity toward human VEGF and its ability to inhibit VEGF activity *in vitro*, as well as its ability to block binding of VEGF to its receptors, has been described previously (12, 13). Anti-mouse IgG–fluorescein isothiocyanate (FITC) conjugate was purchased from Sigma (St. Louis, MO). Plastic cover slips used for *in vitro* studies were obtained from Nunc, Inc. (Naperville, TN). All cell culture reagents were obtained from the Cell Culture Facility, UCSF.

Dual-Chamber Permeability Assay. HUVECs were used after two to five passages. They were seeded at 2×10^4 cells on 0.4-μm pore–size inserts and grown to confluence as a monolayer in a six-well culture chamber in the presence or absence of OVCAR-3 cells in the lower culture well (see schematic illustration in Fig.1). OVCAR-3 cells were seeded (2×10^5) on plastic six-well culture plates. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a 37°C incubator in a humidified atmosphere of 95% air/5% carbon dioxide (CO_2). HUVECs were cultured at 37°C in 95% air/5% CO_2 using complete media (Clonetics, Walkersville, MD), according to the manufacturer's recommendations. When HUVEC monolayers reached confluence, they were co-cultured in the presence or absence of OVCAR-3 cells in the lower culture chamber. Either control medium or test medium was added (1.5 ml) to the apical chamber and 2.6 ml was added to the basal chamber. These volumes yielded no hydrostatic gradient across the cultures. As some types of ovarian cancer cells express little *VEGF* mRNA, including HEY-A8 and OCC1 cells (14), either HEY-A8 or OCC1 cells were seeded in the lower chamber instead of OVCAR-3 cells and used as controls. Permeability of the monolayer was assessed by quantifying the movement of 10-kDa dextran-rhodamine across it, as described below. After a 16-hr (overnight) co-culture period, a 0.05% solution of dextran-rhodamine was placed in the apical chamber of the transwell system. Three hours after adding the dextran-rhodamine solution, medium from the basal chamber was removed and the amount of dextran-rhodamine present was determined with a spectrophotofluorometer with excitation and emission wavelengths of 555 nm and 580 nm, respectively. The amount of diffused tracer was calculated from a standard curve containing known amounts of dextran-rhodamine. The amounts of VEGF mAb (A4.6.1) and VE-cadherin mAb (c175) used in the experiments were 5 μg/ml and 10μg/ml, respectively. These amounts were chosen based on preliminary studies (data not shown) that examined their effects on permeability.

A schematic illustration of the dual-chamber used is illustrated in Figure 1. HUVECs are cultured on a high porosity insert (0.4 μm) in the upper chamber.

VEGF Enzyme-Linked Immunosorbent Assay (ELISA). To confirm that OVCAR-3 cells release high levels of VEGF, media collected from HUVECs co-cultured with and without ovarian cancer cells was centrifuged and stored at –70°C. VEGF protein concentration in the conditioned media was determined using ELISA, as previously described (15).

Immunofluorescence Staining and Analysis. The cell co-culture protocol for immunofluorescence staining was the same as that for the permeability assay, except that HUVECs were cultured on plastic coverslips in the bottom chamber and OVCAR-3 cells were cultured in the upper chamber because of the difficulty in performing immunostaining on the membrane from the upper insert. At the end of the experiment, HUVECs grown on the

Table 1. VEGF Protein Concentration in Culture Medium[a]

	Cell line			
	HUVEC	HUVEC + HEY-A8	HUVEC + OCC1	HUVEC + OVCAR-3
VEGF concentration[b] (pg/ml)	<10	<10	<10	166 ± 49

[a] Media collected from HUVECs cultured with and without ovarian cancer cells.
[b] VEGF protein concentration in the conditioned media was determined using ELISA. Data are presented as mean ± SE.

coverslips in the bottom chamber, co-cultured with and without OVCAR-3 cells, in the presence or absence of the VEGF mAb, were used for immunofluorescence staining. Membranes were washed extensively in phosphate buffered saline (PBS) before and after fixation with 2% paraformaldehyde in PBS for 30 mins at room temperature, followed by immersion for 30 secs in –20°C methanol. Prior to immunostaining, cultures were pretreated with 3% horse serum in PBS plus 0.05% Tween 20 for 1 hr to improve penetration and block nonspecific antibody adsorption. Cultures were rinsed in PBS and incubated with a 1:100 dilution of mouse anti–VE-cadherin for 16 hrs (overnight) on an orbital shaker at 40°C. Cultures were thoroughly washed in PBS before application of a 1:200 dilution of anti-mouse IgG–FITC conjugate diluted in PBS containing 0.05% Tween 20 for 1 hr at room temperature, and finally washed again in PBS. Immunostained HUVEC cultures were examined under a Leica DMRB fluorescence microscope equipped with filter sets appropriate for detection of the FITC fluorophore, a Photonics DEI-470 Charge-Coupled Device (CCD) camera and a RasterOps 24XLTV frame grabber, imported directly into Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA), and stored on an external ZIP 100MB drive (Iomega Corp., Roy, UT). Photomicrographic plates were composed from the original data in Photoshop, without alteration or manipulation, and annotated with rub-on letters and symbols.

Western Blot Analysis. Proteins were extracted from HUVECs. Proteins solubilized in Laemmli buffer were separated on 4%–10% sodium dodecyl sulfate (SDS) polyacrylamide gels (Invitrogen, Carlsbad, CA) and electrotransferred onto polyvinylidene difluoride membranes. Nonspecific binding was blocked by incubation in 5% nonfat dry milk in PBS plus 0.05% Tween 20 (blocking buffer) for 1 hr. VE-cadherin was identified using a mAb, which was diluted 1:1000 in blocking buffer and incubated with membranes overnight at 4°C. Membranes were washed 3 × 10 mins in PBS plus 0.05% Tween 20 and then incubated with a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG. Lastly, membranes were washed 3 × 10 mins in PBS plus 0.05% Tween 20 before development with a standard enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ). Densitometric analysis was used quantify the amount of VE-cadherin protein.

Statistics. Data were analyzed using an unpaired Student's *t* test for comparison between groups. Differences between groups were considered statistically significant at $P < 0.05$. Data are presented as the mean ± SE among at least three independent experiments.

Results

VEGF Concentration in Ovarian Cancer Cells. Table 1 shows the concentration of VEGF in medium from HUVECs cultured alone and co-cultured with OVCAR-3, HEY-A8, or OCC1 cells. The concentration of VEGF from HUVECs co-cultured with OVCAR-3 cells was 166 ± 49 pg/ml. In contrast, the concentrations of VEGF from HUVECs cultured alone or co-cultured with HEY-A8 or OCC1 cells, ovarian cancer cells that do not express VEGF, were below 10 pg/ml.

Alteration of Monolayer Permeability. To confirm that ovarian cancer induces vascular permeability and ascites formation by releasing VEGF, we co-cultured HUVECs with OVCAR-3 cells in the presence and absence of a VEGF mAb. In HUVECs co-cultured with OVCAR-3 cells, monolayer permeability was increased by 235 ± 10%, compared with HUVECs alone. However, HUVECs co-cultured with OVCAR-3 cells in the presence of the VEGF mAb demonstrated a 53 ± 6% decrease in permeability, compared to the untreated co-culture group (Fig. 2A).

To confirm that VEGF release from OVCAR-3 cells modulates monolayer permeability, we used HEY-A8 and OCC1 cells as controls, as they express little VEGF. As shown in Figure 2B, neither HEY-A8 nor OCC1 cells induced significant increases in monolayer permeability.

To determine the extent of VE-cadherin involvement in vascular permeability, we used a function-blocking VE-cadherin antibody (c175) and examined its effect on permeability. As shown in Figure 3, the VE-cadherin antibody increased monolayer permeability by 58 ± 9%.

Expression of VE-Cadherin in HUVECs. To determine both the extent to which VE-cadherin expression is correlated with monolayer permeability and the role of VEGF released from OVCAR-3 cells in modulating VE-cadherin protein expression, we subjected HUVECs grown on plastic coverslips to immunofluorescence staining. Figure 4A demonstrates that VE-cadherin staining is localized to HUVEC intercellular junctions, and that the staining is continuous when OVCAR-3 cells are omitted. In contrast, VE-cadherin staining is discontinuous when HUVECs are placed in the bottom well of the culture chamber and OVCAR-3 cells are placed in the upper

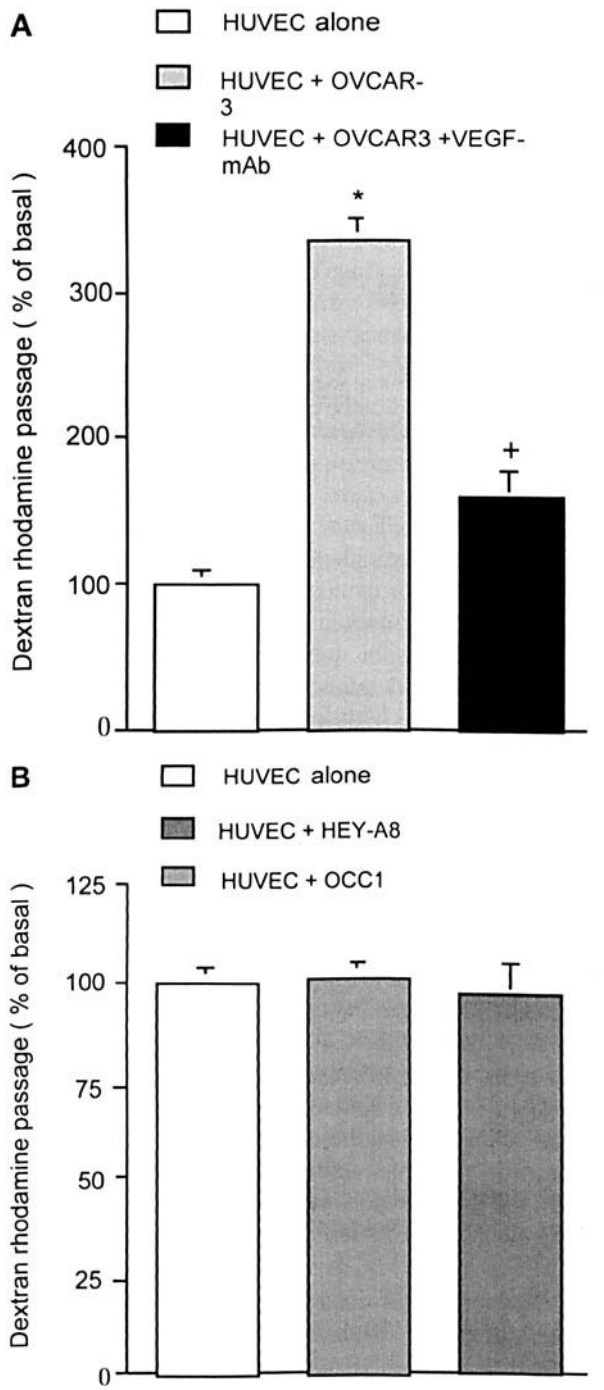

Figure 2. Alteration of monolayer permeability in ovarian cancer. (A) HUVECs co-cultured with OVCAR-3 cells in the presence and absence of a human VEGF mAb (A4.6.1). (B) HUVECs co-cultured with HEY-A8 or OCC1, ovarian cancer cell lines that express little VEGF. Permeability of the monolayer was assessed by quantifying the movement of 10-kDa dextran-rhodamine across it using a newly developed two-chamber permeability assay. Data are expressed as mean ± SE. *, $P < 0.01$ vs. control (HUVEC alone); +, $P < 0.01$ vs. HUVEC + OVCAR-3.

chamber (Fig. 4B). In contrast, VE-cadherin staining is increased when HUVECs are co-cultured with OVCAR-3 cells in the presence of the VEGF mAb (Fig. 4C).

The results of Western blot analysis of VE-cadherin

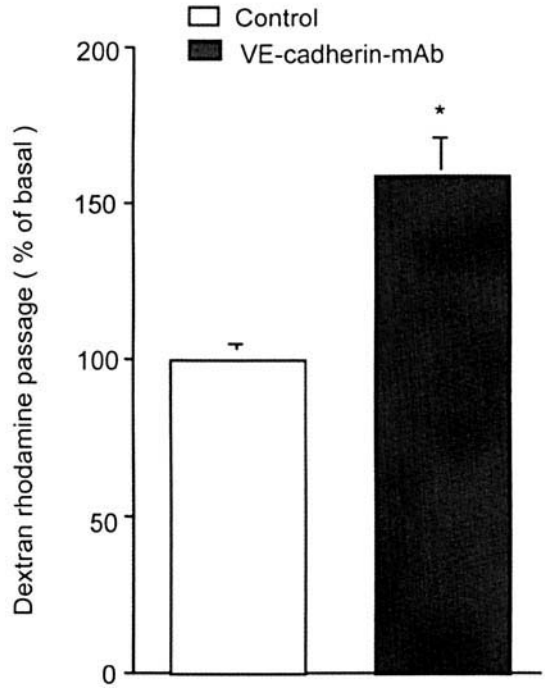

Figure 3. Role of VE-cadherin in vascular permeability. HUVECs were cultured in the upper chamber in the presence or absence of a VE-cadherin function-blocking antibody (c175). Permeability of the monolayer was assessed by quantifying the movement of 10-kDa dextran-rhodamine across the monolayer using a newly developed two-chamber permeability assay. Data are expressed as mean ± SE. *, $P < 0.01$ vs. control.

(Fig. 5) are consistent with our findings on VE-cadherin expression evaluated with immunofluorescence staining of HUVECS co-cultured with and without OVCAR-3 cells in the presence and absence of VEGF mAb (Fig. 4A–C). As anticipated, densitometric analysis, expressed as optical density, indicates that VE-cadherin expression was higher (168.95 ± 86.13) in HUVECs when OVCAR-3 cells were omitted than when HUVECs were co-cultured with OVCAR-3 cells (103.16 ± 79.62; $P < 0.001$). However, although VE-cadherin expression in the presence of the VEGF mAb was higher (115.63 ± 78.68) than that in its absence, in the HUVECs co-cultured with OVCAR-3 cells, the difference did not reach statistical significance.

Discussion

Ascites formation associated with ovarian carcinoma is most likely due to increased vascular permeability. Several lines of evidence indicate that VEGF exerts a key role in vascular permeability (1, 16). The permeability effects of VEGF on vascular endothelial cells and its increased secretion from tumor cells make it an obvious focus of investigation into the mechanisms of malignant ascites formation. Our previous studies of ip human ovarian carcinoma in an athymic mouse model indicate that a mAb to human VEGF could prevent ascites formation (1, 16). Consistent with these studies, the present *in vitro* data demonstrate that VEGF release from OVCAR-3 cells

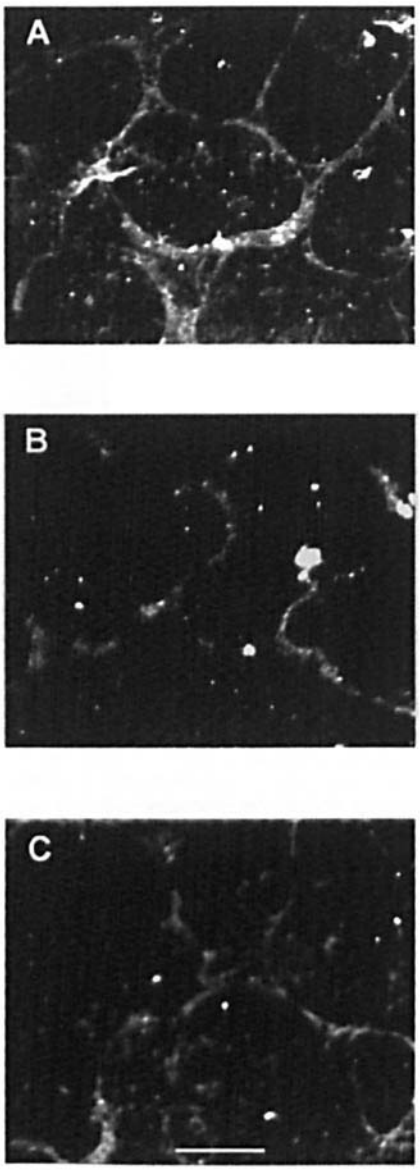

Figure 4. Expression of VE-cadherin in HUVECs. Immunofluorescence staining of the HUVEC monolayer was performed using mouse anti–VE-cadherin and a FITC-conjugated secondary antibody. (A) HUVECs alone cultured in the upper chamber. (B) HUVECs co-cultured with OVCAR-3 cells. (C) HUVECs co-cultured with OVCAR-3 cells in the presence of a human VEGF monoclonal antibody (A4.6.1). Bar, 20 μm.

induces monolayer permeability and the VEGF mAb inhibits this permeability. We extended this study to determine the mechanism by which the VEGF mAb inhibits vascular permeability and ascites formation. We also developed a dual-chamber co-culture model to quantify vascular permeability in tumor cells and demonstrate the paracrine control of vascular permeability by VEGF.

Recent studies indicate that VEGF acts on the adjacent vascular endothelium through paracrine mechanisms (5, 6). VEGF derived from endometrial stromal and epithelial cells diffuses into the interstitial tissue and binds to capillaries and spiral arteries. It exerts its action through a paracrine

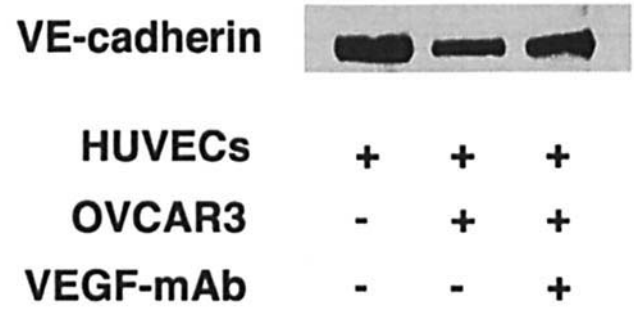

Figure 5. Western blot analysis of VE-cadherin. Expression of VE-cadherin was tested in whole-cell extracts from HUVECS cultured alone, HUVECs co-cultured with OVCAR-3 cells in the presence of VEGF mAb, and HUVECs co-cultured with OVCAR-3 cells in the absence of VEGF mAb. Twenty micrograms of protein was loaded in each lane.

mechanism (5). VEGF may regulate both angiogenesis and tumor cell growth through paracrine mechanisms in prostate cancer (6). Our data demonstrate that VEGF also plays a paracrine role in modulating vascular permeability in ovarian cancer. In the present study, we collected the culture medium and confirmed that, as anticipated, OVCAR-3 cells release high levels of VEGF, whereas VEGF levels in HEY-A8 and OCC1 culture media are virtually undetectable. The results parallel the extent of vascular permeability in the three cell lines. OVCAR-3 cells induce increased monolayer permeability, which is inhibited by the VEGF mAb.

A likely explanation for tumor induction caused by increased vascular permeability is that VEGF derived from ovarian cancer cells plays a paracrine role in modulating permeability after binding to its receptors (Flt1/VEGF-R1 and Flk1/KDR/VEGF-R2) on vascular endothelial cells, where it exerts its effect on vascular permeability. Our finding of a high concentration of VEGF in OVCAR-3 cell cultures supports this thesis, as do previous studies that demonstrate malignant effusions associated with ovarian cancer (2, 17). A previous study indicates that HEY-A8 and OCC1 cells, which express little VEGF, do not cause ascites (12). Using our newly developed permeability assay, we demonstrated that, in contrast to OVCAR-3 cells, neither HEY-A8 nor OCC1 cells induce a significant increase in monolayer permeability.

The molecular mechanisms that underlie the remodeling of host vasculature remain elusive. Recently, the relationship between adhesion junctional organization and permeability has been studied extensively. The intercellular junctions of endothelial cells serve an important barrier function that regulates vascular permeability (5). VE-cadherin is one of the major components of the endothelial cell junction that determines the strength of cell-cell adhesion, thereby influencing monolayer properties (18). Our results are consistent with previous data that indicate that VE-cadherin modulates endothelial monolayer permeability in HUVECs (19), and that VEGF/VPF increases vascular permeability by rearrangement of the endothelial

junctional protein, VE-cadherin (10). The present study demonstrates that VE-cadherin protein is quantitatively correlated with monolayer permeability. Our immunofluorescence staining and Western blot analyses demonstrate decreased production of VE-cadherin protein when HUVECs were co-cultured with OVCAR-3, and apparently increased production when HUVECs were co-cultured with OVCAR-3 in the presence of a VEGF mAb, although this result did not reach statistical significance, perhaps due to the amount of the VEGF mAb. The loss of junctional adhesion molecules in this model provides insight into the increased permeability that we observed: it is likely related to alterations in intercellular adhesion junctional proteins, of which VE-cadherin is a key component (20, 21). The VEGF mAb may increase VE-cadherin expression and maintain the integrity of interendothelial cell junctions, which serve as an important barrier that regulates vascular permeability. This finding suggests that VEGF downregulates expression of VE-cadherin, while the VEGF mAb inhibits vascular permeability by increasing VE-cadherin expression.

Various in *vitro* and *in vivo* models of permeability have been developed (22–25). In this study, we described a dual-chamber co-culture permeability assay. In an effort to approximate the unique tumor environment, we cultured HUVECs in the upper chamber and OVCAR-3 cells in the lower chamber and measured monolayer permeability in the HUVECs. We developed this dual-chamber assay with co-cultured ovarian cancer cells and HUVECs specifically to study tumor vascular permeability, an application that has not been reported previously. Because of the complexity of tumor vessels and the difficulty in culturing them *in vitro*, the regulation of tumor vasculature has been heretofore difficult to study.

There are at least four benefits of our permeability assay: (i) it permits assessment of the level of factors released from tumor cells that can modulate vascular permeability, (ii) it permits quantification of vascular permeability by determining the movement of 10-kDa dextran-rhodamine across the HUVEC monolayer, (iii) it is less time-consuming and less costly than other *in vitro* assays, and (iv) it makes it possible to study the mechanisms by which factors such as vascular endothelial–specific peptides, including VEGF and angiopoietins, modulate vascular permeability. The changes in protein or mRNA levels that are induced by factors that affect permeability can be studied using molecular biological techniques. A potential disadvantage of this method is that it is difficult to perform histologic assessment in the upper chamber, in which there are myriad pores in the thin membrane, and the HUVEC monolayer can be easily peeled off. To overcome this difficulty, we cultured HUVECs on plastic coverslips in the lower chamber and OVCAR-3 cells in the upper chamber. The co-culture protocol for immunofluorescence staining was identical to that of the permeability assay. At the end of the experiment, HUVECs grown on cover slips in the bottom chamber, co-cultured with and without OVCAR-3 cells in the presence or absence of the VEGF mAb, were used for immunofluorescence staining.

Thus, the dual-chamber co-culture permeability assay should serve as a valuable, relatively inexpensive tool for the study of vascular permeability in a variety of pathologic and physiologic states. This newly developed model permits detailed studies of the mechanism of regulation of tumor and likely nontumor–associated vascular permeability.

In summary, our data demonstrate that VEGF derived from ovarian cancer cells significantly induces increased vascular permeability, and the human VEGF mAb inhibits vascular permeability at least in part by altering the expression of VE-cadherin. Our study also indicates that VEGF plays a paracrine role in modulating vascular permeability associated with ovarian carcinoma.

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