Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), has been shown to increase potently the permeability of endothelium and is highly expressed in breast cancer cells. In this study, we investigated the role of VEGF/VPF in breast cancer metastasis to the brain. Very little is known about the role of endothelial integrity in the extravasation of breast cancer cells to the brain. We hypothesized that VEGF/VPF, having potent vascular permeability activity, may support tumor cell penetration across blood vessels by inducing vascular leakage. To examine this role of VEGF/VPF, we used a Transwell culture system of the human brain microvascular endothelial cell (HBMEC) monolayer as an in vitro model for the blood vessels. We observed that VEGF/VPF significantly increased the permeability of the highly metastatic MDA-MB-231 breast cancer cells across the HBMEC monolayer. We found that the increased transendothelial migration (TM) of MDA-MB-231 cells resulted from the increased adhesion of tumor cells onto the HBMEC monolayer. These effects (TM and adhesion of tumor cells) were inhibited by the pre-treatment of the HBMEC monolayer with the VEGF/VPF receptor (KDR/Flk-1) inhibitor, SU-1498, and the calcium chelator 1,2-bis(O-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid (acetoxyethyl)ester. These treatments of the HBMEC monolayer also inhibited VEGF/VPF-induced permeability and the cytoskeletal rearrangement of the monolayer. These data suggest that VEGF/VPF can modulate the TM of tumor cells by regulating the integrity of the HBMEC monolayer. Taken together, these findings indicate that VEGF/VPF might contribute to breast cancer metastasis by enhancing the TM of tumor cells through the down-regulation of endothelial integrity.

Brain metastasis, which is an important cause of cancer morbidity and mortality, occurs in at least 30% of patients with breast cancer. A key event of brain metastasis is the migration of cancer cells through the blood-brain barrier (BBB), which constitutes the endothelium and the surrounding cells (1, 2). To metastasize to the brain, malignant tumor cells must enter into the circulatory system through the endothelium (intravasation) and then attach to microvessel endothelial cells to invade the BBB (extravasation) (1). The precise molecular mechanism of extravasation of tumor cells penetrating the BBB is poorly defined. A widely supported hypothesis is that tumor cell adhesion to endothelium induces the retraction of endothelial cells, which exposes their basement membrane to the tumor cells (3). Tumor cells recognize and bind to components in the vascular membrane, thereby initiating extravasation and the beginning of new growth at secondary organ sites (4). This suggests that the intact endothelium can serve as a “defensive barrier” to the extravasation of tumor cells.

Tumor-bearing blood vessels, however, do not seem to be effective in acting as a defensive barrier for the prevention of tumor cell intravasation, because these blood vessels display high leakage and disrupted integrity (5, 6). Hypoxia is believed to contribute to the leakage of tumor blood vessels by inducing increased vascular permeability (7, 8). Hypoxia is also a strong inducer of VEGF/VPF (9, 10). VEGF/VPF has potent mitotic activity specific to vascular endothelial cells and significant vascular permeable activity (11, 12). VEGF/VPF binds to its cognate receptors, Flt-1, KDR/Flk-1, and neuropilin-1. Among them, KDR/Flk-1 is responsible for the initiation of signal transduction pathways within the cells (11, 12). VEGF/VPF has an essential role in promoting new blood vessel formation (angiogenesis) during tumor development, and inhibition of its function effectively prevents tumor growth through incomplete blood vessel formation (13, 14). Indeed, VEGF/VPF expression has been reported in a number of cancer cell lines growing in several clinical specimens derived from breast, brain, and ovarian cancers (15–18). Although the role of VEGF/VPF as an
angiogenic factor in primary tumor growth and secondary tumor growth (metastatic tumors) is well studied, its role as a vascular permeability factor in metastatic processes is not well elucidated. Tumor cells may more easily penetrate a retracted endothelial monolayer caused by VEGF/VPF than a tightly arranged monolayer, suggesting that the vascular permeability activity of VEGF/VPF contributes an "offensive ability" to the tumor cells, allowing them to penetrate blood vessels. To examine this hypothesis, we constructed a Transwell culture system of the human brain endothelial monolayer as an in vitro model for the BBB. We then evaluated the inter-relationship between the integrity of the endothelial monolayer and the vascular permeability of VEGF/VPF and their effect on the TM of the highly metastatic MDA-MB-231 breast cancer cells, which are known to express high amounts of VEGF/VPF (19).

EXPERIMENTAL PROCEDURES

Materials—Human recombinant VEGFα (VEGF, consists of 165 amino acid residues) and anti-human VEGF/VPF monoclonal antibody were obtained from Genentech, Inc. (San Francisco, CA). Human recombinant bFGF was purchased from R & D Systems, Inc. (Minneapolis, MN). Rhodamine-phalloidin, Dil, and BACEF-AM were from Molecular Probes, Inc. (Eugene, OR) and BDPharMingen, BAPTA-AM, Wortmannin, and SU-1498 were purchased from Calbiochem. Anti-human VE-cadherin monoclonal antibody was from Chemicon International, Inc. (Temecula, CA). Anti-human VEGF/VPF polyclonal antibody and anti-human Csk antibody were from Santa Cruz Biotechnology, Inc. (San Diego, CA).

Cell Culture—Human brain microvascular endothelial cells (HBMECs) were purchased from Cell Systems Inc. (Kirkland, WA). The cells were seeded onto attachment factor-coated culture plates and maintained in culture medium containing 10% fetal bovine serum. T47D cells (ATCC) were cultured in DMEM containing 10% calf serum. MCF-7 cells were obtained from ATCC and maintained in culture medium containing 5% fetal bovine serum and 2 mM L-glutamine. MCF-7 cells were from Clonetics (San Diego, CA) and were cultured in EGM containing von Willebrand factor. Human umbilical vein endothelial cells (HUVECs) were from Clonetics (San Diego, CA) and were cultured in EGM complete medium (Clonetics). MDA-MB-231, MDA-MB-453, and MCF-7 cells were obtained from ATCC and maintained in culture medium containing 10% fetal bovine serum. T47D cells (ATCC) were cultured in RPMI 1640 medium containing 10% fetal bovine serum. All cell lines were incubated in 5% CO2 at 37°C.

Northern Blot Analysis of KDR/Flk-1 Expression in Breast Cancer Cell Lines—mRNAs of breast cancer cell lines were isolated from cellular extracts by using an oligo(dT) column (Invitrogen), according to the manufacturer's protocol. The mRNAs were separated on an agarose gel and transferred to a Hybond N membrane (Amersham Biosciences). The membrane was hybridized with a 32P-labeled probe for KDR/Flk-1 (a generous gift of Dr. Michael Klugbrom, Children's Hospital, Boston, MA), and after washing, the blot was exposed to x-ray film. The blot was also stripped and reprobed for actin mRNA.

Fluorescent Labeling of MDA-MB-231 Cells—MDA-MB-231 cells were incubated with 200 nM Dil for 30 min and then washed twice with PBS. Dil-labeled cells were dispersed in 0.05% trypsin solution and resuspended in culture medium. Alternatively, tumor cells were dispersed in 0.05% trypsin solution and incubated with 1 µM BCECF-AM for 15 min and then centrifuged three times to remove free BCECF-AM.

TM Assay of MDA-MB-231 Cells—HBMECs grown on attachment factor-coated culture plates were dispersed in 0.05% trypsin solution and resuspended in culture medium. Approximately 106 tumor cells were added to the fibronectin-coated 24-well Transculture inserts with pore sizes of 8 µm (Costar Corp.) and grown for 5 days in 5% CO2 at 37°C. After the assays, the cells were fixed with 3.7% formaldehyde and washed extensively with PBS to remove floating tumor cells. Attached tumor cells were observed under a fluorescent microscope and counted from 10 random fields of ×200 magnification.

Retraction Assay of HBMECs—To monitor the extent of endothelial cell retraction, the amount of [3H]Hinulin (Amersham Biosciences) passing across an endothelial monolayer was measured as described (14). Briefly, ~100,000 HBMECs were added to fibronectin-coated 24-well Transwell inserts with pores of 8 µm and grown for 5 days in 5% CO2 at 37°C. The medium was replaced every day with fresh medium. After incubation for 2 h, the wells were fixed with 3.7% formaldehyde, and washed extensively with PBS to remove floating tumor cells. Attached tumor cells were observed under a fluorescent microscope.

VE-Cadherin staining of HBMECs—HBMECs were added to fibronectin-coated 24-well Transwell inserts with pores of 8 µm and grown for 5 days in 5% CO2 at 37°C. After the assays, the cells were fixed with 3.7% formaldehyde and washed extensively with PBS. The transwell inserts were separated carefully from the apical chamber and mounted on a slide, and F-actin staining was observed under a fluorescent microscope.

VE-Cadherin staining of HBMECs—HBMECs were added to fibronectin-coated 24-well Transwell inserts with pores of 8 µm and grown for 5 days in 5% CO2 at 37°C. After the assays, the cells were fixed with 3.7% formaldehyde and washed extensively with PBS. The transwell inserts were separated carefully from the apical chamber and mounted on a slide, and F-actin staining was observed under a fluorescent microscope.

VE-Cadherin staining of HBMECs—HBMECs were added to fibronectin-coated 24-well Transwell inserts with pores of 8 µm and grown for 5 days in 5% CO2 at 37°C. After the assays, the cells were fixed with 3.7% formaldehyde and washed extensively with PBS. The transwell inserts were separated carefully from the apical chamber and mounted on a slide, and F-actin staining was observed under a fluorescent microscope.
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**RESULTS**

**MDA-MB-231 Cells Express mRNA of the VEGF/VPF Receptor, KDR/Flk-1**—It was reported that, in nearly 50% of breast tumors, there was significant expression of KDR/Flk-1 in the tumor epithelial cells, which correlated with the expression of VEGF/VPF by these cells (22). To examine whether various breast cancer cell lines express the VEGF/VPF receptor, KDR/Flk-1, which is responsible for most VEGF/VPF activity, we performed Northern blot analysis with a specific probe for KDR/Flk-1. As shown in Fig. 1A, breast cancer cell lines, such as MDA-MB-231 cells, expressed KDR/Flk-1 mRNA at a low level as compared with HUVEC.

**Western Blot Analysis**—MDA-MB-231 cells were lysed in kinase lysis buffer (New England Biolabs). Proteins were separated by SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes (Millipore, Boston, MA). The membranes were blocked with 5% bovine serum in PBS for 1 h before overnight incubation at 4 °C. The membranes were probed with anti-KDR/Flk-1, which is responsible for most VEGF/VPF activity, enhanced chemiluminescence (Amersham Biosciences). The membranes were blocked with 5% bovine serum in PBS and subsequently incubated with primary antibody for overnight incubation at 4 °C. After incubation for 6 h, the apical chambers were fixed with 3.7% formaldehyde, washed extensively with PBS to remove floatation, and the apical side of the apical chamber was scraped gently with cotton wool. Only the migrating tumor cells were observed under a light microscope and counted from 10 random fields of ×200 magnification. The results are presented as the mean ± S.D. of triplicate samples. **CTL** control.

**Effect of VEGF/VPF on the Invasion of MDA-MB-231 Cells**—Next, to test whether MDA-MB-231 cells respond to exogenously added VEGF/VPF, an invasion assay was performed for 6 h under serum-free conditions. MDA-MB-231 cells were added to a matrigel-coated polycarbonate filter and then VEGF/VPF was added to the basolateral chambers containing 600 μl of DMEM with 1% BSA. The cells were added evenly to the apical and basolateral chambers. After incubation for 6 h under serum-free conditions, the invading cells were assessed under a light microscope. When VEGF/VPF was added to the basolateral chambers, the invasion of MDA-MB-231 cells was not significantly increased, irrespective of KDR/Flk-1 expression (Fig. 1B).

**VEGF/VPF Increases Penetration of MDA-MB-231 Cells across an HBMEC Monolayer**—To test whether VEGF/VPF increases tumor cell penetration, DiI-labeled MDA-MB-231 cells were added to an HBMEC monolayer cultured onto a Transwell apical chamber and then penetrating MDA-MB-231 cells were assessed under a fluorescent microscope. To exclude the chemotactic effect of the added growth factor for the HBMEC monolayer or tumor cells, VEGF/VPF was added evenly to the apical and basolateral chambers. VEGF/VPF treatment led to a dose-dependent increase in the penetration of MDA-MB-231 cells across the HBMEC monolayer as compared with the untreated control (Fig. 2). bFGF is also known as a potent endothelial cell growth factor but does not increase vascular permeability (23). When bFGF was added evenly to the apical and basolateral chambers, the invasion of MDA-MB-231 cells was not significantly increased, irrespective of KDR/Flk-1 expression (Fig. 2B).

**Effect of VEGF/VPF on the Invasion of MDA-MB-231 Cells across a HBMEC monolayer.** HBMECs were added to fibronectin-coated 24-well Transculture inserts with pore sizes of 8 μm (Costar Corp.) and grown for 5 days in 5% CO₂ at 37 °C. 40,000 Dil-labeled MDA-MB-231 cells were added to the apical chamber. To exclude the chemotactic effect of the added growth factor, bFGF or VEGF/VPF was added evenly to the apical and basolateral chambers. After incubation for 6 h, the apical chamber was fixed with 3.7% formaldehyde and washed extensively with PBS. The apical side of the apical chamber was scraped gently with cotton wool. Only the migrating tumor cells were observed by a fluorescent microscope and counted from 10 random fields of ×200 magnification. The results are presented as the mean ± S.D. of duplicate samples and are representative of five individual studies. **CTL** control.

**Fig. 1.** KDR/Flk-1 expression in various breast cancer cells and the effect of VEGF/VPF on the invasion of MDA-MB-231 cells. **Panel A**, the isolated mRNAs of the various breast cancer cell lines (as indicated) were separated on an agarose gel and transferred to a Hybond N membrane. The membrane was hybridized with a probe for KDR/Flk-1, and after washing, the blot was exposed to x-ray film. HUVEC were used as a positive control for the KDR/Flk-1 mRNA. Actin mRNA is shown as an internal control. **Panel B**, MDA-MB-231 cells were trypsinized and suspended at 1.5 × 10⁵ in 100 μl of DMEM containing 1% BSA. The cells were added to the apical chamber of the matrigel-coated Transculture inserts and then VEGF/VPF was added to the basolateral chambers containing 600 μl of DMEM with 1% BSA. After incubation for 6 h, the apical chambers were stained with HEMA-3 solution, and the apical side of the apical chamber was scraped gently with cotton wool. The migrating tumor cells were observed under a light microscope and counted from 10 random fields of ×200 magnification. The results are presented as the mean ± S.D. of triplicate samples. **CTL** control.

**Fig. 2.** VEGF/VPF increases the TM of MDA-MB-231 cells across an HBMEC monolayer. HBMECs were added to fibronectin-coated 24-well Transculture inserts with pore sizes of 8 μm (Costar Corp.) and grown for 5 days in 5% CO₂ at 37 °C. 40,000 Dil-labeled MDA-MB-231 cells were added to the apical chamber. To exclude the chemotactic effect of the added growth factor, bFGF or VEGF/VPF was added evenly to the apical and basolateral chambers. After incubation for 6 h, the apical chamber was fixed with 3.7% formaldehyde and washed extensively with PBS. The apical side of the apical chamber was scraped gently with cotton wool. Only the migrating tumor cells were observed by a fluorescent microscope and counted from 10 random fields of ×200 magnification. The results are presented as the mean ± S.D. of duplicate samples and are representative of five individual studies. **CTL** control.
endothelial monolayer, the monolayers were treated with 50 mM
10 random fields of tumor cells were observed by a fluorescent microscope and counted from
washed extensively with PBS to remove floating tumor cells. Attached
incubation for 2 h, the wells were fixed with 3.7% formaldehyde and
medium were added to each well with or without test samples. After
incubation for 2 h, the wells were fixed with 3.7% formaldehyde and
and with SU-1498 (50 μM), an antagonist of KDR/Flik-1. As
shown in Fig. 4, both treatments against VEGF/VPF signifi-
cantly inhibited the TM of the MDA-MB-231 cells.

VEGF/VPF Increases the Adhesion of MDA-MB-231 Cells to an HBMEC Monolayer—Metastatic tumor cells attach more preferentially to SEB membrane components than to the apical
surface of an intact endothelial monolayer (24). The same phe-
omenon was observed in this study with MDA-MB-231 cells
that attached preferentially to areas where the SEB of the
endothelial cell was exposed (Fig. 3, right panel). Therefore, the
increased TM of the MDA-MB-231 cells induced by VEGF/VPF
might result from the increased adhesion of the cells to the SEB
membrane components of the endothelial cells that were ex-
posed. To test this possibility, DiI-labeled MDA-MB-231 cells
were added to an HBMEC monolayer cultured onto 24-well plates with or without VEGF/VPF, and cell adhesion was then assessed under a fluorescent microscope. At a concentration of
30 ng/ml, VEGF/VPF increased the adhesion of MDA-MB-231
cells to the HBMEC monolayer 3-fold as compared with the untreated control (Fig. 3, left panel), and this effect was blocked by VEGF/VPF monoclonal antibody and SU-1498 (see Fig. 5).
However, bFGF failed to significantly increase the adhesion of
tumor cells to the monolayer as compared with the untreated
control (Fig. 3). These results indicate that the increased TM of
MDA-MB-231 cells induced by VEGF/VPF was at least in part
derived from enhanced tumor cell adhesion onto the exposed
SEB membrane components.
VEGF/VPF Increases the TM and Adhesion of MDA-MB-231 Cells through Calcium Signaling—VEGF/VPF stimulates several molecules mediating intracellular signals in endothelial cells, including mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK), phosphatidylinositol 3-kinase, and calcium (11). To examine which signaling pathways of VEGF/VPF in endothelial cells are responsible for the increased TM and adhesion of MDA-MB-231 cells, the effects of specific inhibitors for various VEGF/VPF signaling pathways were tested. As shown in Figs. 4 and 5, the intracellular calcium chelator (BAPTA-AM) slightly inhibited the increased TM and adhesion of MDA-MB-231 cells stimulated by VEGF/VPF whereas the MEK inhibitor (PD98059) and the phosphatidylinositol 3-kinase inhibitor (Wortmannin) had no effect, indicating that VEGF/VPF increases the TM and adhesion of MDA-MB-231 cells through activation of endothelial calcium signaling.

VEGF/VPF Increases the Permeability of the HBMEC Monolayer—Endothelial cell retraction induces the breakdown of intercellular junctions and leads to an increase in vascular permeability. Therefore, we measured the extent of endothelial cell retraction induced by VEGF/VPF as the degree of permeability change of [3H]inulin through the HBMEC monolayer. After incubation for 2 h, the cells were fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.5% Triton X-100. The VE-cadherin in the cells was stained according to the method described under "Experimental Procedures" and observed by a fluorescent microscope. Panel B, after assaying for the indicated times, the HBMEC monolayer was fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.5% Triton X-100. The VE-cadherin of the cells was stained according to the method described under "Experimental Procedures" and viewed under a confocal microscope. Panel C, 1,000 BCECF-AM-labeled MDA-MB-231 cells were added to each HBMEC monolayer with or without VEGF/VPF. After incubation for 2 h, the cells were fixed with 3.7% formaldehyde in PBS. VE-cadherin in the HBMECs was stained as described under "Experimental Procedures" and viewed under a confocal microscope. MDA-MB-231 cells are visualized by green color, whereas VE-cadherin is viewed as red color, respectively. CTL, control; VEGF/VPF, 30 ng/ml; BAPTA-AM, 10 μM.

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VEGF/VPF induces actin redistribution and VE-cadherin disruption in HBMECs. Panel A, HBMECs were added to fibronectin-coated 24-well Transculture inserts with pore sizes of 0.4 μm and grown for 5 days in 5% CO2, at 37 °C. After assaying for 2 h, the HBMEC monolayer was fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.5% Triton X-100. The F-actin in the cells was stained according to the method described under "Experimental Procedures" and observed by a fluorescent microscope. Panel B, after assaying for the indicated times, the HBMEC monolayer was fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.5% Triton X-100. The VE-cadherin of the cells was stained according to the method described under "Experimental Procedures" and viewed under a confocal microscope. Panel B, after assaying for the indicated times, the HBMEC monolayer was fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.5% Triton X-100. The VE-cadherin of the cells was stained according to the method described under "Experimental Procedures" and viewed under a confocal microscope. Panel C, 1,000 BCECF-AM-labeled MDA-MB-231 cells were added to each HBMEC monolayer with or without VEGF/VPF. After incubation for 2 h, the cells were fixed with 3.7% formaldehyde in PBS. VE-cadherin in the HBMECs was stained as described under "Experimental Procedures" and viewed under a confocal microscope. MDA-MB-231 cells are visualized by green color, whereas VE-cadherin is viewed as red color, respectively. CTL, control; VEGF/VPF, 30 ng/ml; BAPTA-AM, 10 μM.
IgG; WB VEGF/VPF monoclonal antibody (20/H9262) grating tumor cells were observed as described under the apical chambers were fixed with 3.7% formaldehyde, and the mi-
with VEGF/VPF monoclonal antibody and were then added to the apical medium without growth factor. 5,000 DiI-labeled cells were treated 231 cells were dispersed in trypsin solution and resuspended in CSC control.

analyzed by Western blotting using anti-Csk antibody as an internal antibody (Santa Cruz Biotechnology, Inc.). Total protein extracts were analyzed by Western blotting using an anti-VEGF/VPF polyclonal antibody (25). We found that the VE-cadherin of the HBMEC monolayer (Fig. 7B). Furthermore, VE-cadherin was seen to be discontinuous under these conditions, as indicated by the arrows. However, in the presence of VEGF/VPF plus BAPTA-AM, VE-cadherin staining was continuous, as indicated by the arrows, whereas BAPTA-AM inhibited the VEGF/VPF-induced gap formation at this corner (Fig. 7B).

This indicates that calcium signaling governs the disorganization of the endothelial junction induced by VEGF/VPF. These data suggest that the increased TM of MDA-MB-231 cells induced by VEGF/VPF occurs through the loss of junctional proteins, with concomitant gap formation in the endothelial monolayer, as shown in Fig. 7C.

VEGF/VPF Overexpression Increases the TM of MDA-MB-231 Cells—To examine whether endogenously secreted VEGF/VPF modulates the TM of tumor cells, MDA-MB-231 cells were infected with VEGF-Ad, and their TM was examined. As shown in Fig. 8A, VEGF-Ad-infected cells expressed a high amount of VEGF/VPF as compared with the cells infected with the CTL-Ad. Furthermore, VEGF-Ad-infected cells showed increased TM as compared with the cells infected with CTL-Ad. The increased TM of the VEGF-Ad-infected cells was significantly abolished in the presence of VEGF/VPF monoclonal antibody but not control antibody (Fig. 8B). These data indicate that the endogenous expression of VEGF/VPF modulates the TM of tumor cells.

**DISCUSSION**

A key event in cancer metastasis is the TM of tumor cells. Metastasizing tumor cells should penetrate blood vessels twice, by intravasation and extravasation. These tumor cells may possess an offensive ability to penetrate blood vessels. It is well known that increased endothelial cell retraction is closely associated with the enhanced adhesion of tumor cells and their invasion into the endothelial monolayer. Metastasizing tumor cells can induce endothelial cell retraction through the secretion of soluble factors (26, 27) and/or through direct adhesion to the endothelial monolayer where intracellular signals are transduced to induce morphological changes (28). For example, Homn et al. (26) reported that 12(S)-hydroxyeicosatetraenoic acid produced by tumor cells induces retraction of endothelial cells and can enhance tumor cell adhesion to the SEB membrane components of the exposed endothelial monolayer. Kusama et al. (27) also reported that endothelial cell retraction factor secreted by tumor cells increases the TM of tumor cells through enhanced tumor cell adhesion.

In this report, we examined whether VEGF/VPF, which is expressed highly in breast cancer cells, enhances the TM of MDA-MB-231 cells across a monolayer of brain microvascular endothelial cells. VEGF/VPF has been shown to potently induce the retraction of endothelial cells. As expected, VEGF/VPF significantly increased the TM of MDA-MB-231 cells across a monolayer of HBMECs. Our data indicate that the enhanced TM by VEGF/VPF, at least in part, is derived from the increased adhesion of these cells onto exposed SEB membrane components of the monolayer. The mitogenic effect of VEGF/VPF on endothelial cells does not seem to be related to the increased TM and adhesion of the MDA-MB-231 cells, because bFGF and the MEK inhibitor PD98059 failed to stimulate the TM and adhesion of these cells. bFGF has been shown to stimulate the growth of endothelial cells through the ERK pathway as does VEGF/VPF but does not increase vascular permeability (23). However, the permeability effect of VEGF/VPF seems to be related to the increased TM and adhesion of MDA-MB-231 cells, because inhibition of VEGF/VPF-induced vascular permeability by the calcium chelator BAPTA-AM prevented the TM and adhesion of these cells.

Calcium, as a multifunctional modulator, also regulates permeability in the vascular system (29). Many inflammatory agents including histamine and thrombin, as well as VEGF/VPF, are known to increase vascular permeability through calcium up-regulation in endothelial cells (30–32). Although the mechanism of how this transient increase in calcium concentration alters vascular permeability has not been fully elucidated, increased calcium in endothelial cells can induce the activation of myosin light chain kinase, and phosphorylated myosin light chain can contribute to the contraction of endothelial cells upon the increased actin-myosin interaction (31). Our data, as well as other studies, have shown that VEGF-induced actin rearrangement and gap formation in interendothelial junctions result in increased permeability (33) and that these effects were significantly blocked by the intracellular calcium chelator BAPTA-AM (Fig. 7, A and B) (33). Interestingly, after adhesion to the endothelial monolayer, malignant tumor cells can transiently increase the calcium concentration of endothelial cells in the contact area and stimulate endothelial cell retraction by breaking the intercellular junctions (28). In the TM of leukocytes, leukocytes migrate across the endo-

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**Fig. 8. Overexpression of VEGF/VPF increased the TM of MDA-MB-231 cells.** Panel A, MDA-MB-231 cells were infected with VEGF-Ad or CTL-Ad for 48 h. The VEGF/VPF expression of these cells was analyzed by Western blotting using an anti-VEGF/VPF polyclonal antibody (Santa Cruz Biotechnology, Inc.). Total protein extracts were analyzed by Western blotting using anti-Csk antibody as an internal control. Panel B, after labeling with DiI, adenovirus-infected MDA-MB-231 cells were dispersed in trypsin solution and resuspended in CSC medium without growth factor. 5,000 DiI-labeled cells were treated with VEGF/VPF monoclonal antibody and were then added to the apical chamber containing the HBMEC monolayer. After incubation for 18 h, the apical chambers were fixed with 3.7% formaldehyde, and the migrating tumor cells were observed as described under "Experimental Procedures." The results are presented as the mean ± S.D. of triplicate samples. rh-VEGF, recombinant human VEGF/VPF (25 ng); VEGF-Ab, VEGF/VPF monoclonal antibody (20 μg/ml); CTL-IgG, control mouse IgG; WB, Western blotting.
the endothelial monolayer by inducing an enhanced intracellular calcium concentration of the endothelial cells (34–37), and pretreatment of the endothelial monolayer with BAPTA-AM potently blocked the TM of the leukocytes (37). These studies suggest the possibility that cells penetrating through blood vessels have the ability to elicit changes in intracellular calcium concentration in endothelial cells.

VEGF/VPF may also enhance the TM of MDA-MB-231 cells through increased adhesion onto the apical surface of endothelial cells, as well as onto exposed SEB membrane components. Recently, Kim et al. (38, 39) reported that VEGF/VPF significantly increased the expression of E-selectin in HUVEC. Endothelial E-selectin has been shown to mediate increased adhesion onto an endothelial monolayer and the TM of some tumor cells across the layer (40, 41). These studies suggest that the vascular permeability of VEGF/VPF might contribute predominantly to the TM of tumor cells as compared with the up-regulation of endothelial adhesion molecules by VEGF/VPF.

The hepatoprotective agent Malotilate intensifies the cell-to-cell contact of endothelial cells and increases the integrity of the endothelial monolayer (42, 43). Interestingly, this compound significantly inhibits the in vitro TM and in vivo metastasis of some carcinoma cells (42, 43). Furthermore, some peptides and antibodies that are able to block the adhesion of tumor cells to SEB membrane components have exerted anti-metastatic effects (44–46). Taken together, our data, as well as other studies, indicate that the integrity of blood vessels and the progression of tumor metastasis are closely related.

In addition to its vascular permeability activity in endothelial cells, VEGF/VPF induces intracellular signaling-mediated proliferation and invasion in VEGF/VPF receptor-expressing breast cancer cells (47, 48). Thus, it is possible that exogenously added VEGF/VPF affects the TM of breast cancer cells through the altered invasive property of the tumor cells. However, this possibility can be excluded for the following reasons. First, VEGF/VPF (or bFGF) was added evenly to the apical and basolateral chambers to exclude the chemotactic effect of the added growth factor. Second, VEGF/VPF failed to significantly increase the invasion of MDA-MB-231 cells across a matrigel-coated polycarbonate filter (Fig. 1B). Third, VEGF/VPF monochlonal antibody did not have any effect on the survival and invasion of MDA-MB-231 cells (data not shown). Last, the expression of KDR/Flik-1 in MDA-MB-231 cells was very weak as compared with its expression level in endothelial cells (Fig. 1A).

In this study, we also examined whether endogenous expression of VEGF/VPF modulates the TM of tumor cells. MDA-MB-231 cells were infected with VEGF-Ad to overexpress VEGF/VPF, and then their TM was examined. As expected, VEGF-Ad-infected cells showed increased TM as compared with the cells infected with the CTL-Ad. Furthermore, the increased TM of the VEGF-Ad-infected cells was significantly abolished in the presence of VEGF/VPF monochlonal antibody but not control antibody (Fig. 8B). These data indicate that the endogenous expression of VEGF/VPF modulates the TM of tumor cells.

Next, to reduce VEGF/VPF expression, we transfected MDA-MB-231 cells with antisense VEGF/VPF cDNA and selected the stable MDA-MB-231 cell clones. Interestingly, antisense VEGF/VPF stable-transfected MDA-MB-231 cell clones showed reduced VEGF/VPF expression and increased apoptosis as compared with the parental cells.2 Similar to our observations, the reduction in endogenous VEGF/VPF expression has been reported to induce apoptosis in hematopoietic stem cells, although exogenously added VEGF/VPF or soluble VEGF/VPF receptor did not exert phenotypically any effects in the cells (49). Taken together, it is possible that KDR/Flik-1 in hematopoietic stem cells and breast cancer cells might be bound to the endogenous VEGF/VPF, leading to its desensitization to VEGF when the exogenous VEGF/VPF is added. Thus, the survival mechanism of VEGF/VPF in both cell types (hematopoietic stem cells and breast cancer cells) might be different from that of VEGF/VPF in the endothelial cells.

To date, VEGF/VPF has been shown to exert an essential role in tumor angiogenesis. However, its role as a vascular permeability factor in metastatic processes, such as TM, has not been defined. Melnyk et al. (50) reported that neutralization of the function of VEGF/VPF potently blocked tumor metastasis irrespective of its angiogenic property. However, their study did not demonstrate the inter-relationship between tumor metastasis and the functions of VEGF/VPF in blood vessels. Thus, functional blocking of VEGF/VPF is a potentially effective therapeutic approach to delay or prevent tumor metastasis through the inhibition of multiple steps of tumor progression, such as neovascularization (angiogenesis) in tumor-bearing tissue and the TM of tumor cells. In conclusion, we report that VEGF/VPF modulates the TM of MDA-MB-231 breast cancer cells through the down-regulation of brain microvascular endothelial cell permeability.

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