Vascular Endothelial Growth Factor Modulates Neutrophil Transendothelial Migration via Up-regulation of Interleukin-8 in Human Brain Microvascular Endothelial Cells*

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Hypoxia, a strong inducer for vascular endothelial growth factor (VEGF)/vascular permeable factor (VPF) expression, regulates leukocyte infiltration through the up-regulation of adhesion molecules and chemokine release. To determine whether VEGF/VPF is directly involved in chemokine secretion, we analyzed its effects on chemokine expression in human brain microvascular endothelial cells (HBMECs) by using a human cytokine cDNA array kit. Cytokine array analysis revealed that VEGF/VPF induced interleukin-8 (IL-8) expression in HBMECs, a result similar to that described previously in other endothelial cells. Interestingly, we also observed that VEGF/VPF induced interleukin-8 (IL-8) expression in HBMECs and that IL-8 mRNA was maximal after 1 h of VEGF/VPF treatment of the cells. Enzyme-linked immunosorbent assay data and immunoprecipitation analysis revealed that although VEGF/VPF induced IL-8 expression at the translational level in HBMECs, basic fibroblast growth factor failed to induce this protein expression within 12 h. VEGF/VPF increased IL-8 production in HBMECs through activation of nuclear factor-KB via calcium and phosphatidylinositol 3-kinase pathways, whereas the ERK pathway was not involved in this process. Supernatants of the VEGF/VPF-treated HBMECs significantly increased neutrophil migration across the HBMEC monolayer compared with those of the untreated control. Furthermore, addition of anti-IL-8 antibody blocked this increased migration, indicating that VEGF/VPF induced the functional expression of IL-8 protein in HBMECs. Taken together, these data demonstrate for the first time that VEGF/VPF induces IL-8 expression in HBMECs and contributes to leukocyte infiltration through the expression of chemokines, such as IL-8, in endothelial cells.

Brain edema is a major life-threatening complication in a variety of brain injuries, including those caused by stroke and tumors. The common form of brain edema is defined by the disruption of the blood-brain barrier (BBB) (1). The BBB, which constitutes the endothelium and its surrounding cells, is an anatomic structure that is defined mainly by tight junctions between the brain endothelial cells. These junctions strictly regulate the flow of ions, nutrients, and cells into the brain.

Hypoxia is believed to be a precondition for edema development in the brain and mediates leukocyte infiltration through the up-regulation of adhesion molecules and chemokine release (2, 3). Hypoxia has been shown to contribute to the leakage of the BBB by inducing increases in vascular permeability (4, 5). Hypoxia is a strong inducer of vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) (6, 7).

VEGF/VPF has shown potent vascular permeable activity (8, 9) and significant mitotic activity specific to vascular endothelial cells (10). VEGF/VPF induces signaling mediated by its receptors, the Flt-1 and Flk-1/KDR tyrosine kinases, whose expressions are restricted almost exclusively to endothelial cells (11). Expression of VEGF/VPF has been identified in cells correlated with brain inflammation, including microglial cells and reactive astrocytes, suggesting its central role in inflammation of the central nervous system (12, 13). For example, enhanced expression of VEGF/VPF and its receptors was shown to be induced in the rat brain after focal cerebral ischemia (14–16) and to mediate brain injuries through leakage of the BBB in the ischemic brain (17). However, edema formation and brain damage after ischemia or stroke were reduced significantly by antagonism of VEGF/VPF or by inhibition of its signaling pathway (18, 19), indicating that VEGF/VPF is a key mediator in the pathogenesis of these disorders.

VEGF/VPF induces expression of the monocyte chemoattractant protein-1 (MCP-1) in bovine retinal endothelial cells (20) and of the SDF-1 receptor CXCR4 in human umbilical vein endothelial cells (21). These reports suggest that VEGF/VPF not only mediates brain injuries through the leakage of the BBB, but also may have a relevant role in the recruitment of leukocytes through the up-regulation of chemokines. However, its role in the regulation of chemokines is not well elucidated because of the redundancy of chemokines. Chemokines are a superfamily of small, cytokine-like proteins that induce the directional migration of various hematopoietic cells through their interaction with G protein-coupled receptors (22, 23). To date, 44 chemokines and 21 chemokine receptors have been described (23).

In this study, we examined the effects of VEGF/VPF on chemokine expression in human brain microvascular endo-
lial cells (HBMECs) using a human cytokine cDNA array kit containing 34 chemokines and 14 chemokine receptors. We isolated total RNA from unstimulated and VEGF/VPF-stimulated HBMECs and analyzed the gene expression of both samples by hybridizing them with the cytokine cDNA array. Our results demonstrate that VEGF/VPF significantly increased MCP-1 and CXCR4 expression in HBMECs, similar to results reported in other endothelial cell types (20, 21). Interestingly, we also found that VEGF/VPF induced interleukin-8 (IL-8) expression in HBMECs. IL-8, which belongs to the CXC chemokine family, is a potent chemoattractant for neutrophils and T lymphocytes but not monocytes (22). In this report, we have characterized IL-8 expression and secretion upon VEGF stimulation of HBMECs and determined the role of IL-8 in modulating neutrophil transendothelial migration.

Based on these observations we suggest that, in addition to its angiogenic activity and vascular permeable property, VEGF/VPF has a role as an indirect leukocyte migrating factor through the up-regulation of chemokines including MCP-1 and IL-8.

EXPERIMENTAL PROCEDURES

Materials—VEGF/VPF, bFGF, the human cytokine cDNA array kit, IL-8, IL-8 antibody, the IL-8 enzyme-linked immunosorbent assay (ELISA) kit, quantitative IL-8 mRNA kits, and the SDF-1α ELISA kit were purchased from R&D Systems (Minneapolis). TMB-8, PD98059, LY294002, chelerythrine chloride, cycloheximide, t-NAME, and SU-4186 were purchased from Calbiochem. Fibronectin was purchased from Roche Molecular Biochemicals. Von Willebrand factor was purchased from Dako (Carpinteria, CA). Matrigel and PDTC were purchased from Sigma. Acetylated low density lipoprotein (AcLDL) was purchased from Biomedical Technologies, Inc. (Stoughton, MA).

Cell Culture—HBMECs were purchased from Cell Systems, Inc. (Kirkland, WA). The cells were seeded onto attachment factor-coated culture plates and maintained in CSC-complete medium according to the protocol of the manufacturer.

Cytokine cDNA Array—HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. The cells were starved in 0.5% FBS-containing serum-free CSC medium for 4 h and then stimulated with 30 ng/ml VEGF for 5 h. Total RNA preparation and hybridization procedures were performed according to the manufacturer’s protocol. Briefly, equal amounts (2 μg) of total RNA from unstimulated or VEGF-stimulated HBMECs were used for the cytokine cDNA array analysis, with a commercially available membrane (R&D Systems). The membranes were prehybridized at 68 °C for 2 h in a hybrid solution (R&D Systems) containing 100 μg/ml freshly boiled salmon sperm DNA. cDNA probes were hybridized onto the membranes at 68 °C for 18 h. The membranes were washed three times in low stringency washing buffer (2 × SSC and 1% SDS) and three times in high stringency washing buffer (0.1 × SSC and 0.5% SDS) at 68 °C for 20 min each. The membranes were exposed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed using ArrayVision® software (Imaging Research Inc., Ontario, Canada).

Measurement of IL-8 mRNA—The amount of IL-8 mRNA was measured using quantitative IL-8 mRNA kits as directed by the manufacturer. Briefly, HBMECs were grown subconfluently in CSC-complete medium in gelatin-coated 60-mm dishes. The medium was replaced by serum-free CSC medium containing 0.5% FBS, and the cells were starved for 4 h. After incubation with VEGF/VPF for the indicated times, total RNA isolation was performed, and equal amounts (2 μg) were evaluated for IL-8 content using quantitative IL-8 mRNA kits.

Measurement of IL-8 or SDF-1α by ELISA—Chemokine release was quantified by ELISA as directed by the manufacturer. Briefly, HBMECs were grown subconfluently in CSC-complete medium in gelatin-coated 24-well plates. The medium was replaced by serum-free CSC medium containing 0.5% FBS, and the cells were starved for 4 h. Synthetic inhibitors were added to the cells for 1 h before the assay was initiated. After incubation for the indicated times, the culture supernatant was removed and evaluated for IL-8 content using the ELISA kit.

Immunoprecipitation and Western Blot Analysis of IL-8—HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. The cells were starved in 5 ml of 0.5% FBS containing serum-free CSC medium for 4 h and then stimulated with 30 ng/ml VEGF or 30 ng/ml bFGF for 12 h. After incubation, 5 ml of supernatant from HBMECs was harvested and incubated with 5 μg of human IL-8-specific goat IgG at 4 °C. After overnight incubation, protein G-Sepharose at 1:100 was added to the reaction mixture and incubated for 6 h. The immune complexes were precipitated with the Sepharose beads, washed, and resuspended in SDS-PAGE sample buffer with reducing agent. The samples were separated by 15% SDS-PAGE and transferred onto nitrocellulose membrane (Millipore, Boston, MA). The membranes were blocked with 5% nonfat dried milk in phosphate-buffered saline and subsequently incubated with goat anti-human IL-8 antibody for 2 h at room temperature. Bound antibodies were detected by horseradish peroxidase-conjugated anti-goat IgG and enhanced chemiluminescence (Amersham Biosciences, Inc.).

Adenoviral Infection of HBMECs—An adenoviral construct encoding a constitutively active form of Akt (Ad-myrAkt) was constructed as described previously (24). HBMECs were grown subconfluently in CSC-complete medium in gelatin-coated six-well plates. The medium was replaced by serum-free CSC medium containing 0.5% FBS, and the cells were starved for 4 h. HBMECs were infected with Ad-myrAkt at a multiplicity of infection of 100. A synthetic inhibitor of nuclear factor-xB (NF-xB), PDTC (20 μM), was added to the cells for 1 h before infection. After incubation for 24 h, the culture supernatant was removed and evaluated for IL-8 content using the ELISA kit. Alternatively, the cells were lysed, and 50 μg of total protein was resolved by 12% SDS-PAGE and subjected to Western blot analysis by using rabbit anti-human phospho-Akt antibody (New England Biolabs, Beverly, MA). To verify the amount of loaded proteins, blots were reprobed with rabbit anti-human C-terminal SRC kinase antibody.

Isolation of Neutrophils—Human neutrophils were purified from normal donors by dextran sedimentation and Ficoll gradient centrifugation followed by hypotonic lysis of erythrocytes (25). Neutrophils were then resuspended in serum-free CSC medium containing 0.5% FBS. The purity of neutrophils prepared in this way was >98% as judged by the morphology of stained cytocentrifuged preparations, and the viability was >98% as judged by trypan blue exclusion.

Transendothelial Migration Assay—HBMECs were grown confluen-
tly onto gelatin-coated 100-mm dishes. The cells were starved in 5 ml of 0.5% FBS containing serum-free CSC medium for 4 h and then stimulated with 30 ng/ml VEGF for 12 h. After incubation, 5 ml of supernatant from HBMECs was harvested and stored frozen at −20 °C before use. Transendothelial migration of neutrophils was performed using Transwell culture chambers with pore size of 3 μm (Costar Corp., Corning, NY). Approximately 100,000 HBMECs were added to fibronectin-coated 24-well Transwell chambers and grown for 4 days in 5% CO2 at 37 °C. The medium was replaced every day with fresh medium. 0.6 ml of medium from untreated or VEGF/VPF-treated HBMECs was added to the lower compartment. Media that were not exposed to cells were used as the control. 20 μg/ml human IL-8-specific goat antibody or 20 μg/ml normal goat antibody was added to the VEGF/VPF-treated supernatant for 1 h before the assay was initiated, as indicated. In the upper compartment, 1 × 106 neutrophils in 0.1 ml of 0.5% FBS containing serum-free CSC medium were added onto the HBMEC monolayer. The chambers were incubated for 2 h at 37 °C in 5% CO2. The cells in the lower compartments were counted on a hemocytometer. The results are presented as the means ± S.D. of three separate experiments and are expressed as the increase in the number of cells migrating toward the lower compartment.

RESULTS

Characterization of HBMECs—The HBMECs formed tubular-like networks on Matrigel (Fig. 1A) and had the ability to

FIG. 1. Characterization of HBMECs. 50,000 HBMECs were cultured onto 24-well plates coated with 150 μl of Matrigel for 12 h, and then 50 μg of AcDL was added to the wells. After 2 h, the cells were washed and fixed with 3.7% formaldehyde. Tubular-like networks (panel A) and AcDL uptake (panel B) were observed under an inverted fluorescent microscope equipped with a light lamp.

HBMECs was harvested and incubated with 5 μg of human IL-8-specific goat IgG at 4 °C. After overnight incubation, protein G-Sepharose at 1:100 was added to the reaction mixture and incubated for 6 h. The immune complexes were precipitated with the Sepharose beads, washed, and resuspended in SDS-PAGE sample buffer with reducing agent. The samples were separated by 15% SDS-PAGE and transferred onto nitrocellulose membrane (Millipore, Boston, MA). The membranes were blocked with 5% nonfat dried milk in phosphate-buffered saline and subsequently incubated with goat anti-human IL-8 antibody for 2 h at room temperature. Bound antibodies were detected by horseradish peroxidase-conjugated anti-goat IgG and enhanced chemiluminescence (Amersham Biosciences, Inc.).
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Control

VEGF/VPF

Fig. 2. Cytokine cDNA array of HBMECs. HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. The cells were starved in 0.5% FBS containing serum-free CSC medium for 4 h and then stimulated with 30 ng/ml VEGF for 5 h. Total RNA preparation and hybridization were performed as described under “Experimental Procedures.” The hybridized membranes were exposed on a PhosphorImager and analyzed by using ArrayVision™ software. CK indicates the region containing cDNA spots corresponding to the chemokines and their receptors. The numbers indicate spots that were increased significantly in the VEGF/VPF-treated HBMECs. 1, IL-8; 2, MCP-1; 3, CXCR4.

uptake AcLDL (Fig. 1B), indicating that these cells maintained the general properties of endothelial cells. The cells also produced von Willebrand factor (data not shown), an endothelial-specific marker. During the course of the experiment, the cells were used until passages 4 to 10 and checked repeatedly for expression of von Willebrand factor.

VEGF/VPF Predominantly Up-regulates IL-8, MCP-1, and CXCR4 mRNA Expression—To examine whether VEGF/VPF induces chemokines or their receptors in HBMECs, we used a cytokine cDNA array containing 34 chemokines and 14 chemokine receptors. We isolated total RNA from unstimulated and VEGF/VPF-stimulated HBMECs (5-h stimulation). The cytokine cDNA membranes were hybridized and exposed on a PhosphorImager and then analyzed using ArrayVision™ software. The results showed that VEGF/VPF predominantly up-regulated IL-8, MCP-1, and CXCR4 mRNA expression in HBMECs (Fig. 2 and Table I). Our data are consistent with previous studies showing that VEGF/VPF induces expression of MCP-1 and CXCR4 in other endothelial cells. Interestingly, we also found that VEGF/VPF significantly increased IL-8 mRNA expression in HBMECs (Fig. 2 and Table I). To determine further whether VEGF/VPF regulates IL-8 expression at the mRNA level, HBMECs were exposed to VEGF/VPF for various times, and the amount of IL-8 mRNA was determined by using a quantitative IL-8 mRNA kit. The results showed that VEGF/VPF induced IL-8 mRNA expression. This expression reached a maximal level of transcription at 1 h (13-fold increase compared with control) and decreased to control levels at 12 h (Fig. 3). These data indicate that VEGF/VPF induced expression of IL-8 mRNA in a rapid and transient manner.

VEGF/VPF but Not bFGF Induces Rapid Secretion of IL-8 Protein in HBMECs—Next, to determine whether IL-8 expression was regulated at the protein level, the supernatants of VEGF/VPF-treated HBMECs were collected and analyzed for IL-8 secretion by ELISA. When HBMECs were exposed to different concentrations (1, 10, 30, 100 ng/ml) of VEGF/VPF for 24 h, increased IL-8 production with a maximal level of translation at 30 ng/ml was observed (data not shown). At this concentration, VEGF/VPF increased IL-8 production by as early as 3 h, and this increase was sustained for up to 32 h (Fig. 4). We also determined whether the leukocyte chemoattractant SDF-1α was induced by VEGF/VPF. When the supernatants of the 30 ng/ml VEGF/VPF-treated HBMECs were tested by specific SDF-1α ELISA, we observed no increase in SDF-1α protein in these supernatants compared with the untreated control (data not shown). These results are in agreement with the results of the cytokine cDNA array shown in Fig. 2 and Table I.

In addition, we examined whether bFGF, known as a potent mitogen to endothelial cells, increased IL-8 or SDF-1α production in HBMECs. Within 12 h of 30 ng/ml bFGF treatment, we observed no increase in IL-8 protein in the samples compared with the untreated controls. However, after 12 h of bFGF treatment, the amount of IL-8 in the samples was greater than that in the untreated controls, although it was much smaller than in the VEGF/VPF-treated samples (Fig. 4). On the other hand, we observed no increase in SDF-1α protein in the supernatants of the bFGF-treated HBMECs compared with the untreated control (data not shown).

To confirm the molecular characterization of the VEGF/VPF-induced IL-8 protein expression, we performed immunoprecipitation and Western blot analysis. For these studies, supernatants from untreated, VEGF/VPF-, or bFGF-treated HBMECs were incubated with goat anti-human IL-8 antibody, and the immune complexes precipitated with protein G-Sepharose were then analyzed by Western blotting. As seen in Fig. 5, a significant increase in the band corresponding to a molecular mass of ∼8 kDa was detected in the VEGF/VPF-treated conditioned medium. This VEGF/VPF-induced band migrated to a position similar to that of the human recombinant IL-8. These results support the molecular identity of the VEGF/VPF-induced IL-8 as being comparable with that of the recombinant IL-8. Therefore, VEGF/VPF stimulated the secretion of IL-8 protein in HBMECs.

Cycloheximide and SU-1498 Inhibit VEGF/VPF-induced IL-8 Production in HBMECs—To confirm further that VEGF/VPF up-regulated IL-8 expression via new protein synthesis in HBMECs, the protein synthesis inhibitor cycloheximide was used to treat HBMECs for 12 h, and VEGF/VPF-induced IL-8 production was then evaluated by ELISA. As shown in Fig. 6, 10 μM cycloheximide was enough to block VEGF/VPF-induced IL-8 production in HBMECs potently. In addition, VEGF/VPF-induced IL-8 production was also inhibited significantly by 10 μM SU-1498, an inhibitor of its receptor Flk-1/KDR (Fig. 6). These data demonstrate that VEGF/VPF increased IL-8 production at the translational level through its receptor in HBMECs.

NF-κB Mediates VEGF/VPF-induced IL-8 Production in HBMECs—VEGF/VPF has been shown to stimulate several molecules mediating intracellular signals, including mitogen-activated protein/extracellular signal-regulated kinase (ERK)
ArrayVision membranes were exposed on a PhosphorImager and analyzed using isolated from unstimulated and VEGF/VPF-stimulated HBMECs. The membranes were exposed on a PhosphorImager and analyzed using ArrayVision™ software. Bold lettering indicates expression (in untreated or VEGF/VPF-treated HBMECs) of chemokines and their receptors that have sVOL values over 2 and that are visualized as spots on the scanned images, as shown in Fig. 2. Asterisks indicate chemokine or chemokine receptor expression that is increased significantly in the VEGF/VPF-treated HBMECs. sVOL is the subtracted volume value derived by subtracting the background volume value from the volume value of the spot. Genomic DNAs represent the positive controls for the hybridizations. The results are presented as the means of two spots.

### TABLE I

| Chemokines and chemokine receptors | Control sVOL | VEGF sVOL | VEGF/control ratio (VOL) |
|-----------------------------------|-------------|----------|------------------------|
| Midkine                           | 22.56       | 27.30    | 1.21                   |
| MIG                               | -0.12       | 0.82     | -7.85                  |
| MIP-1α                            | -0.32       | 0.58     | -1.80                  |
| MIP-1β                            | -0.12       | 0.98     | -8.13                  |
| MIP-3α                            | 0.60        | 0.22     | 0.36                   |
| MIP-3β                            | 0.60        | 0.18     | 0.29                   |
| SDF-1                             | 3.12        | 3.66     | 1.17                   |
| Eotaxin                           | 0.40        | 0.50     | 1.24                   |
| TARC                              | 1.32        | 0.34     | 0.25                   |
| Eotaxin-2                         | 1.08        | 0.86     | 0.79                   |
| TECK                              | 0.60        | 0.50     | 0.84                   |
| Decorin                           | 0.68        | 0.50     | 0.73                   |
| Fractalkine                       | 1.24        | -0.06    | 0.15                   |
| GRO-α                             | 1.12        | 0.22     | 0.19                   |
| GRO-β                             | 2.52        | 0.90     | 0.36                   |
| GRO-γ                             | 2.96        | 2.30     | 0.78                   |
| HCC-1                             | 2.20        | 2.42     | 1.00                   |
| HCC-2                             | 0.96        | 1.34     | 1.39                   |
| I-309                             | 0.88        | 1.98     | 2.25                   |

Kinase (MEK), phosphatidylinositol 3-kinase (PI3-kinase), protein kinase C, and calcium in endothelial cells (26, 27). Because there are multiple VEGF/VPF signaling pathways, we examined which of these pathways were responsible for the increased IL-8 production in HBMECs. As shown in Fig. 7A, the intracellular calcium chelator TMB-8 (10 μM) and PI3-kinase inhibitor

Next, we examined the effect of PDTC, the inhibitor of NF-κB, on VEGF/VPF-induced IL-8 production in HBMECs. As shown in Fig. 7A, the intracellular calcium chelator TMB-8 (10 μM) and PI3-kinase inhibitor

### FIG. 3. Induction of IL-8 mRNA by VEGF/VPF in HBMECs. The amount of IL-8 mRNA was measured as described under "Experimental Procedures." Briefly, HBMECs were grown subconfluent in CSC-complete medium in gelatin-coated 24-well plates. The medium was replaced by serum-free CSC medium containing 0.5% FBS, and the cells were incubated for 4 h. After incubation with VEGF/VPF for the indicated times, total RNA was isolated, and equal amounts (2 μg) of total RNA were evaluated for IL-8 content using a quantitative IL-8 mRNA kit. One atmol of human IL-8 was considered to be 524 fg according to the protocol of the manufacturer. The results are presented as the means ± S.D. of triplicate samples.

### FIG. 4. VEGF/VPF but not bFGF induces rapid secretion of IL-8 protein in HBMECs. HBMECs were grown subconfluent in CSC-complete medium in gelatin-coated 24-well plates. The medium was replaced by serum-free CSC medium containing 0.5% FBS, and the cells were incubated for 4 h. After incubation with VEGF/VPF or bFGF for the indicated times, the culture supernatants were removed and evaluated for IL-8 secretion using the ELISA kit. The results are presented as the means ± S.D. of duplicate samples and are representative of three individual studies.

### FIG. 5. Immunoprecipitation and Western blot analysis of VEGF/VPF-induced IL-8 expression in HBMECs. HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. The cells were incubated with 5 ml bFGF for 12 h. After incubation, 5 ml of supernatant from the HBMECs was immunoprecipitated with anti-IL-8 antibody and applied to SDS-PAGE. CTL, control; rhIL-8, recombinant human IL-8 (20 ng); IP, immunoprecipitation; WB, Western blotting. LY294002 (10 μM) significantly blocked IL-8 production stimulated by VEGF/VPF, whereas the protein kinase C inhibitor chelerythrine chloride (2 μM) and nitric oxide synthase inhibitor L-NAME (1 mM) moderately inhibited its production. However, the MEK inhibitor PD98059 (10 μM) had no effect on IL-8 production in HBMECs stimulated by VEGF/VPF.
**FIG. 6.** Cycloheximide and SU-1498 inhibit VEGF/VPF-induced IL-8 production in HBMECs. HBMECs were grown subconfluently in CSC-complete medium in gelatin-coated 24-well plates. The medium was replaced by serum-free CSC medium containing 0.5% FBS, and the cells were starved for 4 h. Cycloheximide and SU-1498 were added to the cells for 1 h before the assay was initiated. After incubation with VEGF/VPF for 12 h, the culture supernatants were removed and evaluated for IL-8 content using the ELISA kit. The results are presented as the means ± S.D. of triplicate samples and are representative of two individual studies. **CTL**, control; **CHX**, cycloheximide; **DMSO**, dimethyl sulfoxide.

IL-8 expression in endothelial cells upon treatment with various stimuli, such as tumor necrosis factor-α (28, 29), whereas the intracellular calcium chelator TMB-8 has been shown to inhibit the activation of NF-κB (30, 31). As expected, the NF-κB inhibitor PDTC (20 μM) significantly blocked IL-8 production in HBMECs stimulated by VEGF/VPF (Fig. 7A). We further examined whether the PI3-kinase pathway mediates VEGF/VPF-induced IL-8 production in HBMECs through NF-κB activation. To test this possibility, HBMECs were infected with adenovirus. However, the NF-κB inhibitor PDTC (20 μM) potently inhibited Ad-myrrAkt-induced IL-8 production in HBMECs (Fig. 7B). Expression of phosphorylated Akt protein was confirmed by Western blotting (Fig. 7C).

These results indicate that VEGF/VPF induces IL-8 production in HBMECs through activation of the transcription factor NF-κB via PI3-kinase and calcium signaling pathways. VEGF/VPF-induced IL-8 production increased the transendothelial migration of human neutrophils—IL-8 is an potent chemoattractant for neutrophils. To determine whether VEGF/VPF induced the production of functional IL-8, the supernatants were harvested from HBMECs treated with VEGF/VPF for 12 h and added to the lower compartment as described under “Experimental Procedures.” VEGF/VPF-treated supernatants significantly increased neutrophil migration across an HBMEC monolayer compared with untreated samples (Fig. 8A). To test specifically the role of IL-8 in migration, 20 μg/ml human IL-8-specific goat antibody was added to the VEGF/VPF-treated supernatants for 1 h before the assay was initiated. The results demonstrated that anti-IL-8 antibody blocked the neutrophil migration across the HBMEC monolayer that was induced by the VEGF/VPF-treated supernatant, thereby indicating that VEGF/VPF-induced functional IL-8 protein in HBMECs. In addition, we examined the amount of IL-8 in these VEGF/VPF-treated supernatants using ELISA and compared its functional activity with that of human recombinant IL-8. The sample contained ~3 ng/ml IL-8, and the transendothelial migration of neutrophils induced by this sample was comparable with that induced by human recombinant IL-8 (Fig. 8B). Thus, the secreted IL-8 in HBMECs treated with VEGF/VPF was functionally active.

**DISCUSSION**

In this study, we examined the role of VEGF/VPF in chemokine expression in HBMECs. We used a cytokine cDNA array containing 34 chemokines and 14 receptors. This array is an excellent tool to identify chemokines expressed differentially in response to external stimuli. By using the cytokine cDNA array, we observed that VEGF/VPF significantly increased MCP-1 and CXCR4 expression in HBMECs similar to that described in other endothelial cells (20, 21). Interestingly, VEGF/VPF also induced IL-8 production at the transcriptional level, and IL-8 mRNA expression was induced in a rapid and transient manner (Fig. 3). Furthermore, VEGF/VPF increased IL-8 protein production by as early as 3 h, and this increase was sustained for up to 32 h (Fig. 4). In addition, the protein synthesis inhibitor cycloheximide significantly inhibited IL-8 synthesis (Fig. 6), indicating that VEGF/VPF regulates IL-8 expression.

We also examined whether bFGF induces increased IL-8 production in HBMECs. bFGF has been shown to induce neovascularization like VEGF/VPF, but it does not induce vascular permeability (32). Within 12 h of bFGF treatment, we observed no increase in IL-8 protein expression in the samples compared with the untreated controls. However, after 12 h of bFGF treatment, the amount of IL-8 protein in these samples was greater than that in the untreated controls, although it was much smaller than in the VEGF/VPF-treated samples (Fig. 4). Thus, bFGF induced IL-8 production in the later time periods compared with VEGF/VPF. However, IL-8 has been shown to be secreted rapidly in endothelial cells upon treatment with various stimuli (33, 34). Therefore, bFGF might induce IL-8 production in HBMECs via another mechanism such as up-regulation of the IL-8 inducer. Based on our data and a previous report showing that bFGF induces VEGF/VPF expression in vascular endothelial cells through autocrine and paracrine mechanisms (35), we suggest that bFGF might induce IL-8 production in HBMECs via the up-regulation of VEGF/VPF.

VEGF/VPF is involved in intracellular signaling mediated by ERK, PI3-kinase, protein kinase C, calcium, and NF-κB in endothelial cells. We observed that the intracellular calcium chelator TMB-8, the NF-κB inhibitor PDTC, and the PI3-kinase inhibitor LY294002 significantly blocked IL-8 production in HBMECs stimulated by VEGF/VPF. The transcriptional factor NF-κB has been shown to induce IL-8 expression in endothelial cells upon treatment with various stimuli, such as tumor necrosis factor-α (28, 29). Also, the intracellular calcium chelator TMB-8 has been shown to inhibit the activation of NF-κB (30, 31). Therefore, it is possible that the PI3-kinase pathway may mediate VEGF/VPF-induced IL-8 production in HBMECs through NF-κB activation. Indeed, when HBMECs were infected with an adenovirus encoding constitutively active Akt, the increased expression of IL-8 protein in HBMECs was observed, whereas the NF-κB inhibitor PDTC potently blocked this IL-8 protein synthesis induced by activated Akt. These data indicate that VEGF/VPF induces IL-8 synthesis in HBMECs through activation of the transcriptional factor NF-κB via PI3-kinase and calcium signaling pathways. However, the MEK inhibitor PD98059 had no effect on IL-8 production in HBMECs stimulated by VEGF/VPF. These results also indicate that the ERK pathway did not mediate IL-8 production in HBMECs and are comparable with the results for bFGF, a potent inducer of the ERK pathway in endothelial cells (32), which failed to induce IL-8 within 12 h of treatment.

IL-8 belongs to the CXC chemokine family and is a potent chemoattractant for neutrophils and T lymphocytes, but not monocytes. In humans, it was reported that intradermal injection of IL-8 induced the perivascular infiltration of neutrophils...
within 1 h, which was sustained for several hours (36). IL-8 has been shown to be produced in a variety of cell types including monocytes and endothelial cells by stimuli such as the inflammatory cytokines, tumor necrosis factor-α and interleukin-1β (22, 33). In particular, activated endothelial cells are a source of a number of chemotactic molecules including IL-8. Enhanced
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chemotactic molecules in the endothelium elicit the movement of leukocytes from blood into tissues, a characteristic feature of inflammation. Our data suggest that VEGF/VPF can mediate leukocyte infiltration in endothelial cells through the up-regulation of chemokines, including IL-8, because supernatants of VEGF/VPF-treated HBMECs significantly increased neutrophil migration across an HBMEC monolayer compared with the untreated control, and that anti-IL-8 antibody blocked this migration (Fig. 8A).

Hypoxia is a strong inducer of VEGF/VPF expression. Hypoxia, an important factor in the pathophysiology of vascular and inflammatory diseases, contributes to leukocyte infiltration through the up-regulation of adhesion molecules and chemokine release (2, 3). During systemic hypoxia in vivo, VEGF/VPF was shown to be expressed in glial cells as well as in neurons in the hippocampus and dentate gyrus (37). However, VEGF/VPF expression does not appear to be localized in endothelial cells, although marked receptor expression was noted, thus suggesting that a paracrine action is involved in this condition (37). Therefore, our data suggest that hypoxia might at least in part contribute to leukocyte infiltration through the up-regulation of VEGF/VPF, which in turn induces the secretion of chemokines such as IL-8 in endothelium through a paracrine mode.

Recently, we and others reported that VEGF/VPF up-regulates intercellular adhesion molecule-1 (ICAM-1), the receptor for neutrophil adhesion molecules CD11/CD18 (MAC-1), in rat brain microvascular endothelial cells (38) and in human umbilical vein endothelial cells (39). Together with these studies, we suggest that VEGF/VPF might be responsible for the accumulation of neutrophils within hypoxic areas through the simultaneous up-regulation of IL-8 and its receptor ICAM-1 in endothelium.

In conclusion, this is the first report that VEGF/VPF induces IL-8 expression in human brain microvascular endothelial cells. We therefore suggest that VEGF/VPF contributes to leukocyte infiltration through the up-regulation of chemokines in endothelial cells.

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